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Resumo

O consumo de tabaco continua a ser a principal causa evitável de morbidade e mortalidade humana. Tem também sido demonstrado que exposição *in utero* a fumo de tabaco ou outros poluentes ambientais são capazes de afetar a etiologia e aumentar a suscetibilidade a doenças mais tarde na vida. Este estudo tem o objetivo de compreender melhor os efeitos de exposição pré-natal a fumo de tabaco em amostras de sangue de cordão umbilical, onde serão avaliados os níveis de dano genético analisados por citometria de fluxo através do ensaio de micronúcleos e fosforilação de H2AX.

Uma vez que as amostras de sangue de cordão umbilical foram congeladas, um processo de otimização foi implementado para determinar os valores de γH2AX e % de micronúcleos formados em amostras frescas e congeladas de células mononucleares de sangue periférico. A influência da estimulação na indução de danos no DNA e na resposta a danos no DNA foi também determinada com fitohemaglutinina antes e após o tratamento com vários compostos genotóxicos capazes de induzir quebras duplas na cadeia de DNA. Resultados mostraram também que o processo de congelamento causa um aumento nos níveis de H2AX fosforilada; um aumento que pode ser revertido com estimulação com fitohemaglutinina ou um repouso das células durante a noite, em células com níveis elevados de dano. Foi também observado que estimulação com fitohemaglutinina antes das células serem tratadas com compostos genotóxicos aumenta os níveis de γH2AX comparativamente com células frescas ou congeladas não estimuladas.

Em controlos negativos, verificou-se que o processo de congelamento faz com que a % de micronúcleos decresça, no entanto, estimulação com fitohemaglutinina aumenta a % de micronúcleos tanto em células frescas como em congeladas comparativamente com células não estimuladas. Após descongelamento, verificou-se também que o acondicionamento ou estimulação das células faz com que a % de micronúcleos aumente.

Um total de 499 amostras de sangue de cordão umbilical foram consideradas na análise final; de acordo com os níveis de cotinina registados em urina materna, 83 participantes foram consideradas fumadoras e 416 não fumadoras. Verificou-se que existe uma correlação baixa, mas estatisticamente significativa, entre os

biomarcadores utilizados neste estudo. Em relação aos níveis de γH2AX, não se verificaram diferenças significativas nas diferentes variáveis analisadas, nomeadamente idade, nacionalidade, área de residência, nível de educação, ocupação, consumo de álcool, paridade, consumo de tabaco e exposição a fumo de tabaco ambiental. No entanto, verificou-se uma diferença estatisticamente significativa na frequência de micronúcleos das participantes em diferentes grupos etários (p=0.034), sendo as mulheres mais jovens (<25 anos e 25-29 anos) as que apresentaram maior frequência de % micronúcleos.

Os resultados demonstraram que não existe uma associação entre exposição a fumo de tabaco e o dano genético, mas sugerem que idade, ocupação, e educação podem afetar os níveis de dano no DNA.

Palavras-chave: fosforilação de H2AX; exposição pré-natal; micronúcleos; citometria defluxo; genotoxicidade; fumo de tabaco.

Abstract

Tobacco smoking continues to be the leading preventable cause of human morbidity and mortality. *In utero* exposure to tobacco smoke and other environmental pollutants have shown to affect the etiology and susceptibility to diseases later in life. This study has the aim to better understand the effects of prenatal exposure to tobacco smoke using umbilical cord blood samples to evaluate the levels of genetic damage assessed by H2AX phosphorylation and micronuclei assay, by flow cytometry.

Since the umbilical cord blood samples were frozen, an optimization process was implemented to assess the γH2AX levels and % of micronuclei formed in fresh and frozen peripheral blood mononuclear cells samples. The influence of stimulation on the levels of DNA damage was also determined with phytohemagglutinin (PHA) stimulation before and after treatment with various genotoxic compounds known to cause DSB induction. Results showed that the freezing process (at -80ºC) causes an increase in γH2AX levels; an increase that can be reversed with PHA stimulation or overnight recovery incells with high levels of damage. It has also been observed that cell stimulation with PHA before treatment increases γH2AX levels when compared to unstimulated cells in both fresh and frozen cells.

In negative controls, it was shown that the process of freezing reduces the % of micronuclei, however, stimulation with PHA increases the % of micronuclei in both fresh and frozen cells when compared to unstimulated cells. After thawing, it was verified that a cell recovery period or stimulation with PHA increases the % of micronuclei.

A total of 499 samples of umbilical cord blood samples were considered in the final analysis; according to the cotinine values previously determined in maternal urine, 83 participants were considered smokers and 416 non-smokers. It was verified that there was a low but statistically significant correlation between the different biomarkers used in this study. Regarding γH2AX levels, no significant differences were observed for the different variables analyzed, namely age, nationality, area of residence, education level, occupation, