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Licenciada em Bioquímica

Occupational exposure to hexavalent Chromium: biomarkers of genotoxicity in human peripheral blood

Dissertação para obtenção do Grau de Mestre em Bioquímica

Orientador: Doutora Maria João Silva, Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

Co-orientador: Doutora Maria Henriqueta Louro, Departamento de Genética Humana do Instituto Nacional de Saúde Doutor Ricardo Jorge, I.P.

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ABSTRACT

The hexavalent Chromium [Cr(VI)] is a human carcinogen, which is still authorized for use in several industrial settings because it has been difficult to replace. This was the reasoning to select it as a priority chemical by the European Human Biomonitoring Initiative (HBM4EU, https://www.hbm4eu.eu/), which aims to bridge chemicals human exposure to their possible impact on health. For that purpose, not only exposure was evaluated, but also early effect biomarkers were done to reflect potential health outcomes in several countries across Europe.

In Portugal, the study was developed in one aircraft maintenance company since the substitution of Cr(VI) is not expected in the near future. Following the company agreement and the volunteers informed consent, an individual questionnaire was filled in order to obtain personal information, as well as lifestyle habits and occupational issues. Personal air samples were collected in order to assess occupational exposure to Cr(VI) soluble and insoluble compounds. Sampling for effect biomarkers analyses involved blood samples from 50 workers and 26 healthy individuals (controls). Biomarkers of effect involving the analysis of chromosome alterations (micronucleus assay) and DNA damage (comet assay) were studied; the results were statistically compared.

Cr(VI)-exposed workers display a significantly higher frequency of micronucleated binucleated cells ($p < 0.001$) and an increased level of DNA breaks (comet assay) ($p < 0.001$) when compared with the non-exposed group. Only in the workplaces dedicated to painting exterior surfaces the values (0.4 mg/m^3) were higher than the Occupational Exposure Limit (OEL of 0.010 mg/m^3) currently proposed by the Directive (EU) 2019/130, 16/01/2019.

The present results suggest a potential health risk for this group of workers given that an association between an increased micronucleus frequency and cancer risk has been shown. Also, these findings should promote the investment in new risk management measures and the effective application of the ones already in place, such as adequate local ventilation and a frequent use of protective equipment.

Keywords: Alkaline Comet assay; Cytokinesis-block Micronucleus assay; HBM4EU; Hexavalent Chromium; Human Biomonitoring.

RESUMO

O Crómio hexavalente [Cr(VI)], sendo um agente carcinogénico humano, ainda é utilizado em vários ambientes industriais devido à sua difícil substituição. Consequentemente, foi selecionado como substância química prioritária pela Iniciativa Europeia de Biomonitorização Humana (HBM4EU, https://www.hbm4eu.eu/), que visa reduzir a exposição humana a compostos químicos e os seus possíveis impactos na saúde. Para esse fim, em vários países da Europa, não só a exposição a Cr(VI) foi avaliada, como também biomarcadores de efeito foram analisados de modo a refletir os possíveis danos para a saúde.

Em Portugal, o estudo foi desenvolvido numa empresa de manutenção de aeronaves, dado que a substituição de Cr(VI) não é esperada num futuro próximo. Após o contacto e acordo da empresa e o consentimento informado dos voluntários, foi preenchido um questionário individual de modo a obter informações pessoais, bem como hábitos de vida e questões ocupacionais. Foram recolhidas amostras de ar e fluidos biológicos (sangue e urina) a fim de avaliar a exposição ocupacional a compostos solúveis e insolúveis de Cr(VI). Foram também estudados biomarcadores de efeito envolvendo a análise de alterações cromossómicas (ensaio de micronúcleos) e danos no DNA (ensaio de cometa). Para isso, foram colhidas amostras de sangue de 50 trabalhadores e de 26 indivíduos saudáveis não expostos a Cr(VI) (controlos); os resultados foram comparados estatisticamente.

Relativamente aos resultados, somente nos locais de trabalho dedicados à pintura de superfícies externas, os valores (0,4 mg/m^3) foram superiores ao Limite de Exposição Ocupacional (OEL de 0,010 mg/m^3) atualmente proposto pela Diretiva (UE) 2019/130, 16/01/2019. Os trabalhadores expostos a Cr(VI) apresentam uma frequência significativamente maior de células binucleadas com micronúcleos ($p < 0,001$) e um nível aumentado de quebras de DNA ($p < 0,001$) quando comparados com o grupo não exposto.

Os resultados sugerem um potencial risco para a saúde dos trabalhadores, uma vez que se encontra demonstrada uma associação entre aumento da frequência de micronúcleos e o risco em desenvolver cancro. Posto isto, deve promover-se o investimento em novas medidas de gestão de riscos e a aplicação das já existentes, como ventilação adequada do local de trabalhado e uso frequente de equipamentos de proteção.

Palavras-chave: Biomonitorização humana; Crómio hexavalente; Ensaio do cometa em meio alcalino; Ensaio dos micronúcleos em células com bloqueio da citocinese; HBM4EU.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS and ACRONYMS

·OH	Hydroxyl radical
ALS	Alkali-labile sites
ASC	Ascorbate
ATSDR	Agency for Toxic Substances and Diseases Registry
BN	Binucleated
CBMN	Cytokinesis-Block Micronucleus Assay
CBPI	Cytokinesis-Block Proliferation Index
Cyt-B	Cytochalsin-B
DMSO	Dimethyl Sulfoxide
EBC	Exhaled breath condensate
EMS	Ethyl Methanesulfonate
EU	European Union
FBS	Fetal bovine serum
GSH	Glutathione
HBM	Human Biomonitoring
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HepG2	Hepatoma cell line
IARC	International Agency for Research on Cancer
MMC	Mitomycin C
MN	Micronucleus
MNBN	Micronucleated Binucleated cell(s)
NBUD	Nuclear bud
NIOSH	National Institute of Occupational Safety and Health
NPB	Nucleoplasmic bridge
OECD	Organisation for Economic Co-operation and Development
OEL	Occupational exposure limit
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PHA	Phytohaemagglutinin
RBC	Red blood cells
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RI	Replication Index
SCGE	Single Cell Gel Electrophoresis
SD	Standard Deviation
SOD	Superoxide dismutase
SSB	Single-strand breaks
U-Cr	Chromium in urine
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

1. INTRODUCTION

1.1 Human Biomonitoring

No matter where we live, we are daily exposed to environmental substances. Most of these substances occur naturally in the environment, but others are produced by humans. The large-scale growth of economic activities has been accompanied by parallel increases in the consumption of nonferrous metals, some of which are recognized human carcinogens. They could be in the air, water, food, and in the products we use, and although the amounts may be small, and their effects sometimes poorly understood, continuous exposure to a mixture of these substances over long periods could have consequences for the health and well-being of individuals and society as a whole (Salnikow and Zhitkovich, 2008).

Exposure limit values used in chemical safety regulations are derived using animal models, being these extrapolated to human exposure limit values. Thus, there is a lack of harmonization among the various cohort and human biomonitoring studies resulting in a shortage of accepted exposure limit values based on human biomonitoring data (Steckling et al., 2018). Therefore, there is a need to obtain biologically effective dose values for the compounds of interest (Sarigiannis et al., 2019). Thus, the interest of Human Biomonitoring (HBM) reside in its preventative nature, in order to reduce and, whenever possible, prevent human exposure to chemical substances that may lead to morbidity or mortality (Sexton et al., 2004).

HBM can be defined as "(…) the assessment of human exposure to chemicals or their effects by measuring these chemicals, their metabolites or reaction products in human specimens" (CDC, 2005). Samples used in human biomonitoring include body fluids (blood, urine, exhaled air, saliva, sweat, semen, faeces, etc) and tissues (skin, mucosa, parenchymal tissue, bone, hair, nails, etc) and depend on the type of biomarker used (exposure, effect and/or susceptibility) (Manno et al., 2010; WHO, 2015).

The analysis of the levels of certain substances in the body through biomarkers of exposure provides an unequivocal indication that both exposure and absorption have occurred (Angerer et al., 2007; Sexton et al., 2004). However, measuring uniquely exposure biomarkers does not provide information on preclinical effects that may allow establishing a link between exposure and health effects. The inclusion of biomarkers of effect in HBM studies give information on early biological alterations before the onset of disease (Ladeira and Viegas, 2016). Given that, these biomarkers reflect reversible alterations in the organism, the effects detected are likely to be prevented, if exposure to the critical substance (or mixture of substances) is reduced or ceased. Therefore a relationship between exposure biomarkers and effect biomarkers provide meaningful data for a comprehensive risk assessment (Manno et al., 2010; WHO, 2015).

Therefore, the biomonitoring of human populations using biomarkers is considered a useful tool for the identification of potential risk groups (WHO, 2015). Thus, the collected information from biomarkers of exposure and effect in HBM can be used to improve health risk assessment and reinforce the scientific basis to implement preventive policies in occupational and environmental settings (Annangi et al., 2016; Den Hond et al., 2015). The human biomonitoring has been considered as a beneficial approach for the health risk management under Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation (Boogaard et al., 2011).

1.2 The HBM4EU Project

The European Human Biomonitoring Initiative (HBM4EU) is a project that builds on existing scientific information and develops new data for the purpose of establishing a European human biomonitoring platform, involving 28 partner countries and European Environment Agency (Bopp et al., 2018). The HBM4EU aims to provide comparable European data on human exposure to chemicals and mixtures of chemicals, in the environmental and occupational settings to develop new priorities and investments regarding risk management, in order to reduce exposure to toxic chemicals in Europe (Bopp et al., 2018; Ganzleben et al., 2017).

The general aim of the initiative is to coordinate and advance HBM in Europe in order to provide better evidence of the actual internal exposure of citizens to chemicals and its impact on health, to support policy making in relevant chemical regulatory domains (Ganzleben et al., 2017). This project aims to: 1) harmonizing procedures for HBM across countries and thus to provide policy makers with comparable data on human internal exposure to chemicals at the EU level; 2) linking data on aggregate internal exposure to chemicals to external exposure and identifying exposure pathways and upstream sources; 3) generating scientific evidence on the causal links between human exposure to chemicals and adverse health outcomes; 4) adapting chemical risk assessment methodologies to use HBM data to account for the contribution of multiple exposure pathways to the total chemical body burden (Bopp et al., 2018; Santonen et al., 2019).

Within the HBM4EU project, the substances or groups of substances that will be considered with priority were selected in consortium with primarily policy makers and regulators in chemical/food safety and occupational health in European Union Member States. The criteria used to select the priority compounds included public concern, scientific evidence and the urgency to regulate in order to safeguard occupational and consumer health (Sarigiannis et al., 2019).

Several of those compounds are also relevant at European workplaces, such as hexavalent Chromium, Cr(VI) (Santonen et al., 2019), a human carcinogen (IARC, 2012). Therefore, there is a need to collect new data on human exposure in the European countries.

1.3 Chromium

Any metallic element with relatively high density as compared to water and toxic even at low concentrations is termed as “Heavy Metal”. Heavy metals are a defined group of chemical elements that include transition metals and some metalloids, and they are widely distributed in the Earth's crust (WHO, 1996). Several metals are considered essential nutrients and are necessary for the correct functioning of various physiological processes. Cobalt, Copper, Chromium, Iron, Magnesium, Manganese, Molybdenum, Nickel, Selenium and Zinc belong to the heavy metals group and their presence in the body below or above certain concentrations may cause some disorders (Fraga, 2005). Most of the heavy metals are well known for their toxicity, non-biodegradability, persistence in nature and bioaccumulation tendency (Garg et al., 2007).

The exposure to most metals has been linked to the induction of adverse health effects and different human pathologies, since metals have the ability to target cell nuclei and interact with DNA. Consequently, various metal compounds are associated with genotoxic and carcinogenic effects (IARC, 2012, 2006). This genotoxic and carcinogenic potential of the metals is mainly dependent on their oxidation state, as it affects their absorption, intracellular transport, distribution and bioavailability. For this reason the metals can induce oxidative stress; damage in cellular components, including DNA; inhibition of major DNA repair systems, resulting in genomic instability; deregulation of cell proliferation by induction of signalling pathways or inactivation of growth controls such as tumour suppressor genes (Annangi et al., 2016; Beyersmann and Hartwig, 2008; Koedrith and Seo, 2011).

Chromium is one of the heavy metals, it exists in many chemical and physical forms that influence its environmental behaviour and biological activity. Cr(0), Cr(III) and Cr(VI) are the major oxidative states of Chromium, and are most usually found in the workplace and general environment (IARC, 2012; Salnikow and Zhitkovich, 2008).

Cr(0) is normally found in its metallic form in alloys with other metals, such as Nickel, Iron and Cobalt, with stainless steel being the product containing more Cr(0). Metallic Chromium is chemically and biologically inert, but exposure to Cr(0) containing dust may cause irritation in the respiratory tract. Although it is stable to oxidation by atmospheric oxygen under ambient conditions, high temperature processes such as welding or exposure to corrosive chemicals lead to the formation of higher oxidation states, namely Cr(III) and Cr(VI) (IARC, 2018; Salnikow and Zhitkovich, 2008; Zhitkovich, 2005).

The most common and stable form of Chromium is the trivalent form, Cr(III). This substance is thermodynamically stable and is the final oxidative form found in all biological systems. Chromium compounds in the +3 oxidation state are relatively non-toxic because they are not well absorbed into the body (Sun et al., 2015). According to the World Health Organization (WHO, 1998), “Trivalent Chromium is not considered to be carcinogenic”. The International Agency for Research on Cancer (IARC) evaluated that “There is inadequate evidence in humans for the carcinogenicity of Cr(0) and Cr(III) compounds”. Also based on the results of animal carcinogenicity studies, the overall evaluation was that Cr(0) and Cr(III) “are not classifiable as to their carcinogenicity to humans (Group 3)” (IARC, 2012, 1990).

1.3.1 Hexavalent Chromium

Cr(VI) is the second most stable oxidation state of Chromium (Costa, 1997). Cr(VI) has been known to be an occupational carcinogen for more than a century (Langrrd, 1990).

Several regulatory and nonregulatory agencies, including the IARC evaluated that “there is sufficient evidence in humans for the carcinogenicity of Cr(VI) compounds”. The overall evaluation was that “Cr(VI) is carcinogenic to humans (Group 1)” (IARC, 2012, 1990). The U.S. Environmental Protection Agency (USEPA) has also classified hexavalent Chromium in Group A, that is a human carcinogen (USEPA, 1998), and the Agency for Toxic Substances and Diseases Registry (ATSDR), also consider it as toxic and carcinogenic to humans (ATSDR, 2012). Furthermore, extensive studies and data collection on the industrial exposure to Chromium compounds have led the National Institute of Occupational Safety and Health (NIOSH) to identify these compounds as one of the leading causes

of occupational lung cancer (NIOSH, 2013).

The biological effects associated with Cr(VI) exposure are diverse. Exposure to these compounds can cause nosebleeds, ulcers and holes in the nasal septum, inflammatory respiratory problems, skin irritation and rashes from allergic dermatitis, upset stomachs, kidney and liver damage (ATSDR, 2012; Rakhunde et al., 2012). Cr(VI) may also be involved in the cause of a broad spectrum of cancers, including prostate cancer, lymphoma, leukaemia, and bone, stomach, brain, kidney and testicle cancers (ATSDR, 2012; Costa, 1997; De Flora, 2000).

The respiratory tract is the main target for Cr(VI) carcinogenesis in individuals who perform certain occupational activities, for this reason respiratory problems such as fibrosis, nasal septum perforation, nasal polyp development, and lung cancer are very common (ATSDR, 2012; ECHA, 2019; IARC, 2012; NIOSH, 2013). The most common form of cancer among workers exposed to Cr(VI) is squamous cell carcinoma of the lung. This tumours are located where there is an accumulation of Chromium, bronchial bifurcations exhibit the highest levels of Chromium in workers exposed to chromates. The sedimentation of chromate particles with 1-3 μm in diameter is probably the main reason for the high Chromium deposition in the areas of bifurcations (Salnikow and Zhitkovich, 2008). However, ingestion or inhalation of Cr(VI) compounds results in Chromium distribution to every organ of the body, hence, all organs are potentially susceptible to the toxic and carcinogenic effects of Cr(VI) (ATSDR, 2012; Costa, 1997).

1.3.2 Chromium toxicokinetics and toxicodynamics

In humans, the absorption, retention, and elimination of Chromium compounds depend on the solubility and particle size of the compound. Chromates of low water solubility are less carcinogenic than chromate compounds of greater water solubility. Additionally, Cr(VI) compounds of high water solubility are likely to penetrate throughout the body and induce a wide variety of cancers (Costa, 1997; NIOSH, 2013).

Inhaled Cr(VI) is readily absorbed from the respiratory tract. The degree of absorption depends on the physical and chemical properties of the compound and the extent of reduction of the hexavalent form to trivalent Chromium, which is absorbed to a much lesser extent. The same factors mentioned above apply to absorption from the gastrointestinal tract, although absorption by this route is generally much lower compared with that in the respiratory tract (IARC, 2012; NIOSH, 2013). Cr(VI) can also penetrate human skin to some extent (Shelnutt et al., 2007). In humans, absorbed Cr(VI) is distributed in nearly all tissues, with the highest concentrations found in the kidney, liver, and bone (NIOSH, 2013).

After exposure by inhalation, excretion occurs predominantly via the urine. Inhaled Cr(VI) that is not absorbed in the lungs may enter the gastrointestinal tract, much of this Cr(VI) is rapidly reduced to Cr(III) and excreted in the faeces. The remaining 3% to 10% of the Cr(VI) is absorbed from the intestines into the blood stream, distributed throughout the body, transported to the kidneys, and excreted in the urine (ATSDR, 2012; NIOSH, 2013).

At physiological pH most Cr(VI) compounds are tetrahedral oxyanions that can cross cell membranes, while trivalent Chromium compounds are mainly octahedral structures to which the cell membrane is practically impermeable, therefore its toxicity is very low (ATSDR, 2012). This way the

major difference between Cr(VI) and Cr(III) is their different capacity to enter cells, which is the basis of the almost one thousand fold difference in their toxicities (NIOSH, 2013). At neutral pH Cr(VI) exists as a mixture of chromate (CrO_4^{2-}) or hydrochromate ($HCrO_4^-$) anions. This chromates are isostructural with physiological sulphate and phosphate ions and because of this molecular mimicry, Cr(VI) is taken up by cells via the non-specific anionic transport system and accumulated in cells instead of sulphate or phosphate (Zhitkovich, 2005).

The hexavalent Chromium is a pro-carcinogen that by itself does not react with DNA. However, in the biological systems Cr(VI) undergoes a series of reduction reactions producing thermodynamically stable Cr(III) (ATSDR, 2012; Ray, 2016). When this occurs extracellularly, reduction acts as the detoxification process due to the production of low permeability Cr(III) complexes (IARC, 2012). However, when intracellular reduction of Cr(VI) occurs, it does not require enzymatic steps but is mediated by direct electron transfer from ascorbate (Asc) and non-protein thiols, such as glutathione (GSH) and cysteine (IARC, 2012; O'Brien et al., 2001).

The respective cellular availability and reaction rate with Cr(VI) determines which is the primary reducing agent. There are studies supporting the significance of both Asc and GSH in Cr(VI) reduction however, most data show that ascorbate is the dominant kinetically favoured biological reductant responsible for around 90% of Cr(VI) reduction reactions *in vivo* (O'Brien et al., 2001). When ascorbate is the reductant, two electrons are transferred, and Cr(IV) but not Cr(V) is generated as the first intermediate, whereas with cysteine as a reductant, predominantly Cr(V) is formed due to one-electron transfers. In both cases, the final product is trivalent chromium (O'Brien et al., 2003).

The intracellular Cr(VI) reduction process can generate various amounts of unstable Cr(V) and Cr(IV) intermediates and the final product of Cr(VI) reduction, Cr(III), molecular oxygen radicals, and other free radicals. The molecular oxygen is reduced to superoxide radical, which is further reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 reacts with Cr(V), Cr(IV) or Cr(III) to generate hydroxyl radicals ($^{\cdot}OH$) via the Fenton reaction, and it undergoes reduction-oxidation cycling (NIOSH, 2013).

Trivalent Chromium and the unstable Cr(IV) and Cr(V), the oxygen radicals and other free radical species formed by the reduction of Cr(VI), induce various types of DNA damage. Structural genetic lesions include DNA adducts, DNA strand breaks, DNA-protein crosslinks, oxidized bases, and DNA inter and intrastrand crosslinks (Ding and Shi, 2002). The damage induced by Cr(VI) can also lead to dysfunctional DNA replication and transcription, altered gene expression, aberrant cell cycle checkpoints, dysregulated DNA repair mechanisms, inflammatory responses, and the disruption of key regulatory gene networks responsible for the balance of cell survival and cell death, and promoting genomic instability. Such lesions could lead to mutagenesis and ultimately to carcinogenicity. In fact, some studies have been published regarding the possible epigenetic effect of Cr(VI) and consequently carcinogenicity. As example, potassium dichromate is able to induce DNA methylation, thus, silencing some genes. Actually, biomonitoring studies proved that Cr(VI) is capable of induce DNA methylation of p16 and MLH1 genes and augment lung cancer risk (Arita and Costa, 2009; Kondo et al., 2006; Takahashi et al., 2005).

1.3.3 Human exposure to Cr(VI)

1.3.3.1 Exposure of the general population

Hexavalent Chromium occurs naturally in the earth's crust, although most of Cr(VI) compounds are human-made, products or by-products of large industrial emissions (NIOSH, 2013). For this reason, the general population resident in the vicinity of anthropogenic sources of Cr(VI) may be exposed by the oral route, contaminated food and drinking-water, and by inhalation of ambient air. Cigarette smoking is another important source of exposure to Cr(VI) (Annangi et al., 2016; ATSDR, 2012).

1.3.3.2 Occupational exposure

According to the World Health Organization, "there is no reason, to be concerned that Chromium in the air presents a health problem, except under conditions of industrial exposure" (WHO, 1998).

Under the European regulation (EC 1907/2006) on the registration, evaluation, authorization and restriction of substances Chemicals (REACH), the Cr(VI) compounds [chromates, Chromium trioxide and dichromium tris (chromate)] are authorized for occupational activities (ECHA, 2019).

Workers in industries that use Chromium are one segment of the population that is especially at high risk to Chromium exposure (ATSDR, 2012). Inhalation of dusts, mists or fumes, and dermal contact with Chromium-containing products are the main routes of occupational exposure. In Europe the estimated number of Cr(VI)-exposed workers in 2012 was approximately 786000 (IARC, 2012).

Workers have potential exposures to airborne Cr(VI) compounds in many industries, including production, use and welding of Chromium-containing metals and alloys (stainless steels), electroplating, production and use of Chromium-containing compounds, such as pigments, catalysts, corrosion inhibitors and wood preservatives (ATSDR, 2012; IARC, 2018; NIOSH, 2013). During these processes, extremely high temperatures are used resulting in the oxidation of the metallic forms of Chromium to Cr(VI). During the welding process, both are heated to the melting point, and a fraction of the melted metal vaporizes. Any vaporized metal that escapes the welding area quickly condenses and oxidizes into welding fume, and an appreciable fraction of the Chromium in this fume is Cr(VI) (IARC, 2018; NIOSH, 2013). Dermal exposure to Cr(VI) may occur with any task or process in which there is potential for splashing, spilling or other skin contact with material containing liquid forms of Cr(VI), such as in electroplating baths or during preparation of chrome inks. Dermal exposure may also occur because of the contamination of workplace surfaces or equipment (NIOSH, 2013). But most occupational and environmental exposures are actually co-exposures with other carcinogens (Salnikow and Zhitkovich, 2008). Stainless steel welding also produces fumes of Nickel, inorganic Nickel compounds are lung carcinogens, and Manganese, a neurotoxic substance. Companies performing Cr(VI) electroplating, may also apply Nickel electroplating (Annangi et al., 2016).

1.3.4 Occupational Exposure Limit for hexavalent Chromium

The Directive (EU) 2019/130, 16/01/2019 proposed an Occupational Exposure Limit (OEL) for the Cr(VI) of 0.010 mg/m^3. The introduction of an EU-wide OEL could have a significant positive long-

term impact on workers' health. However the OEL may not be sufficiently protective to prevent all occurrences of lung cancer and other adverse health effects among workers exposed for a working lifetime. Exposure to Cr(VI) compounds should be eliminated from the workplace where possible because of the carcinogenic potential of these compounds. Where possible, less-toxic compounds should be used instead Cr(VI) compounds. Where elimination or substitution of Cr(VI) compounds is not possible, it is recommended that all reasonable efforts be made to reduce exposures to Cr(VI) compounds below the OEL (NIOSH, 2013).

Compliance with the OEL for Cr(VI) compounds is currently achievable in some industries and for some job tasks. It may be difficult to achieve the OEL during certain tasks, including welding, electroplating, spray painting, and atomized-alloy spray-coating operations. Thus, during the performance of these tasks the use of respiratory protection will be required (NIOSH, 2013).

1.4 Biomarkers

According to the U.S. National Academy of Sciences Committee on Biological Markers, biomarkers are an alteration in cellular or biochemical components, processes, structure or functions that is measurable in a biological system or sample (CBM, 1987).

A biomarker can be any substance, structure or process that can be monitored in tissues or fluids and can contribute to quantitative long-term risk assessment by helping to: determine the forms of dose-time-response relationships; assess the biologically effective dose; make interspecies comparisons of effective dose, relative potency, and effects; resolve the quantitative relationships between human interindividual variability; and identify subpopulations that are at enhanced risk (Manno et al., 2010; Schulte and Mazzuckelli, 1991; WHO, 2015). A goal in the use of biomarkers is to identify early adverse effects of environmental/occupational exposure through chemical and biochemical markers, which may modulate disease risk, e.g., metabolic diseases, immune deficiencies and cancer. These markers can avoid disease progression and minimize problems at advanced stages (Kyrtopoulos, 2006).

Ideally, biomarkers used for environmental and occupational health risk assessments should be accessible, measured in a relatively non-invasive way, non-destructive, easy and cheap to measure (Manno et al., 2010; WHO, 2015), relevant and valid (Schulte and Mazzuckelli, 1991; WHO, 2001).

Biomarkers can be classified into three main categories, exposure, effect, and susceptibility, depending on their significance (Duffus et al., 2007; WHO, 2001). A biomarker of exposure is the identification of an exogenous substance, its metabolite or the product of an interaction between a xenobiotic agent and some target molecule. A biomarker of effect is a measurable biochemical, structural, functional, behavioural or any other kind of alteration in an organism that, according to its magnitude, can be associated with established or potential health impairment or disease. A biomarker of susceptibility is an indicator of an inherited or acquired ability of an organism to respond to the challenge of exposure to a chemical (CBM, 1987; Manno et al., 2010).

1.4.1 Effect Biomarkers

Effect biomarkers can be obtained as a result of interaction of the organism with several different environmental factors including chemical, physical and biological. This type of biomarker indicates early biochemical or functional changes, including a wide range of biological responses, ranging from physiological adaptation to disease. These biomarkers are able to monitor disease progression and prognosis. When combining these with other biomarkers, an improvement in epidemiology and risk assessment can be obtained (Manno et al., 2010; WHO, 2015; Ladeira and Viegas, 2016).

An important group of effect biomarkers which have been used in occupationally exposed populations, are genotoxicity biomarkers that are used in the assessment of biological effects that result from exposure to known or putative genotoxic agents. They are considered as biomarkers of early carcinogenic effects, and are used in specific occupational and environmental exposures, to predict the risk of disease, or to monitor the effectiveness of exposure to chemicals (Manno et al., 2010; WHO, 2001).

Among the available genotoxicity biomarkers, the cytokinesis-block micronucleus assay (CBMN) in peripheral blood lymphocytes (PBL) and the alkaline comet assay. These are recognized due to their robustness, sensitivity and statistical power. These tests may be effective in distinguishing individuals exposed to genotoxic chemicals from non-exposed individuals (Araldi et al., 2015; Manno et al., 2010). Currently studies show that the association of alkaline comet assay and CBMN in peripheral blood lymphocytes are the best battery of tests to evaluate the mutagenic potential, thus, being considered as a gold standard among genotoxic tests (Araldi et al., 2015). This way, effect biomarkers used as early predictors of clinical disease that can improve occupational health risk assessment and contribute to implement new effective disease prevention policies in occupational and environmental settings (Bonassi et al., 2001; WHO, 2001).

1.4.1.1 Cytokinesis-block micronucleus assay

The micronucleus assay is one of the most important *in vitro* and *in vivo* tests to assess genetic damage and characterize genotoxicity of a specific compound or chemical (Fenech, 2007, 2000).

Micronuclei consist of chromosome fragments (acentric fragments) and/or whole chromosomes that are unable of migrating to the poles of the cell during anaphase. At telophase, a nuclear membrane is formed around the genetic material and this corpuscle remains in the cytoplasm and gradually assume similar morphology to main nuclei but smaller, which gave origin to the term “micronucleus” (MN) (Figure 1.1) (Fenech, 2000).

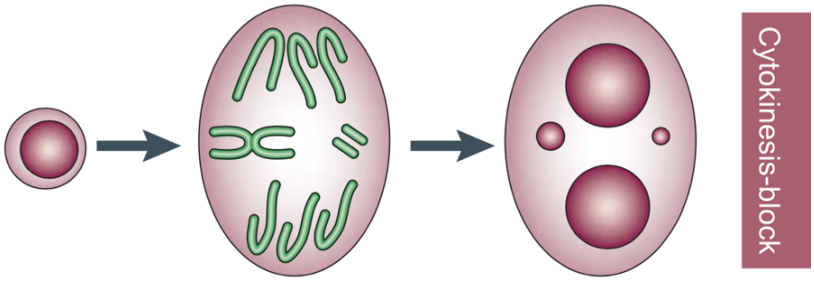

Figure 1.1 - MN formation in cells undergoing nuclear division. MN originate from either lagging whole chromosomes or acentric chromosome fragments. These events can only be observed in cells completing nuclear division, which are recognized by their binucleated appearance after cytokinesis blocking with Cytochalasin-B (Fenech, 2007).

A cell that has suffered a DNA-damaging event can only express such damage as a MN if it completes at least one nuclear division after such an event so it is important to restrict and distinguish cell population between dividing and non-diving cells (Fenech, 1997; Fenech and Morley, 1985a). To overcome this kinetic problem, Fenech and Morley in 1985 developed a method to identify cells that have completed nuclear division by their binucleated appearance by using cytochalasin-B (Cyt-B), an inhibitor of cytokinesis (Fenech and Morley, 1985a, 1985b). The *in vitro* CBMN is an efficient methodology, with international validation and described in OECD Guideline 487 as standard procedure for detect genotoxic events at the chromosome level (OECD, 2016).

The CBMN in peripheral blood lymphocytes has become one of the most widely used methods for measuring structural and numerical chromosomal changes in human cells *in vitro* and *in vivo* (Kirsch-Volders et al., 2014; Nersesyan et al., 2016). In this technique, MN are scored only in lymphocytes that have completed one nuclear division following phytohaemagglutinin (PHA) stimulation. Cyt-B is essential to individualize the two daughter cells in cytokinesis phase, resulting in a binucleated cell (Fenech, 2007, 2000). There is evidence that MN frequency in peripheral blood lymphocytes is predictive of cancer risk, suggesting that increased MN formation is associated with early events in carcinogenesis (Bonassi et al., 2001; Fenech et al., 2016).

CBMN is also called the cytome assay because it also enables the measurement of other chromosomal abnormalities such as nucleoplasmic bridges (NPB), a biomarker of DNA misrepair and/or telomere end-fusions; nuclear buds (NBUD), a biomarker of elimination of amplified DNA and/or DNA repair complexes. It also allows the evaluation of loss of cell viability (necrotic and apoptotic aspect) and cytostatic effects. The NBUD are formed from dicentric chromosomes that are pulled to opposite poles of the cell in anaphase, the NBUD and MN have homologous structure, but NBUD are still linked to the main nucleus (Figure 1.2). Cytostasis represents the ratio between mononucleated, binucleated and multinucleated cells in a population (Fenech, 2007, 2000). All of these parameters allows to measure genome damage which may be induced by endogenous and environmental genotoxins (Fenech, 2007; Kirsch-Volders et al., 2018).

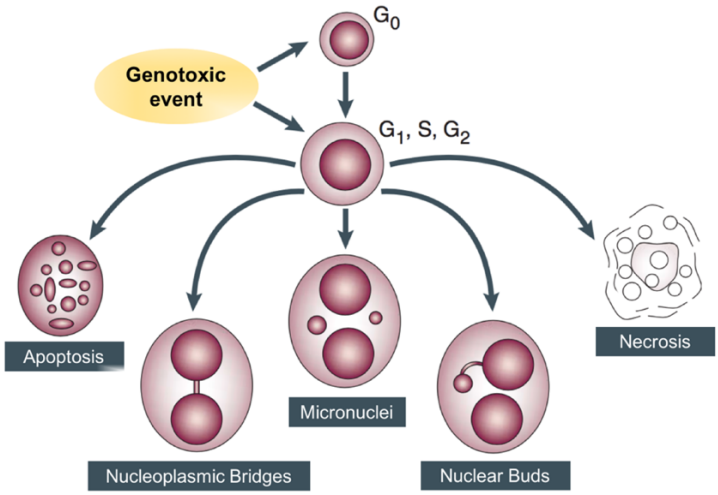

Figure 1.2 - The various possible fates of cultured cytokinesis-blocked cells following exposure to cytotoxic/genotoxic agents (Fenech, 2007).

1.4.1.2 Alkaline Comet Assay

The Single Cell Gel Electrophoresis (SCGE) or Comet Assay has become one of the most popular method for measuring DNA damage (Azqueta and Collins, 2013; Tice et al., 2000). For many reasons, the comet assay is an essential tool in toxicological research. It allows to understand background levels of DNA damage in different types of tissues and the ability of cells to respond to a toxic agent and their repair capacity (Collins, 2004; Glei et al., 2016).

The comet assay *in vivo*, has already been reported in OECD Guideline 489 as a standard test to execute the "Testing of Chemicals" (OECD, 2014). This assay is widely used in HBM studies to measure DNA damage in whole blood or isolated peripheral blood mononuclear cells (PBMC) as a marker of effect from exposure to genotoxic agents, and can usefully contribute to the "biological effect dosing" of occupational and environmental exposures (Bausinger and Speit, 2014; Collins et al., 2013; Faust, 2004). The Comet assay has demonstrated high sensitivity for detecting low levels of DNA damage, requires small numbers of cells per sample, is flexible, has low cost, is easy to employ, allows to conduct studies using relatively small amounts of a test substance and the study can be completed in a short time (Tice et al., 2000).

The method relies on the migration of lysed cells embedded in agarose on a microscope slide, where an electric current is applied. Agarose assures that DNA is immobilized for the electrophoresis run (Collins and Azqueta, 2012a). Tice et al. 2000 reported that the optimal version of the Comet assay for identifying agents with genotoxic activity was the alkaline, pH 13, version of the assay developed by Singh et al. 1988. The alkaline version is capable of detecting DNA single-strand breaks (SSB), alkali-labile sites (ALS), DNA-protein cross-linking, and SSB associated with incomplete excision repair sites (Singh et al., 1988; Tice et al., 2000). Under electrophoretic conditions, the DNA that contain breaks migrates at a higher rate through the agarose gel, forming a tail (where the DNA fragments are present) with a head (undamaged DNA molecule) looking a comet, when viewed under fluorescence microscopy following staining with a DNA-binding fluorescent dye (Collins and Azqueta, 2012b; Tice et al., 2000).

The percentage of DNA in tail corresponds to the intensity of the comet tail, and it is directly related to the DNA breakage frequency (Glei et al., 2016). The DNA lesions quantified by the Comet assay correspond to primary and reversible lesions that can be repaired or, on contrary, lead cell to death if it is highly damaged. Each individual comet is scored to give a measure of DNA damage; it is often necessary to pool the results of 100 comets to obtain the overall damage level of a population of cells (Azqueta and Collins, 2013). According the OECD, comets should be scored quantitatively with an automated or semiautomated image analysis system (OECD, 2014).

1.5 The hexavalent Chromium occupational study

The general aim of the HMB4EU hexavalent Chromium occupational study is to cooperate for the establishment of a sound and valid scientific basis to propose biological limit values for occupational exposure to Cr(VI) in industrial settings across Europe. Moreover, the study will provide reference values for the general population (from data collected from controls) and use this data as scientific evidence for regulatory risk assessment and decision-making under EU chemical legislation and under occupational safety and health legislation (Santonen et al., 2019).

Most occupational studies are performed with a low number of workers who can be recruited in national studies. These studies are conducted by different researchers in individual countries, so analytical methodologies and data collection are different, which makes it difficult to compare results and use of the data in regulatory risk assessment at the European level (Santonen et al., 2019). Thus, this ongoing project is the first that will show data from multiple European countries using harmonized protocols for data gathering, sampling and biochemical analyses. This will improve and bring EU-added value for the data collected from different European countries (Santonen et al., 2019). Figure 1.3 shows the project workflow.

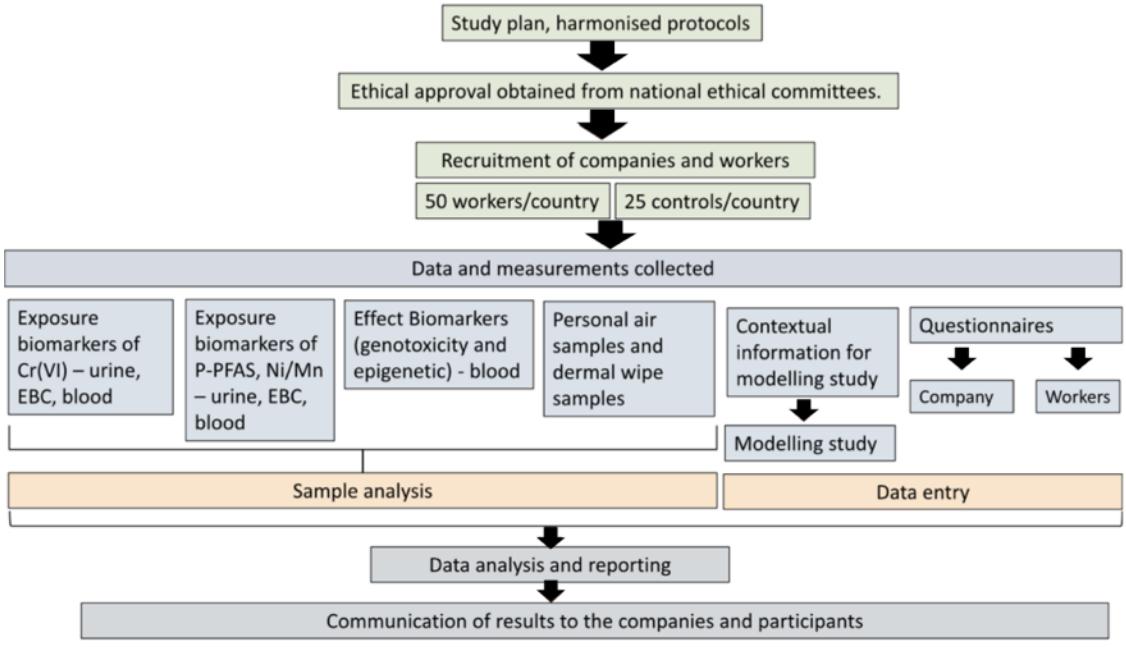

Figure 1.3 - Schematic illustration of the workflow (Santonen et al., 2019).

In this study a group of workers exposed to Cr(VI) and a control group non-exposed, is being recruited from eight countries: Belgium, Finland, France, Italy, Poland, Portugal, The Netherlands and United Kingdom. Following participants acceptance in the study, several biomarkers are being used (Santonen et al., 2019). It should be taken into account that inhalation is the main route of concern for occupational exposure to Cr(VI). Inhaled Cr(VI) enters the respiratory system, where it can remain, be reduced or enter the bloodstream. Cr(VI) can be reduced to Cr(III) in the lungs or plasma and excreted as Cr(III) in the urine. Cr(VI) that is not reduced in plasma can enter red blood cells and lymphocytes. This Cr(VI) distribution allows the biomonitoring of total Chromium in urine (U-Cr), the common biomarker used for the biomonitoring of Chromium exposure at the workplace. 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) (Santonen et al., 2019).

Within HBM4EU project, several matrices will be used to identify Cr(VI) exposure. Hexavalent Chromium in exhaled breath condensate (EBC) has been proposed as a new biomarker-matrix combination which can give specific information on the Cr(VI) levels in the main target tissue, the lungs (Leese et al., 2017). Blood samples are being collected from each participant for measuring Cr(VI) in red blood cells (RBC) and plasma. Cr(VI) levels in RBC reflects mainly the exposure to Cr(VI) since their compounds easily passes through cell membranes, while Cr(III) does not (Ray, 2016). In order to evaluate if the workers are exposed by dermal route, are used wipe samples to recover the mass of total Chromium from the hands of volunteers participating in the workplace. Also, air samples will be analysed for determination of the inhalable (total) and respirable (alveolar) dust fraction and respective total Chromium or Cr(VI) amount, in order to assess workplace exposure. The correlations between these biomarkers allows further study of the fate and transformation of Cr(VI) to trivalent form when entered to the body (Santonen et al., 2019).

Also, effect biomarkers are being analysed, such as CBMN in human peripheral blood lymphocytes and micronucleus assay in peripheral blood reticulocytes, which is considered very sensitive for the monitoring of genetic damage in humans (Abramsson-Zetterberg, 2018). Alkaline comet assay that allows the identification of a broad spectrum of primary DNA lesions, such as single and double-strand breaks (Azqueta and Collins, 2013) is also being used. Epigenetics, oxidative stress and telomere length analyses will also be done. These biomarkers will be performed to bridge the gap between exposure and health effect, because they provide information on early biological alterations before the onset of disease (Annangi et al., 2016).

An association between the levels of exposure to Cr(VI) and the early effects characterized in workers, if existent, is expected to predict potential health outcomes for Cr(VI) exposed workers, particularly, on the long-term. All the information generated will help to support new priorities and provide significant data for a comprehensive risk assessment (Santonen et al., 2019)

2. OBJECTIVE

The aim of this work was to evaluate the genotoxic effects from exposure to Cr(VI) in workers from a Portuguese aircraft maintenance company, who are occupationally exposed to this heavy metal. For this purpose, two relevant biomarkers of genotoxicity were characterized in blood cells from a group of exposed and non-exposed (control group) individuals: the CBMN was used for measuring chromosomal break or chromosomal loss events, and the alkaline comet assay to quantify DNA strand breaks.

3. METHODOLOGY

3.1 Company and workers recruitment, ethical approvals

For this work, the target population were workers exposed to Cr(VI), from a Portuguese aircraft maintenance company performing electroplating, welding, surface treatment by painting, quality control and machining. Workers inclusion and exclusion criteria for the study are given in Table 3.1 (Santonen et al., 2019). Additionally, a non-exposed group (control group) was recruited, this group was selected from the same company and among volunteers from an University nearby with no known occupational exposure to Cr(VI).

Recruitment of the company and workers followed the standard operating procedure developed under HBM4EU for the selection of participants, recruitment, and obtaining informed consent. The participants had to fill a questionnaire with the following information: personal information (gender, age, medical history, health status), lifestyle habits (smoking habits, alcohol consumption, diet), and occupational questions (years of exposure and job description) (Annex 1).

Study protocols were submitted for approval by two ethics committees one from each coordinating entity, in Portugal (IPL and INSA) and in each participating country, with the necessary approvals being granted.

Table 3.1 - Worker inclusion and exclusion criteria.

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.
Participants should be in good health and present at work during the planned period of the study.

3.2 Collection and transport of blood samples

The collection of blood samples was done by personnel trained in phlebotomy knowing the special precautions related to the handling of biological material, according to European rules. One blood sample was collected from each participant (exposed and non-exposed), following signed informed consent. For effect biomarkers analysis, a 6 mL blood tube per participant (with sodium heparin) was required (Figure 3.1). All Portuguese blood samples were collected on Thursdays at the end of the shift and arrived at our laboratory on the same day. All tubes were transported at 4ºC (max 10ºC) and were protected from light (wrapped in aluminium foil). In order to reproduce the conditions of the assays performed with samples from other countries, all procedures performed with the Portuguese samples were started on Monday of the following week.

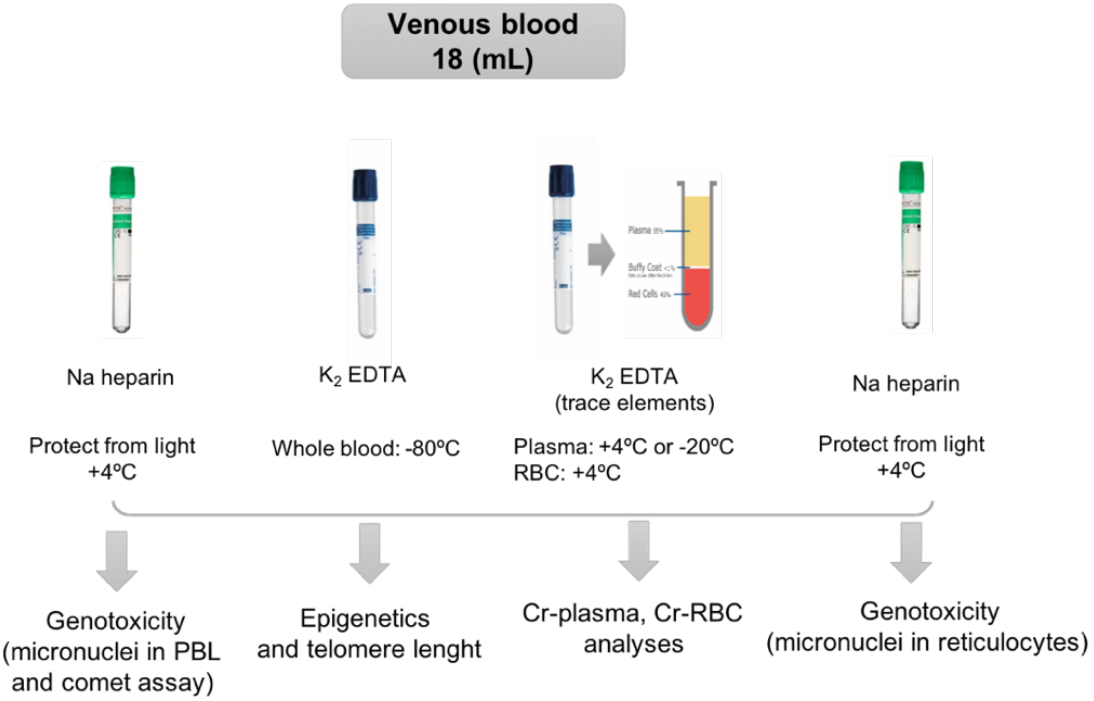

Figure 3.1 - Blood samples collected (Santonen et al., 2019).

3.3 Reagents

3.3.1 Micronucleus Assay

Culture medium RPMI 1640 + GlutaMAX + HEPES, Fetal Bovine Serum, Penicillin/Streptomycin mix (Pen/Strep; with 10000 units/mL of penicillin and 10000 µg/mL of streptomycin), Phytohaemagglutinin, Phosphate-Buffered Saline and Gurr's phosphate buffer (pH 6.8) were purchased from Thermo Fisher Scientific (United Kingdom); Mitomycin C and Cytochalasin-B were purchased from Sigma-Aldrich (United Kingdom); Methanol, KCl, Giemsa Stock Solution and Entellan Mounting Medium were purchased from Merck (Germany) and Acetic Acid was purchased from VWR (France).

3.3.2 Comet Assay

DMSO, Tris-HCl, Trizma-base, Triton X-100, Ethyl Methanesulfonate, Ethidium Bromide and Low Melting Point agarose were purchased from Sigma-Aldrich (United Kingdom); NaCl, NaOH and HCl were purchased from Merck (Germany); $Na_2EDTA.2H_2O$ was purchased from Calbiochem (Germany); Normal Melting Point agarose was purchased from GE Healthcare Life Sciences (Sweden) and Phosphate-Buffered Saline was purchased from Gibco (United Kingdom).

3.4 Cytokinesis-Block Micronucleus Assay

To carry out this procedure 600 µL of whole blood was added to 4.3 mL of culture medium RPMI 1640 + GlutaMAX + 25 mM HEPES supplemented with 15% fetal bovine serum, 1,5% Penicillin/Streptomycin mix (Pen/Strep; with 10000 units/mL of penicillin and 10000 µg/mL of streptomycin) and was also added phytohaemagglutinin a mitogen, which predominantly stimulates the division of T-lymphocytes. Cultures were maintained at 37ºC, placed in ≈ 30º angle inclination, for 43 hours (Fenech, 2007, 2000).

Mitomycin C (MMC) was chosen as the positive control since it produces chromosome breaks (Fenech, 2007; OECD, 2016). MMC was added to 43 hours cultures at a final concentration of 0.1 µg/µL (diluted in PBS) and incubated for 1 hour before Cyt-B addition. Cyt-B is an inhibitor of actin polymerisation required for the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis, so it is essential for individualize the two daughter cells in cytokinesis phase and thus its effect results in a binucleated cell following the mitosis (Fenech, 2007). Cyt-B at a final concentration of 5 µg/mL was added to each sample at 44 hours post PHA stimulation, in order to prevent cytokinesis.

The lymphocytes were harvested 24 hours after adding Cyt-B, they were centrifuged for 5 minutes at 1200 rpm, the supernatant was discarded and the pellet was resuspended. Cells were submitted to a hypotonic treatment by addition of 5 mL of pre-warmed KCl (0.1M) solution to each 15 mL tube, drop-by-drop, while vortexing. These tubes were immediately centrifuged at 1200 rpm during 5 minutes. The supernatant was discarded and the pellet was resuspended. The cells were fixed two times using a 5 mL previously cold fix solution (3:1 methanol: acetic acid) drop-by-drop, while vortexing. Lastly the cells were fixed using a 5 mL previously cold fix solution (97:3 methanol: acetic acid) drop-by-drop, while vortexing, prior to transfer to slides and air-dried.

The staining was executed with a 4% Giemsa solution (Giemsa stock solution was filtered with a Millipore paper and diluted in Gurr's phosphate buffer). Slides were mounted with 2 drops of Entellan and a coverslip (Fenech, 2007, 2000). Slides were coded before analysis so that the scorer was not aware of the identity of the slide. Microscopic analysis of the slides was performed using an optical microscope (Axioskop 2 Plus, Zeiss, Germany) with a final magnification of 100x (Fenech, 2007).

The criteria for cell selection and scoring in the CBMN assay has been described and well characterized by Fenech, (2000) and taking this into account it will not be described in detail in this thesis. However, there are some important issues to consider: for each sample 2000 binucleate cells (2 independent cultures per sample, 1000 cells per culture) should be analysed for each culture; two different scores need to be made, 1000 cells for percentage of mononuclear, binucleate and multinucleate cells and a total of 2000 binucleated cells for the frequency of MN, NBUD and NPB. Cells should have a well-limited cytoplasm and normal nucleus morphology. The MN should only be considered if had until 1/3rd diameter and the same colour intensity as the main nuclei (Fenech, 2007, 2000).

Based on mono, bi and multinucleated index, it is possible to quantify the cell proliferation capacity or cytotoxic effects caused, the Cytokinesis-Block Proliferation Index (CBPI) or the Replication Index (RI) are recommended to estimate the cytotoxic and cytostatic activity of a sample by comparing

values in the sample and control cultures. The Cytokinesis-Block Proliferation Index (CBPI) and Replication Index (RI) were calculated as presented in equations below (OECD, 2016):

$$\boldsymbol{CBPI} = \frac{(Mononucleate\ cells) + (2x\ Binucleate\ cells) + (3x\ Multinucleate\ cells)}{Total\ number\ of\ cells}$$

$$\boldsymbol{RI} = \frac{\left(\frac{(Binucleate\ cells) + (2x\ Multinucleate\ cells)}{Total\ number\ cells}\right) Treated}{\left(\frac{(Binucleate\ cells) + (2x\ Multinucleate\ cells)}{Total\ number\ of\ cells}\right) Control} \times 100\%$$

3.5 Alkaline Comet Assay

A previously warmed (38ºC) 0.7% low melting point (LMP) agarose was added to 20 µL of blood sample, homogenised and placed on a pre-coated microscope slide with 1% normal melting point (NMP) agarose. Coverslips (20x20) were immediately added to each drop and placed on a cold box to assure agarose solidification in order to form a thin gel. Ethyl Methanesulfonate (EMS) was selected as the positive control, to induce DNA damage. The EMS was diluted in PBS at a final concentration of 30 mM. Then the cells were exposed during 30 minutes at 37ºC. At the end of exposure, the cells were centrifuged for 2 minutes, 1400 rpm, 4ºC. Supernatant was discarded and the pellet was gently resuspended, and washed twice with PBS. Then, 20 µL were embedded in LMP agarose and placed on microscope slides previously coated with 1% NMP agarose (Collins, 2013).

After 10 min, coverslips were carefully removed and slides transferred to a copplin jar, protected from light and with fresh cold lysis solution consisting of high salts concentration and detergents [89% NaCl (2.5 M), Na2EDTA.2H2O (100 mM), Tris-HCl (10 mM), NaOH (10 M); 1% Triton X-100; 10% DMSO]. Incubation proceeded for 1-14 hours, at 4ºC. This lysis step allows the removal of the cellular membranes, allowing soluble cell and nuclear components to diffuse away, and detach histones from the DNA. The residual structures, containing highly condensed DNA, still resemble nuclei but are now known as nucleoids (Collins, 2013; Collins and Azqueta, 2012a).

After lysis, the slides placed in an electrophoretic cell surrounded by ice. Slides were covered with electrophoresis buffer [NaOH (300 mM); Na2EDTA.2H2O (1 mM); pH>13] during 20 minutes, to allow unwinding and to produce single stranded DNA and to express ALS as SSB (Collins, 2013; Collins and Azqueta, 2012a). After this time, amperage and voltage were defined at 300 mA and 25 V, respectively. Electrophoresis was run during 20 min, at 4ºC, since the use of a lower temperature is thought to provide increased reproducibility. During electrophoresis, DNA, being negatively charged, attracted to the anode, but it only moves appreciably if it contains breaks (Collins and Azqueta, 2012a).

The DNA is supercoiled because it was wound around the histone cores of nucleosomes. Although the histones are no longer present, the supercoiling remains because the DNA loops are constrained by their matrix attachment. A strand break relaxes supercoiling, and so broken loops are

able to extend towards the anode, and it is these loops that form the comet tail. The relative size of the tail (most conveniently measured as the percentage of total fluorescence in the tail) reflects the number of DNA loops and therefore the frequency of DNA breaks (Collins, 2013). After electrophoresis, the slides were washed three times with neutralization buffer [Trizma-base 0.4M in deionized water, with 9.5% vol HCl 4M; pH=7.5] for 5 minutes at 4ºC.The slides were kept in a box, protected from the light, to dry at room temperature until analysis.

Microgel staining was performed with Ethidium Bromide (125 µg/mL). Slides were covered with coverslips and placed in a humid box protected from light and let incubate for 1 hour, 4ºC. The analysis of the slides was done under a fluorescence microscope (Leica DM 2500), with the assistance of specific image-analysis software (Comet Assay IV, from Perceptive Instruments). In each slide 50 nucleoids were randomly analysed per microgel, which results in 100 nucleoids per sample (Collins, 2013; Collins and Azqueta, 2012a). An important aspect to take into account is the comet scoring is that only cells with a clear head and tail should be scored. According to Tice et al., 2000 and Azqueta and Collins, 2013, the comets near the edges of the gel should not be scored (Azqueta and Collins, 2013; Tice et al., 2000).

3.6 Statistical analysis

The general processing of the data was performed in Microsoft Office Excel. The statistical analysis of the results was performed with IBM SPSS Statistics 25. In the cytokinesis-block micronucleus assay a Chi-square, Fisher's exact test was applied, and the *p*-value was considered significant when less than 0.05. For the alkaline comet assay the distribution of the data was checked for normality using the Kolmogorov Smirnov test, and the homogeneity of the variance was verified by the Levene test. ANOVA and Student's t-test were used when the results assumed a normal distribution; on the other hand, when the results don't follow a normal distribution, the non-parametric tests were applied, such as Kruskal-Wallis (more than two variables) and Mann-Whitney (two variables), and the *p*-value was considered significant when lower than 0.05.

4. RESULTS

4.1 Population characteristics

During the chromate occupational study, micronucleus analysis in PBL was carried out for 78 individuals from Belgium, 76 from Portugal, 71 from Poland, 44 from Finland and 30 from Netherlands, making a total of 299 samples. For the comet assay 76 samples from Portugal, 39 from Belgium, 28 from Finland and 8 from Poland were analysed, making a total of 151 samples. In the present work only the results of the 76 Portuguese samples were analysed and presented.

A total of 50 healthy workers exposed in a daily basis to Cr(VI) were included in the exposed group. All of them are employees from one Portuguese aircraft maintenance company. The remaining 26 participants were selected from the general population and were not occupationally exposed to Chromium compounds or any known physical or chemical agent of concern in the workplace. Interested participants receive an information leaflet (Annex 2), explaining the aims and objectives of the study. All participants accepted to participate by signing an informed consent previously to the blood withdrawal. Also, the participants had to fill a questionnaire with several information (Annex 1). Detailed personal information and lifestyle characteristics of participants are presented in Table 4.1.

Table 4.1 – The general information and lifestyle factors of the study population.

	Exposed	**Non-exposed**
Population, n (%)	50 (65.8)	26 (34.2)
Gender, n (%)		
Female	5 (10)	3 (11.5)
Male	45 (90)	23 (88.5)
Age (years, mean ± SD)	45.9 ± 10.2	41.7 ± 7.9
Age (min - max)	27 - 64	29 - 57
Smoking habits, n (%)		
Smokers	19 (38)	6 (23.1)
Non-smokers	31 (62)	20 (76.9)
Occupation, n (%)		
Electroplating	5 (6.5)	-
Welding	4 (5.2)	-
Painting exterior surfaces	26 (33.8)	-
Painting interior surfaces	5 (6.5)	-
Quality control	5 (6.5)	-
Machining	5 (6.5)	-
Occupational exposure, mean years (min - max)	18.8 (1 - 43)	-
≤ 10 years, n (%)	19 (38)	-
11 - 20 years, n (%)	9 (18)	-
≥ 21 years, n (%)	22 (44)	-

For this study, the participants have to fulfill some inclusion criteria (Table 3.1). This way, the participants were divided into groups according to their age, smoking habits, job function, and years of

occupational exposure, based on the questionnaire that they filled out. The occupationally exposed group (n=50) consisted of 45 males (90%) and 5 females (10%), aged 27–64 years (mean age, 45.9 ± 10.2). The volunteers in the control group (n=26) consisted of 23 males (88.5%) and 3 females (11.5%), with ages between 29 and 57 years (mean age, 41.7 ± 7.9). In terms of statistical analysis of this work, the gender was not considered because there are few women, so there would be no statistical power. The exposed workers and the control group were divided according to smoking habits. Of the 50 occupationally exposed worker group, 31 were non-smokers and 19 were smokers and of the 26 in the control group, only 6 were smokers, and the remaining 20 were non-smokers.

The occupationally exposed workers were categorised into six activities , according to the main task they performed. A group of five workers performed electroplating or less commonly Chromium plating in bath. Electroplating is a technique by which a thin layer of chromium is deposited onto a metal object. The chromed layer can be decorative, provide corrosion resistance or increase surface hardness. During this process, workers put and remove the metal pieces into the baths and they also prepare the baths adding chromium when needed. In the welding group there are four workers. Welding is a term used to describe a wide range of processes for joining any materials by fusion or coalescence of the interface. A major source of workers' exposure to Cr(VI) occurs during "hot work" such as welding on stainless steel and other alloy steels containing Chromium metal. The only soluble species of Chromium in welding fumes is Cr(VI). The painters were divided into two groups: twenty six are exterior surface painters and five are interior surface painters. Exposure to Cr(VI) can occur during the spray painting of aircraft exteriors, interiors or parts, and during the removal of chromate-based coatings, by sanding and abrasive blasting. The difference between exterior surface painters and interior surface painters is that the paints used in exterior of the aircrafts have a higher content of Chromium. In quality control there is a group of five workers. These workers remove samples from plating baths to guarantee that the content of chromium is correct and they evaluate the quality of the mixtures (e.g. Alodine) that have Chromium and are use in all the company sectors. In machining there was also a group of five workers who performed various processes in which a piece of raw material is cut into a desired final shape and size. Exposure to Chromium happens when workers perform these process to pieces that had been treated with Chromium plating.

The duration of occupational exposure ranged from 1 to 43 years. The workers were categorized into three time ranges, nineteen worked for less than 10 years, nine worked between 11 and 20 years, twenty two others worked for at least 21 years.

4.2 Micronucleus assay

The cytokinesis-block micronucleus assay is accurate to identify permanent damages in DNA , that results from DNA breakage or loss and remains in daughter cells after division. For each sample, 2000 binucleated cells were scored and analysed by two independent readers, which gives a total of 152000 binucleated cells. Examples of micronucleated cells scored during micronucleus analysis are shown in Figure 4.1.

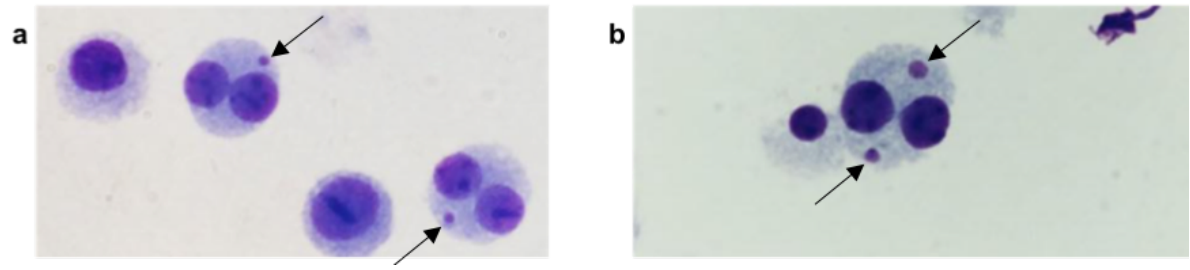

Figure 4.1 - Pictures of chromosomal damage in micronucleated cells (black arrows). Image a shows two binucleated cells with one micronucleus each, image b shows a binucleated cell with two micronucleus (ampliation x400).

Table 4.2 shows the frequency of micronucleated binucleated cells (MNBN) per 1000 binucleated cells (BN). The exposed workers presented a significantly higher frequency of micronucleated cells compared with the controls (6.60 ± 2.49 versus 4.08 ± 1.77; $p < 0.001$) (Annex 3). *p* values of CBMN experiments were calculated by Fisher exact test.

Table 4.2 - Frequency of binucleated cells with one or more micronuclei per 1000 binucleated cells.

	MNBN/1000 BN (mean ± SD)				
	n	Exposed	n	Non-exposed	*p* value
	50	6.60 ± 2.49	26	4.08 ± 1.77	< 0.001
Age (years)					
≤ 46	25	6.11 ± 2.65	18	3.97 ± 1.81	0.004
≥ 47	25	7.30 ± 2.59	8	4.17 ± 1.33	0.009
Smoking habits					
Smokers	19	5.78 ± 1.51	6	2.80 ± 1.29	0.01
Non-smokers	31	7.17 ± 2.88	20	4.21 ± 1.85	< 0.001
Occupation					
Electroplating	5	6.00 ± 1.80		-	
Welding	4	6.50 ± 2.86		-	
Painting exterior surfaces	26	6.46 ± 2.52		-	
Painting interior surfaces	5	6.40 ± 2.21		-	
Quality control	5	6.90 ± 2.62		-	
Machining	5	7.90 ± 2.85		-	
Years of occupational exposure					
≤ 10 years	19	5.56 ± 2.57		-	
11 - 20 years	9	5.89 ± 2.91		-	
≥ 21 years	22	7.64 ± 2.53		-	

In the exposed workers there was a statistically significant increase in the frequency of MNBN for ages below 46 years when compared to the control group ($p = 0.004$). The same was observed for

participants with more than 47 years (p = 0.009). An age-dependent increase in MNBN frequency was not observed in exposed workers (6.11 ± 2.65 versus 7.30 ± 2.59; p = 0.13) and age does not have either a statistically significant influence on the frequency of MNBN in the control group (3.97 ± 1.81 versus 4.17 ± 1.33; p = 0.91).

Regarding the smoking habits, the exposed workers who are smokers, showed a statistically significant increase in the frequency of MNBN when compared to smokers from the control group (p = 0.01). The same was observed for participants who are non-smokers (p < 0.001). In the exposed group, no significant difference was observed between smokers and non-smokers (p = 0.07). Among the control group it was possible to see that the frequency of MNBN was higher in non-smokers, but there wasn't statistical significance between the two sub-groups (p = 0.14).

Taking into account the occupation of the exposed workers, the frequency of MNBN ranged between (6.00 ± 1.80) in electroplating and (7.90 ± 2.85) in machining. Stratified analysis on job categories showed that no significant differences in MNBN frequencies among tasks (p > 0.05). For example, there wasn't a statistically significant difference between workers who performed welding or machining, or workers who performed quality control or airplane interior painting (p > 0.05). The same occurred in the control group (p > 0.05).

As for the duration of exposure, the frequency of MNBN does not have a statistically significant influence (p > 0.05) among workers with less than 10 years of exposure (5.56 ± 2.57), those working between 11-20 years (5.89 ± 2.91) and those working for more than 20 years (7.64 ± 2.53). There were also no statistically significant differences between ≤ 10 and 11-20 years of exposure, or between 11 - 20 and ≥ 21 years of exposure (p = 0.73 and p = 0.10, respectively). However, there was a statistically significant increase in the frequency of MNBN between workers who performed their activities less than 10 years and those who were exposed more than 21 years (p = 0.02).

MMC was chosen as a positive control, displaying significant difference in the frequency of MNBN comparatively to all performed assays in the exposed group without MMC (18.83 ± 4.42 versus 6.60 ± 2.49) and comparatively to non-exposed group without MMC (15.67 ± 3.25 versus 4.08 ± 1.77), showing a genotoxic effect (Annex 3).

In terms of statistical analysis of this work, other chromosomal abnormalities such as NBP and NBUD were not considered, since the number of NBP and NBUD found in our population was very low, so there would be no statistical power. The total number of NBP found in exposed group was 7, in non-exposed group was 0 and in all assays performed with MMC was 2. The total number of NBUD found in exposed group was 4, in non-exposed group was 0 and in all assays performed with MMC was 22.

The CBMN assay can also provide information about cytotoxic effects by the analysis of proliferation index. By the CBPI analysis (mean ± SD), alterations on the cell cycle progression were not verified between exposed and non-exposed groups (1.96 ± 0.10 versus 1.84 ± 0.15). A difference was seen in the CBPI values when comparing the values for MMC-treated samples with the mean values of the exposed workers (1.74 ± 0.11 versus 1.96 ± 0.01) and comparing MMC with non-exposed group (1.55 ± 0.09 versus 1.84 ± 0.15).

4.3 Comet assay

For each sample 100 cells were randomly chosen, representative images are shown in Figure 4.2. Round nucleoids are seen when no damage occurs on DNA strands. The increase in the length and intensity of fluorescence of the comet's tail is proportional to the levels of damage to DNA. During this work, most of the analysed nucleoids were similar to the first picture, without or with a very low level of DNA damage. Comets with longer tails, as exemplified in the last picture, are typical of positive control (in this work it was used EMS).

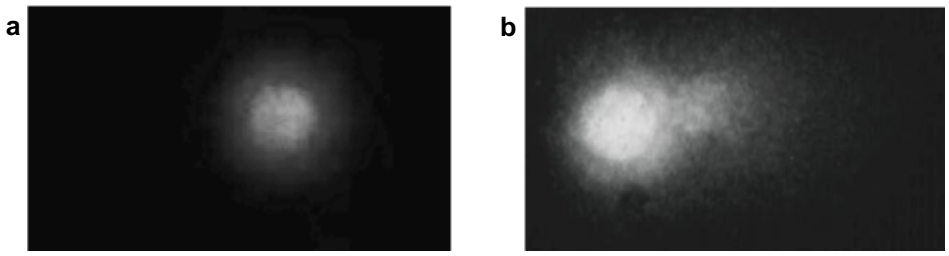

Figure 4.2 - Examples of DNA damage that can be found during Comet Assay analysis. Image a show a cells without DNA damage and image b show a cells with DNA damage caused by EMS (ampliation x100).

As mentioned above, in order to reproduce the conditions of the assays performed with samples from other countries, all procedures performed with the Portuguese samples were started on Monday of the following week (approximately 96 hours after blood collection). This way, a small optimization experiment, with 7 blood samples, was done to ensure that the results of comet assay were not under or overvalued. So this assay was performed on the day of blood collection and 96 hours after the collection. The results are given in Table 4.3.

Table 4.3 - Percentage of DNA in tail, on day of blood collection, and 96 hours after collection.

	% of tail intensity (mean ± SD)	
	On day of blood collection	**96 hours after the blood collection**
Sample 1	4.09 ± 5.59	3.59 ± 7.76
Sample 2	2.91 ± 4.57	2.53 ± 4.08
Sample 3	4.09 ± 6.27	3.59 ± 10.30
Sample 4	5.27 ± 7.68	5.61 ± 6.24
Sample 5	4.33 ± 10.35	4.70 ± 4.82
Sample 6	4.73 ± 5.22	3.83 ± 4.25
Sample 7	3.38 ± 10.29	3.15 ± 4.34

The mean and standard deviation of the tail intensity on the day of blood collection and 96 hours after blood collection are 4.18 ± 0.81 and 4.26 ± 1.39 respectively. The mean values are similar, so all assays were performed 96 hours after blood collection.

The results of comet assay in 76 participants, expressed in terms of the percentage of DNA in the comet tail, are given in Table 4.4. The percentage of DNA in tail is directly related to the DNA breakage frequency. After statistical analysis, it is possible to conclude that in exposed workers there was a significant increase in mean values of the percentage DNA in tail ($p < 0.001$) when compared with controls (5.68 ± 1.77 versus 2.73 ± 1.73) (Annex 4).

Table 4.4 - DNA damage among workers and control group is expressed as the mean of the percentage of DNA in tail.

	% of tail intensity (mean ± SD)				
	n	**Exposed**	**n**	**Non-exposed**	***p* value**
	50	5.68 ± 1.77	26	2.73 ± 1.73	< 0.001
Age (years)					
≤ 46	25	5.00 ± 1.78	18	3.04 ± 1.71	< 0.001
≥ 47	25	6.36 ± 1.75	8	2.02 ± 1.18	< 0.001
Smoking habits					
Smokers	19	5.29 ± 1.32	6	2.55 ± 1.44	< 0.001
Non-smokers	31	5.92 ± 2.00	20	2.78 ± 1.88	< 0.001
Occupation					
Electroplating	5	6.38 ± 1.25		-	
Welding	4	8.28 ± 2.20		-	
Painting exterior surfaces	26	5.55 ± 1.83		-	
Painting interior surfaces	5	4.90 ± 1.26		-	
Quality control	5	4.95 ± 1.89		-	
Machining	5	5.12 ± 1.96		-	
Years of occupational exposure					
≤ 10 years	19	4.66 ± 1.80		-	
11 - 20 years	9	6.39 ± 2.19		-	
≥ 21 years	22	6.28 ± 1.77		-	

Considering the age of the participants, there was a statistically significant increase of percentage DNA in tail, between exposed workers with less than 46 years when compared to the control group matched for age (5.00 ± 1.78 versus 3.04 ± 1.71; $p < 0.001$). The same was observed for participants older than 47 years (6.36 ± 1.75 versus 2.02 ± 1.18; $p < 0.001$). The age of the exposed group have a statistically significant influence on the percentage DNA in tail, the subgroup under 46 years has less DNA damage (5.00 ± 1.78 versus 6.36 ± 1.75; $p < 0.05$). While an age-dependent increase in percentage DNA in tail was not observed in the control group (3.04 ± 1.71 versus 2.02 ± 1.18; $p > 0.05$).

Among the smokers, a statistically significant difference in percentage DNA in tail was observed between the exposed group and non-exposed group (5.29 ± 1.32 versus 2.55 ± 1.44; $p < 0.001$). The same was observed for participants who are non-smokers (5.92 ± 2.00 versus 2.78 ± 1.88; $p < 0.001$).

In the exposed group, a significant difference was not observed between smokers and non-smokers ($p > 0.05$). Similarly, there was not a significant effect of tobacco in the control group ($p > 0.05$).

Regarding the occupation of the exposed group, the percentage DNA in tail ranged between (4.90 ± 1.26) in airplane interior painting and (8.28 ± 2.20) in welding. The tasks performed by this group does not have a statistically significant influence on the percentage tail intensity ($p > 0.05$). The same occurred in the control group ($p > 0.05$).

DNA damage was further found to be significantly higher in participants with more years of exposure (4.66 ± 1.80 versus 6.39 ± 2.19 versus 6.28 ± 1.77; $p = 0.008$). There were also statistically significant differences between ≤ 10 and 11-20 years of exposure, and between ≤ 10 and ≥ 21 years of exposure ($p = 0.02$ and $p = 0.001$, respectively).

As mentioned above, EMS was used as positive control, presenting significant increases of DNA breaks comparatively to the all performed assays without EMS (18.42 ± 2.29 versus 4.67 ± 2.24) (Annex 4).

5. DISCUSSION

Health concerns in the workplace among occupationally exposed groups are important issues all over the world. Hexavalent Chromium is an occupational carcinogen that belongs to group 1 in IARC classification and has been shown to cause lung cancer in humans (Deng et al., 2019; Cole and Rodu, 2005; Luippold, 2003; Gibb et al., 2000). Occupational exposure to carcinogenic isoforms of Chromium occurs among several professional activities, such as welding (Aksu et al., 2018), electroplating (Wultsch et al., 2017; Goldoni et al., 2010) and other surface treatment activities such as paint application and removal of old paint containing Cr(VI) (NIOSH, 2013).

This work aims to present data on effect biomarkers obtained in one aircraft maintenance company in Portugal, since the substitution of Cr(VI) in this sector is not expected in the near future. There are several HBM studies conducted in aircraft companies, which confirm the potential occupational risk of Cr(VI) exposure. As example, the case study reported by Gherardi et al., 2007, aimed to evaluate the occupational exposure level to Cr(VI) in 10 painters of an Italian aircraft industry, where chromate is added to primer as a corrosion inhibitor of aircrafts surfaces. Thus, the application of this primer by using electrostatic guns (the most used methodology), exposes painting and coating workers to high concentrations of aerosols containing Cr(VI). Gherardi and colleagues measured the exposure to Cr(VI) during the painting operations using total urinary Chromium and showed that painters had higher levels of Cr(VI) than in the control group (Gherardi et al., 2007). In another study, Genovese et al., 2015, assessed Cr(VI) in assembly workers. The task of "assembler" in an aircraft industry consists in the drilling of the modules forming the aircraft, followed by their assembly. This task does not imply the direct handling of chemicals or compounds containing Cr(VI), hence a low Cr(VI) exposure is expected. However, since in assembly areas of this industry, were found compounds containing strontium chromate, calcium chromate and chromium trioxide, urine samples were analysed by atomic absorption spectroscopy and the results showed statistically significant differences between urinary Chromium levels in workers and controls. Although this task does not involve the direct use of Cr(VI), the results show a slight occupational exposure to Cr(VI), which should be carefully considered. These two studies are examples that aircraft maintenance companies have detectable amounts of Cr(VI) in their workers, thus, justifying the continuously necessity of HBM studies in this type of industries. Regarding to workplace exposure assessment, different working conditions can imply higher exposures. In fact, results from HBM4EU project (data not shown) revealed that external surfaces painters have higher exposure to Cr(VI) - 0.4 mg/m^3. This value was higher than the OEL of 0.01 mg/m^3 currently proposed by the Directive (EU) 2019/130, 16/01/2019. Concerning the other occupations referred in our study, the exposure values were all lower than the OEL.

The work here presented is part of a bigger project, the chromate occupational study, that emerged from the necessity of harmonization of methodologies and collection of relevant data on Cr(VI) internal exposure and early biological effects in occupational settings. Thus, we investigated the genotoxic effects associated with occupational exposure to Cr(VI) using the CBMN and Comet assay (whole blood). Although these biomarkers of effect are valuable tools for biomonitoring studies, it should be taken into account that all humans respond differently to a specific xenobiotic or mixture of xenobiotics (Araldi et al., 2015). In fact, there is a large interindividual variation observed within human populations exposed to similar levels of diverse xenobiotics. Interindividual variation may be explained

by different lifestyle factors including environmental exposure or individual susceptibility factors. Age and gender in combination with lifestyle factors, such as smoking habits, are crucial and should also be taken into account when conducting biomonitoring studies (Gajski et al., 2018).

The micronucleus assay has been employed as a biomarker of genomic instability given that it reflects chromosome breakage or loss. Usually, this instability of the genetic material is measured in whole blood or isolated lymphocytes, but can also be monitored using exfoliated epithelial cells from nose and oral cavity. In fact, a few studies conducted with Indian Chromium platers (Sudha et al., 2011a), Bulgarian Chromium platers (Benova et al., 2002) and Indian welders (Danadevi et al., 2004) showed an increase in frequency of MN in exfoliated cells from exposed workers. On the contrary, a study with Mexican welders showed that MN frequencies and the rest of nuclear abnormalities did not differ between exposed and non-exposed participants (Jara-Ettinger et al., 2015). This findings are consistent with Wultsch et al., 2014, who studied the induction of chromosomal alterations in exfoliated cells of 22 Austrian welders. This study showed an increased frequency of MN in the buccal cells of the workers, but this effect failed to reach statistical significance.

Our results show that the MN frequency is significantly higher in the exposed group when compared to the control group, which is a clear evidence of genotoxicity. Thus, the greater prevalence of MN in the exposed group implies that they are more exposed to a carcinogen and therefore are at a greater risk of developing cancer. These results are in agreement with those presented by other authors. Sudha et al., 2010, studied the frequency of micronuclei in 93 welders from South India, and revealed a significant induction of the frequency of binucleated cells with MN when compared with 60 participants from the control group. A study with 40 Bulgarian Chromium platers, showed a significant increase in the number of micronucleated cells with respect to the values obtained from the control group (Vaglenov et al., 1999). Two other studies were carried out in tanneries, in Morocco (Hilali et al., 2008) and Portugal (Medeiros et al., 2003) and a significant increase in the MN frequency in human lymphocytes were observed in the exposed group compared with non-exposed. Also, a study from China (Xiaohua et al., 2012) showed that the MNBN frequency of the exposed group was statistically higher when compared with the control group. Xiaohua et al., 2012 also evaluated the correlation between chromate exposure and MNBN frequencies. The median of the Chromium concentrations in air of the exposed group was 9.04 $\mu g/m^3$, higher than that of the control group: 0.064 $\mu g/m^3$. A correlation between the concentration of Chromium in air and MNBN frequencies were found, showing that MNBN frequencies were positively correlated with occupational chromate exposure. Novotnik et al., 2016 used another *in vitro* approach and found a statistically significant increase in MN frequency when human hepatoma cell line (HepG2 cells) were exposed to Cr(VI). Curiously, the authors showed that Cr(VI) does not influence the frequency of nuclear buds or nucleoplasmic bridges, another type of nuclear abnormalities usually found in the CBMN. These results are in accordance with ours, since the number of nucleoplasmic bridges and nuclear buds found in our population was very low. For that reason, we did not consider this type of abnormality in our statistical analysis.

Age can be considered as a confounding factor. However, in our study, the age of exposed and control groups does not influence the frequency of MNBN. This may mean that the increase in MNBN frequency in the exposed group was not due to the age of participants, but due to exposure to Cr(VI).

Other authors observed similar results. Iarmarcovai et al., 2005, studied the occupational risk in 27 welders, and did not find a relationship between age and the frequency of MNBN. Also Aksu et al., 2018 did not find significant correlation between buccal MN frequency and age of 48 welders from Turkey. On the contrary, a few other authors showed that age might be influencing MN frequency. Briefly, studies from India (Sudha et al., 2010; Danadevi et al., 2004) and Turkey (Şener and Eroğlu, 2013) showed an age-dependent accumulation of genome damage in exposed workers.

Lifestyle factors, such as smoking habit, can also be considered as confounding factors in Cr(VI) exposure assessment and is known to affect the levels of chromosome damage. The significant difference between exposed and non-exposed groups is also observed when a stratification is made regarding the smoking status. However, there were no significant differences between smokers and non-smokers within each exposure group. This suggests that the frequency of MNBN is higher in the exposed group, owing to exposure to Cr(VI), and is not the smoking habits of the participants. Similar results were obtained by other researchers (Aksu et al., 2018; Iarmarcovai et al., 2005)

Regarding the occupation of the exposed group, the workers who paint the exterior surfaces of the aircraft are the group with the highest exposure to Cr(VI). As stated before, these painter were the only occupational group where the levels detected were higher than OEL. These values might be related with the fact that paints used outside the aircraft have a higher content of this compound. Since, the number of participants in each occupational group is too small, we cannot discuss the effect of these particular exposures on MNBN frequencies. In fact, there was no statistically significant differences between the tasks that different workers performed.

In this work a significant correlation was observed between MNBN induction and duration of exposure, considering workers who perform their activities less than 10 years and those who are exposed more than 21 years. These results provide evidence that higher periods of exposure to Cr(VI) compounds may result in induction of chromosome instability. Other two studies were carried out in India (Danadevi et al., 2004; Sudha et al., 2010), and both revealed a significant induction of MN in welders with more years of exposure. These studies are in line with our results, thus the years of exposure to the Cr(VI) is a factor that should be considered.

Our data show an increase of MNBN frequency in exposed group, revealing that Cr(VI) can induce genotoxicity in exposed workers. Thus, our results are in agreement with studies mentioned above, showing that population exposed to Cr(VI) exhibit chromosomal instability.

Another biomarker used during this work was the Comet Assay using whole blood. Our results, demonstrated that Cr(VI)-exposed workers display an increased level of DNA breaks in blood white cells when compared with the non-exposed group. The results suggest that comet assay is valid for monitoring the effects of genotoxic compounds like Cr(VI) in occupationally exposed groups. These data also suggest a potential health risk for this group of workers. In line with these findings, other authors observed the same results. A study with 30 French welders, showed increased levels of DNA damage in lymphocytes of exposed group when compared with 22 controls (Botta et al., 2006). The percentage of DNA in tail in lymphocytes of tannery workers from India was also analysed (Balachandar et al., 2010), and it was observed a significant increase in the DNA breaks of exposed workers relative to people living in the surrounding area and control group. Other biomarker of genotoxicity namely,

chromosome aberrations was used in this study, and in concordance with results obtained in the comet assay, the number of chromosomal aberrations in tannery workers was significantly higher than in control group. All these studies that used the comet assay in lymphocytes or leukocytes have shown that population exposed to Cr(VI) exhibit a higher level DNA strand breaks.

In our results, a relationship was found between the level of DNA in tail and age of exposed workers. These DNA breaks may indicate a decreased DNA repair capacity with the increasing age or may be related to a longer exposure period to chromate. Similar results were reported in a study with 157 Chinese electroplaters (Zhang et al., 2011) and in a study with 93 Indian welders (Sudha et al., 2010). Nevertheless, the majority of published studies have not detected an age-dependent increase in DNA damage (Faust, 2004). This was also observed during this study, since the age of the non-exposed group had no significant influence on the DNA breaks.

When a stratification was made regarding the smoking habits, a significant difference between exposed and non-exposed groups was observed. However, the smoking status within each exposure group are not statistically influencing the DNA breaks. According to Faust, 2004 only nine human biomonitoring studies with alkaline comet assay have found a significant relationship between DNA damage and smoking habits. In this work smoking did not have a significant effect on DNA damage, indicating that DNA damage may be due to possible exposure to Cr(VI). Several studies conducted with 52 Indian (Sardas et al., 2010) and 48 Turkish (Aksu et al., 2018) welders, showed that smoking habits of the exposed group had not a statistically significant influence on the DNA breaks and the same was obtained in the control group.

The influence of duration of exposure was also analysed. DNA damage was significantly higher in participants with more years of exposure to Chromium compounds. These results are in line with studies conducted by Gambelunghe et al., 2003 with 19 Italian chrome-plating workers, by Danadevi et al., 2004, with 102 Indian welders and by Al-Shoeb et al., 2014, with 50 workers in a tannery from Bangladesh, revealed a significant induction of damage in DNA for workers with more years of exposure to Cr(VI).

Exfoliated epithelial cells from the nose and oral cavity can also be used for Comet assay. A study conducted by Sudha et al., 2011b showed that 66 Indian welders was a significant increase in DNA mean tail length when compared with non-exposed group. This study was important to show that other matrices can be used to perform comet assay. Exfoliated epithelial cells are easily collected and the technique is less invasive than blood withdraw. However, this technique is not standard procedure.

The results obtained in both biomarkers of effect were concordant. The present results suggest that both, the frequency of micronuclei and the level of DNA damage assessed by the comet assay in whole blood cells, are sensitive as early effect biomarkers in the case of occupational exposure to Cr(VI). These findings also suggest a potential health risk for this group of workers, given that an association between an increased micronucleus frequency and cancer risk has been shown.

6. CONCLUSION

The aim of this work was to evaluate the genotoxic effects of Cr(VI). Taking this into account, two relevant biomarkers of genotoxicity were used, the cytokinesis-block micronucleus assay and the alkaline comet assay, both in whole blood cells. The present results suggest that they were reliable early effect biomarkers in case of occupational exposure to Cr(VI). Moreover, the findings also suggest that occupational exposure to Cr(VI) may induce genotoxic effects, indicating a potential health risk for workers. Workers in many occupational settings are exposed to genotoxic agents and may not be aware of it. Therefore, there is a strong need to educate those who work with potentially hazardous compounds about its adverse effects and highlight the importance of using protective measures. This way, is our belief that preventive measures to reduce the exposure of workers are needed, in order to avoid adverse health effects that might be caused by genetic damage. These results should also promote the investment in new risk management measures, such as the modification of working procedures, the adoption of adequate personal and collective protection equipment, as well as performing education courses aimed to increase risk perception and the effective application of the risk management measures already in place, such as adequate local exhaust ventilation and a more frequent and correct use of personal protective equipment. In addition, these results together with the results from other workers received from different occupational settings in several countries are expected to contribute to establish improved exposure limits to Cr(VI), if needed, thus impacting also in new regulation for occupational health.

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8. ANNEXES

ANNEX 1: Questionnaire filled by workers.

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

QUESTIONÁRIO PARA OS LOCAIS DE TRABALHO (auto-administrado)

Agradecemos que responda a este breve questionário sobre as atividades da sua empresa relacionadas com o crómio hexavalente e outros produtos químicos. Quando terminar, queira entregá-lo diretamente ao investigador.

Informações sobre a empresa e a saúde no trabalho

Nome e cargo do representante da empresa:

Nome da empresa/organização:

Nome do departamento:

Endereço do sítio Web:

País:

Setor industrial:

Código NACE Rev.2 (a preencher pelo investigador):

Descrição do local de trabalho (natureza da atividade, o que é fabricado, a forma como o trabalho é organizado):

Descreva as práticas gerais de formação, acções de monitorização da exposição e da saúde e segurança no trabalho relacionadas com a exposição a produtos químicos perigosos na sua empresa:

Nome e endereço do serviço de saúde no trabalho: .....

Pessoa de contacto e dados de contacto (endereço de correio eletrónico, número de telefone) do departamento responsável pela saúde e segurança no trabalho:

1

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Condições operacionais

(Selecione as operações aplicáveis: cromagem, pulverização/pintura ou soldadura.)

Operação	**É efetuada nas suas instalações? (Assinale, se aplicável)**	**Responda às perguntas nas secções**
Cromagem	☐	1 e 4
Pulverização/pintura	☐	2 e 4
Soldadura	☐	3 e 4

1. Cromagem por imersão

a. Quantidades de crómio hexavalente, em % (ou g/l), utilizadas na solução? (Queira assinalar a caixa correspondente/especificar)

☐ ≤ 5; ☐ > 5-10; ☐ > 10-50 % ou ..... gramas/litro (g/l)

b. Frequência das operações de cromagem com crómio hexavalente? (categorias: diária, dias/semana ou dias/mês)

c. O trabalho inclui igualmente a niquelagem? (queira assinalar com um círculo) Sim / Não / Não sei

d. Se for utilizado níquel, qual é a quantidade utilizada em % (g/l) na solução?

.....% ou ..... gramas/litro (g/l)

e. Frequência das operações de niquelagem? (categorias: diária, dias/semana ou dias/mês)

f. O trabalho inclui igualmente o revestimento com crómio trivalente? (queira assinalar com um círculo) Sim / Não / Não sei

g. Se for utilizado crómio trivalente, qual é a quantidade utilizada em % (g/l) na solução?

.....% ou ..... gramas/litro (g/l)

h. Frequência das operações de cromagem com crómio trivalente? (categorias: diária, dias/semana ou dias/mês)

i. O trabalho inclui igualmente o revestimento com cádmio? (queira assinalar com um círculo) Sim / Não / Não sei

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

j. Se for utilizado cádmio, qual é a quantidade utilizada em % (g/l) na solução?

.....% ou ..... gramas/litro (g/l)

k. Frequência das operações de cadmiagem? (categorias: diária, dias/semana ou dias/mês)

l. Dimensão das peças tratadas? (queira descrever)

m. Quantos empregados realizam estas atividades?

Medidas de gestão de riscos para a cromagem (queira assinalar com um círculo)

Confinamento da solução	Total	...Parcial	Inexistente
Ventilação por exaustão local (LEV) da solução	Sim	Não	
Utilização de dispersores de bolhas à superfície do líquido	Sim	Não	
Utilização de eliminadores de névoa	Sim	Não	

- Utilização de eliminadores de PFAS (substâncias perfluoroalquiladas)
  Sim (queira especificar) Não Não sei

- Utilização de outros eliminadores
  Sim (queira especificar) Não

2. Tratamento de superfícies por pulverização ou pintura

a. Quantidades de crómio hexavalente, em %, utilizadas na tinta? (queira assinalar a caixa correspondente)
☐ ≤ 0,01; ☐ > 0,01-0,1; ☐ > 0,1-0,5; ☐ > 0,5-1; ☐ > 1-5; ☐ > 5-10; ☐ > 10-15; ☐ > 15 %

b. Quantidade média de tinta utilizada mensalmente (litros ou galões)? ..... litros / ..... galões

c. Frequência da pulverização ou da pintura e de operações de maquinagem? (categorias: diária, semanal, mensal, outra)

d. Dimensão das peças pulverizadas ou pintadas? (queira descrever)

e. Quantos empregados realizam estas atividades?

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

3. Soldadura

a. Frequência das operações de soldadura? (categorias: diária, semanal, mensal, outra)

b. Dimensão das peças soldadas? (queira descrever)

c. Quantos empregados realizam estas atividades?

Qual o método de soldadura utilizado? (queira assinalar a caixa correspondente)	☐ MMA (manual por arco metálico) ☐ MAG (em atmosfera ativa) ☐ MIG (em atmosfera de gás inerte) ☐ TIG (em atmosfera inerte com elétrodo de tungsténio) ☐ SAW (soldadura por arco submerso) ☐ Plasma – gás de plasma ☐ Soldadura com fio fundente ☐ Outro (especificar) ..... ☐ .....

4. Medições anteriores

Foi algum dos seguintes tipos de medições efetuado junto dos seus trabalhadores no local?

Medições	**Assinale todas as caixas aplicáveis**	**Anos em que foram colhidas**
Amostras de ar	☐	
Medições de exposição cutânea	☐	
Amostras de sangue de trabalhadores	☐	
Amostras de urina de trabalhadores	☐	
Outra (especificar)	☐	
	☐	

Está disposto a autorizar o acesso dos investigares a esses resultados (de forma confidencial)? (queira assinalar com um círculo) Sim Não

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Em caso afirmativo, queira indicar a pessoa de contacto e os respetivos dados de contacto (endereço de correio eletrónico, número de telefone):

Obrigado por responder ao questionário!

Quando terminar, queira entregá-lo diretamente ao investigador.

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

QUESTIONÁRIO PÓS-TURNO PARA TRABALHADORES (entrevistados pelo investigador)

Informações gerais sobre o trabalhador

Identificação do trabalhador	
Amostra de urina	**Data da colheita:** **Hora:** **Código da amostra**
Amostra EBC	**Data da colheita:** **Hora:** **Código da amostra**
Amostra de sangue	**Data da colheita:** **Hora:** **Código da amostra**
Amostra de ar (pessoal)	**Data da colheita** **Código da amostra:**
Amostra de toalhete (pessoal)	**Data da colheita:** **Código das amostras**
Nome da empresa e nome do departamento	
Nome e função do trabalhador	
Sexo (queira assinalar com um círculo)	**Masculino** **Feminino**
Data de nascimento (dd/mm/aaaa)	

Separar esta folha do questionário

6

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Identificação do Trabalhador		
Altura (cm ou pés/polegadas)	..... **cm /** ..... **pés** ..... **polegadas**	
Peso atual (kg ou pedras/lb)	..... **kg / ..... St ..... lb**	
Profissão	**Descrição livre:**	**Código CITP 08**
O trabalho é feito predominantemente (queira assinalar com um círculo)	**No interior** **No exterior**	
Duração dos turnos (horas) **e das horas extraordinárias normalmente realizadas (horas)**		
Tipo de turnos (queira assinalar a caixa correspondente)	☐ **Diurno fixo** ☐ **Noturno fixo** ☐ **Rotativo diurno** ☐ **Rotativo diurno/noturno** ☐ **Outro** (queira especificar).....	
Endereço privado		
Localização da habitação e características conexas (queira assinalar com um círculo)	**Urbana** **Rural**	
Há unidades industriais, instalações de incineração ou aterros nas imediações da sua habitação? (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, quanto dista aproximadamente da sua habitação o que se encontra mais próximo (km)?**	
Queira descrever a densidade de tráfego rodoviário nas imediações da sua habitação (queira assinalar com um círculo)	**Rua tranquila (baixa densidade)** **Estrada residencial (média densidade)** **Estrada principal (alta densidade).**	

7

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

É fumador?(queira assinalar com um círculo)	**Sim Não Ex-fumador**
É fumador?(continuação)	**Aproximadamente, quantos cigarros fuma por dia?** **Há quantos anos é fumador?** **Se é ex-fumador, há quantos anos deixou de fumar?** **Aproximadamente, quantos cigarros fumava por dia?** **Durante quantos anos foi fumador?**
Identificação do Trabalhador	
Fuma cigarros eletrónicos? (queira assinalar com um círculo)	**Sim Não Ex-fumador de cigarros electrónicos**
Fuma cigarros eletrónicos? (continuação)	**Aproximadamente, quantos cigarros eletrónicos fuma por dia?** **Há quantos anos fuma cigarros eletrónicos?** **Se é ex-fumador de cigarros eletrónicos, há quantos anos deixou de fumar?** **Aproximadamente, quantos cigarros eletrónicos fumava por dia?** **Durante quantos anos fumou cigarros eletrónicos?**
Consome outros produtos do tabaco? (queira assinalar com um círculo)	**Sim Não Ex-consumidor** **Se consome ou consumiu, queira especificar.**
Outros produtos do tabaco (continuação)	**Aproximadamente, quantos produtos do tabaco consome por dia?** **Há quantos anos os consome?** **Se é ex-consumidor, há quantos anos deixou de os consumir?** **Aproximadamente, quantos produtos do tabaco consumia por dia?** **Durante quantos anos os consumiu?**

8

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Tem implantes que possam conter metais (ex. parafusos, placas, anca, articulações do joelho etc)? (queira assinalar com um círculo)	**Sim** **Não** **Não sabe** **Em caso afirmativo, há quanto tempo?** **Sabe qual é o tipo de implante?** (queira especificar)
Nos último 3 meses fez algum exame radiologico (raio X, ressonância magnética)? (queira assinalar com um círculo)	**Sim** **Não**
Identificação do Trabalhador	
Esteve, ou está, a ser tratado de algum cancro?(queira assinalar com um círculo)	**Sim** **Não**
Tem obturações dentárias? (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, sabe de que material são feitas?** (queira especificar)
Consome bebidas alcoólicas?	**Sim** **Não** **Com que frequência consome bebidas alcoólicas?** (queira assinalar com um círculo) **Diariamente semanalmente mensalmente**
Consumo de bebidas alcoólicas (continuação)	**Em média, quantos dias por mês consome pelo menos uma bebida alcoólica?** **Num dia normal em que consome álcool, quantos copos costuma beber?**
Que outras bebidas consome? (queira assinalar com um círculo)	**Café** **Chá** **Bebidas energéticas** **Em média, quantas vezes num dia normal?** **Café ……. Chá ……. Bebidas energéticas …….**
Hábitos alimentares (queira assinalar com um círculo o seu tipo de alimentação)	**Diversificada** **Vegetariano** **Vegan** **Outro** (queira especificar)

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Utiliza suplementos alimentares? (por exemplo, comprimidos dietéticos) (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, queira especificar:**
Atividades de lazer ou passatempos suscetíveis de ocasionar exposição adicional ao crómio (por exemplo, soldadura, pintura à pistola, trabalhos com metais) (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, queira especificar:**

Percurso profissional

Identificação do Trabalhador						
Profissão/designação do cargo	**O trabalho que executava incluía alguma das seguintes atividades? (assinale as opções que se aplicam)**				**Data de início (ano)**	**Data de termo (ano)**
	metalização	**pintura ou pulverização**	**soldadura**	**outros trabalhos com metais**		
	☐	☐	☐	☐		
	☐	☐	☐	☐		
	☐	☐	☐	☐		
	☐	☐	☐	☐		

Descrição das operações

Que operações realizou hoje?

Identificação do Trabalhador		
Operação	**(Assinale, se aplicável)**	**Responda às perguntas da secção**
Cromagem	☐	1
Pulverização/pintura	☐	2
Soldadura	☐	3

10

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

1. Descrição das operações de cromagem por imersão (indique o tipo de tarefas que executou hoje)

	Tarefa	**Duração da tarefa num turno de trabalho** (horas/ minutos)	**Frequência da tarefa** (x vezes por semana)	**Processo tipo** (manual ou automático)	**EPI* utilizado** (adicionar os números correspondentes)	**LEV** utilizado** (sim, não)
1	**Reajuste do eletrólito:** decantação e pesagem, mistura, reenchimento da solução					
2	**Relativamente à solução**: carregamento de gabaritos, pré-tratamento químico, aplicação por imersão breve ou imersão, lavagem e secagem, pós-tratamento químico, limpeza e descarga de gabaritos, limpeza do equipamento, manutenção regular do equipamento					
3	**Atividades de manutenção pouco frequentes**					
4	**Extração de amostras e respetiva transferência para o laboratório**					
5	**Análises laboratoriais**					
6	**Gestão de resíduos**					
7	**Outro** (queira especificar)					

*EPI (equipamento de proteção individual) utilizado:
1. Respirador motorizado ou a ar
2. Respirador de meia máscara ou máscara facial inteira reutilizável (sem respirador motorizado ou a ar)
3. Máscara descartável
4. Outros equipamentos de proteção respiratória (queira especificar)
5. Fato-macaco
6. Luvas reutilizáveis
7. Luvas descartáveis
8. Outro (queira especificar)

** **LEV = ventilação por exaustão local

O seu equipamento de proteção respiratória (máscara) foi testado? (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, quando?**

11

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

Recebeu informações, instruções ou formação sobre a utilização de práticas de trabalho seguras no exercício desta atividade? (queira assinalar com um círculo)	**Sim** **Não**
Instalações de higiene na empresa (queira assinalar, se aplicável)	☐ **Possibilidade de lavar as mãos** ☐ **Possibilidade de tomar duche** ☐ **Local separado para o vestuário de trabalho** ☐ **Local específico para a arrumação do equipamento de proteção respiratória** ☐ **Outro** (queira especificar)..... ☐ .....
As condições de trabalho foram normais durante o dia de trabalho? (queira assinalar com um círculo)	**Sim** **Não** **Em caso negativo, queira especificar (por exemplo, problemas com a máscara ou não funcionamento da extração):**

2. Descrição das operações de tratamento de superfícies com pulverização ou pintura (indique o tipo de tarefas que executou hoje)

	Tarefa	**Duração da tarefa num turno de trabalho** (horas/ minutos)	**Frequência da tarefa** (x vezes por semana)	**EPI* utilizado** (adicionar os números correspondentes)	**LEV** utilizado** (sim, não)
1	**Tarefas de preparação:** Decantação, mistura de tintas, reenchimento de aparelhos				
2	**Pulverização em cabina/zona de pulverização**				
3	**Pulverização fora da zona de pulverização**				
4	**Tratamento de superfícies em túnel de pulverização automática**				
5	**Tratamento de superfícies por laminagem (superfícies de pequena a média dimensão)**				

12

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

6	**Tratamento de superfície com pincel ou caneta (pequenas superfícies/retoques)**				
7	**Secagem/autossecagem (atividades dos trabalhadores no exterior, a um metro de distância do elemento que está a secar), sem LEV**				
8	**Limpeza e manutenção do equipamento**				
9	**Atividades de manutenção pouco frequentes**				
10	**Operações de maquinagem (retificação) em peças que contenham crómio**				
11	**Operações de maquinagem (retificação) em peças cobertas com tinta que contenha crómio**				
12	**Gestão de resíduos**				
13	**Outro** (queira especificar)				

*EPI (equipamento de proteção individual) utilizado:
1. Respirador motorizado ou a ar
2. Respirador de meia máscara ou máscara facial inteira reutilizável (sem respirador motorizado ou a ar)
3. Máscara descartável
4. Outros equipamentos de proteção respiratória (queira especificar)
5. Fato-macaco
6. Luvas reutilizáveis
7. Luvas descartáveis
8. Outra (especificar)
** **LEV = ventilação por exaustão local

O seu equipamento de proteção respiratória (máscara) foi testado? (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, quando?**
Recebeu informações, instruções ou formação sobre a utilização de práticas de trabalho seguras no exercício desta atividade? (queira assinalar com um círculo)	**Sim** **Não**
Instalações de higiene na empresa (queira assinalar, se aplicável)	☐ **Possibilidade de lavar as mãos** ☐ **Possibilidade de tomar duche** ☐ **Local separado para o vestuário de trabalho** ☐ **Local específico para a arrumação do equipamento de proteção respiratória** ☐ **Outro** (queira

13

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

	especificar)..... ☐ .....
As condições de trabalho foram normais durante o dia de trabalho? (queira assinalar com um círculo)	**Sim** **Não** **Em caso negativo, queira especificar (por exemplo, problemas com a máscara ou não funcionamento da extração):**

3. Descrição das operações de soldadura (indique o tipo de tarefas que executou hoje)

	Tarefa	**Duração da tarefa num turno de trabalho** (horas/ minutos)	**Frequência da tarefa** (x vezes por semana)	**EPI* utilizado** (adicione os números correspondentes)*	**LEV** utilizada** 1. Extração fixa 2. Capuz de soldadura móvel 3. Bancada de trabalho isolada 4. Cabina de soldadura isolada 5. Ventilação geral 6. Outra (queira especificar)
1	**Soldadura manual**				
2	**Soldadura descontínua manual**				
3	**Soldadura robotizada**				
4	**Outras tarefas manuais**: Limpeza, retificação, corte, etc.				
5	**Limpeza e manutenção do equipamento**				
6	**Gestão de resíduos**				
7	**Outro** (queira especificar)				

*EPI (equipamento de proteção individual) utilizado:
1. Capacete de soldadura com respirador motorizado ou a ar
2. Capacete de soldadura com respirador de poeira de meia máscara reutilizável
3. Capacete de soldadura com respirador de partículas descartável
4. Capacete de soldadura sem respirador
5. Capacete de soldadura com outros equipamentos de proteção respiratória (queira especificar)
6. Vestuário ignífugo
7. Luvas de soldadura
8. Outras luvas
9. Outro (especificar)

** **LEV = ventilação por exaustão local

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

O seu equipamento de proteção respiratória (máscara) foi testado? (queira assinalar com um círculo)	**Sim** **Não** **Em caso afirmativo, quando?**
Recebeu informações, instruções ou formação sobre a utilização de práticas de trabalho seguras no exercício desta atividade? (queira assinalar com um círculo)	**Sim** **Não**
Instalações de higiene na empresa (queira assinalar, se aplicável)	☐ **Possibilidade de lavar as mãos** ☐ **Possibilidade de tomar duche** ☐ **Local separado para o vestuário de trabalho** ☐ **Local específico para a arrumação do equipamento de proteção respiratória** ☐ **Outro** (queira especificar)…… ☐ ……
As condições de trabalho foram normais durante o dia de trabalho? (queira assinalar com um círculo)	**Sim** **Não** **Em caso negativo, queira especificar (por exemplo, problemas com a máscara ou não funcionamento da extração):**

Condições operacionais de soldadura

Que material foi soldado? (queira assinalar com um círculo)	**Aço inoxidável** **Outro** (queira especificar)
Qual o método de soldadura utilizado? (queira assinalar a caixa correspondente)	☐ **MMA (manual por arco metálico)** ☐ **MAG (em atmosfera ativa)** ☐ **MIG (em atmosfera de gás inerte)** ☐ **TIG (em atmosfera inerte com elétrodo de tungsténio)** ☐ **SAW (soldadura por arco submerso)** ☐ **Plasma – gás de plasma** ☐ **Soldadura com fio fundente** ☐ **Outro** (queira especificar)…… ☐ ……
Qual o teor de níquel e crómio do material soldado?	**Teor de crómio: ……%** **Teor de níquel: ……%** **Não sabe** (queira assinalar com um círculo, se aplicável)

15

ESTUDO sobre a exposição ao crómio hexavalente e a outros produtos químicos no quadro da HBM4UE (WP8)

O material soldado foi pintado com tintas que continham crómio? (queira assinalar com um círculo)	**Sim** **Não** **Não sabe**
Material e tipo da vareta de soldar?	
Material do fluxo de solda?	
Onde costuma soldar? (queira assinalar a caixa correspondente)	☐ **Ao ar livre** ☐ **Ao ar livre, num espaço confinado ventilado de ..... m³** ☐ **sem ventilação/ventilação natural** ☐ **com ventilação forçada** ☐ **com LEV*** ☐ **No interior, num espaço > 1000 m³** ☐ **No interior, num espaço confinado de ..... m³** ☐ **sem ventilação/ventilação natural** ☐ **com ventilação forçada (por exemplo, construção naval)** ☐ **com LEV***

* **LEV = ventilação por exaustão local

16

ANNEX 2: Information leaflet.

HBM4EU science and policy for a healthy future

Estudo de biomonitorização profissional da HBM4EU sobre o crómio e outras substâncias químicas nocivas
Informações para os trabalhadores participantes

Convidamo-lo a participar num estudo de investigação. Leia as informações que seguem para ficar a saber por que motivo a investigação está a ser realizada e o que implica. Temos todo o prazer em responder a quaisquer perguntas ou dúvidas que possa ter.

Acerca da HBM4EU – Iniciativa Europeia de Biomonitorização Humana
Na vida quotidiana, as pessoas estão expostas a uma mistura complexa de produtos químicos. Estes produtos químicos podem ser encontrados no ambiente, nos produtos de consumo, nos alimentos e na água potável, bem como nos locais de trabalho.
A biomonitorização humana implica a colheita de amostras, nomeadamente de sangue, cabelo, saliva ou urina de pessoas, e a medição dos níveis dos indicadores dos produtos químicos visados. A HBM4EU (Biomonitorização Humana para a Europa) é um estudo europeu que visa harmonizar e utilizar a biomonitorização humana para compreender a exposição das pessoas a esses produtos químicos e os riscos para a saúde, a fim de melhorar a gestão dos riscos químicos. Nos locais de trabalho, a biomonitorização humana pode fornecer-nos informações acerca da necessidade de reduzir a exposição. A HBM4EU é financiada pela Comissão Europeia e pelos governos nacionais. Conta com a colaboração de peritos de 28 países e agências da União Europeia e será realizado entre 2017 e 2021. Para mais informações, consulte https://www.hbm4eu.eu/.

Sobre o estudo
Queremos saber se as atuais medidas de controlo e segurança aplicadas nos locais de trabalho pela Europa são eficazes para proteger os trabalhadores da exposição ao crómio hexavalente (Cr(VI)) e a outros produtos químicos nocivos. Além disso, queremos estudar novos métodos para avaliar a exposição a esses produtos químicos.
O estudo foi aprovado pela Comissão de Ética da Escola Superior de Tecnologia da Saúde de Lisboa/Instituto Politécnico de Lisboa e está em conformidade com o regulamento relativo à proteção de dados.

Porque fui escolhido?
Estamos a convidar trabalhadores cujas funções impliquem uma eventual exposição ao crómio hexavalente (Cr(VI)) (soldadura, metalização, tratamento de superfícies) e trabalhadores que não exerçam esse tipo de funções, para comparação. Foi escolhido porque trabalha num local em que pode ser exposto a crómio hexavalente (Cr(VI)) ou porque o consideramos uma pessoa adequada para controlo, dado que o seu trabalho não implica exposições dessa natureza. O seu empregador aceitou participar no estudo e concordou em o autorizar a participar, se assim o decidir.

Que tenho de fazer se aceitar participar?
Não tem de mudar a sua rotina habitual, uma vez que o estudo será realizado durante as horas normais de trabalho. Se decidir participar, deve confirmar o seu acordo, mediante o preenchimento e a assinatura do formulário de consentimento anexo.

1

Em seguida, definiremos um horário adequado para a realização do estudo. Durante a semana de trabalho, irá conhecer os investigadores responsáveis pela colheita de amostras.

Se as suas funções incluem a soldadura de aço inoxidável, a cromagem ou o tratamento de superfícies com crómio, ser-lhe-á pedido que faculte:

- Amostras de ar expirado, que serão colhidas do seguinte modo: deverá respirar normalmente para um tubo esterilizado descartável durante cerca de 15 minutos, no início e no final da sua semana de trabalho.
- Amostras de urina, colhidas antes do seu turno no início da semana de trabalho e após o seu turno no final da semana de trabalho. Fornecer-lhe-emos o frasco para a colheita, que poderá entregar ao investigador.
- Duas pequenas amostras de sangue (16 ml), que serão colhidas de uma veia do braço. As amostras de sangue serão colhidas após o turno, de preferência no final da semana de trabalho ou, pelo menos, após um ou dois dias de trabalho.
- Deverá igualmente responder a um questionário com perguntas sobre as suas tarefas laborais, o seu equipamento de proteção individual e aspetos relacionados com o seu estilo de vida suscetíveis de afetar os resultados das análises químicas.

A alguns (não a todos) participantes, procederemos ainda à colheita de amostras de ar, que serão colhidas mediante o uso, durante um dia de trabalho, de um dispositivo específico de colheita de amostras de ar colocado no seu vestuário de trabalho, enquanto realizam as suas tarefas normais. Poderão ainda ser colhidas amostras dos toalhetes a que limpa as mãos, para medir a contaminação das suas mãos.
Opcionalmente, e apenas se autorizar, poderemos tirar fotografias ou gravar vídeos para documentar o seu espaço e as suas práticas de trabalho. Esse material será desfocado para proteger a sua identidade e a da sua empresa. Contudo, poderá participar no estudo sem estar presente em fotografias ou vídeos.

Se as suas funções não incluem a soldadura de aço inoxidável, a cromagem ou o tratamento de superfícies com crómio, ser-lhe-á pedido que faculte:

- Uma amostra da sua primeira urina da manhã, colhida antes de se encontrar com o investigador. Fornecer-lhe-emos o frasco para a colheita, que poderá entregar ao investigador.
- Uma amostra de ar expirado, que será colhida do seguinte modo: deverá respirar normalmente para um tubo esterilizado descartável durante cerca de 15 minutos.
- Duas pequenas amostras de sangue (16 ml), que serão colhidas de uma veia do braço a qualquer momento, durante a semana de trabalho.

Para todos os elementos que participem no estudo as amostras de sangue e de ar expirado serão colhidas por um profissional de saúde.

O que vai acontecer às minhas amostras, dados e resultados?
O seu nome será substituído por um código, para proteger a sua privacidade, e as amostras serão transferidas para laboratórios especializados, para análise.
As suas amostras serão analisadas para medir a sua exposição ao crómio hexavalente. Consoante as suas tarefas laborais, serão igualmente analisadas para medir o níquel e o manganês (se trabalhar como soldador), níquel (se as suas tarefas incluírem a niquelagem) ou substâncias polifluoradas (se

trabalhar com cromagem por imersão), que podem ser também importantes contaminantes químicos quando se executam estas tarefas. As amostras de sangue e urina podem ainda ser analisadas para detetar os efeitos celulares iniciais e reversíveis do crómio, a fim de estudar a utilidade destas análises na vigilância da saúde dos trabalhadores.

As suas amostras não serão analisadas para medir álcool, medicamentos ou drogas ilegais.
Os seus dados anonimizados, bem como os dos outros participantes, serão armazenados e utilizados para fins de investigação, podendo ser combinados com outros dados de diferentes fontes. As suas amostras anonimizadas serão armazenadas em local apropriado durante 5 anos para serem utilizadas em futuros estudos de biomonitorização eticamente aprovados para estudar a exposição química.

Os seus resultados pessoais relativos ao crómio na urina ser-lhe-ão comunicados num prazo de aproximadamente 6 meses, a menos que nos informe que não deseja recebê-los. Quando os resultados globais do estudo estiverem disponíveis, receberá o resumo dos resultados coletivos de todas as empresas participantes.

O seu empregador receberá os resultados coletivos de todos os trabalhadores, mas não receberá quaisquer resultados individuais. Os resultados coletivos de todas as empresas participantes serão publicados na forma de relatório do estudo, que estará disponível ao público em https://www.hbm4eu.eu/

Como será garantida a minha privacidade?
A HBM4EU está em conformidade com o regulamento da UE relativo à proteção de dados. Garantimos o seu anonimato mediante a substituição do seu nome por um código e a proteção de todos os registos eletrónicos e em papel contra qualquer acesso não autorizado. Os relatórios do estudo publicados não conterão informações suscetíveis de o identificar a si ou à sua empresa. Nem o seu empregador nem terceiros terão acesso aos seus resultados pessoais sem o seu consentimento expresso.

Porque necessitam do meu consentimento por escrito?
O seu consentimento por escrito confirma que se disponibiliza voluntariamente para participar no estudo e que sabe o que lhe é pedido e com que finalidade. Confirma ainda que estamos autorizados a contactá-lo futuramente para o informar acerca dos seus resultados pessoais ou para fins científicos, estatísticos ou históricos.

Que vantagens resultam para mim com a minha participação?
O estudo contribuirá para o desenvolvimento de práticas de trabalho seguras no seu local de trabalho. Ficará a conhecer a sua exposição pessoal ao crómio e ser-lhe-ão fornecidas orientações para a reduzir.

Há riscos inerentes à minha participação na HBM4EU?
Todas as amostras serão colhidas por profissionais de saúde qualificados e com formação específica. Não há quaisquer riscos, além de algum eventual desconforto durante a colheita.

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Poderei abandonar o estudo?
Aconselhamo-lo a discutir connosco quaisquer preocupações que possa ter; contudo, pode abandonar o estudo a qualquer momento e sem consequências. Não obstante, a equipa de investigação reserva-se o direito de utilizar confidencialmente as amostras colhidas antes de abandonar o estudo.

O estudo implica custos?
O estudo não lhe acarreta quaisquer custos. Participará no estudo durante o seu horário normal de trabalho (com exceção da colheita de amostras da primeira urina da manhã). O tempo consagrado ao estudo não será deduzido do seu salário.

Quem devo contactar se tiver dúvidas ou pretender mais informações sobre o estudo relativo ao crómio hexavalente?
Queira contactar: Susana Viegas, Escola Superior de Tecnologia da Saúde de Lisboa
Tel.: +351 21 8980422 **Endereço de correio eletrónico**: susana.viegas@estesl.ipl.pt

ANNEX 3: Results of CBMN assay.

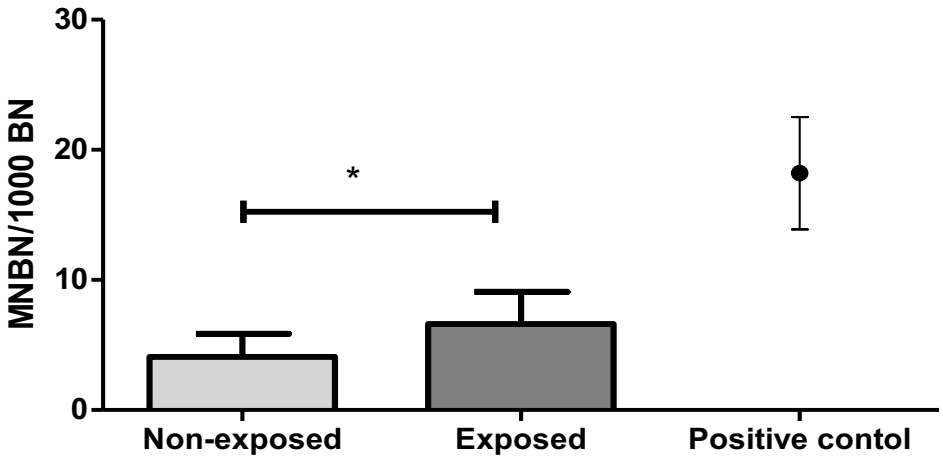

Figure A – Micronucleated binucleated cells (MNBN) frequency per 1000 BN from exposed and non-exposed group. MMC was used as the positive control. Data are shown as mean ± SD. * $p < 0.001$ (Fisher's exact test).

ANNEX 4: Results of Alkaline Comet Assay.

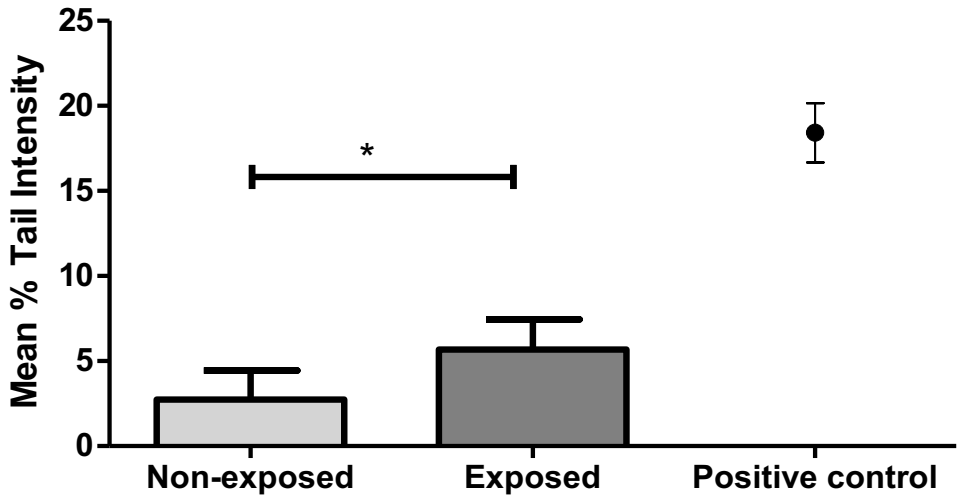

Figure A – Percentage of tail intensity for exposed and non-exposed group. EMS was used as the positive control. Data are shown as mean ± SD. * $p < 0.001$ (Mann-Whitney U test).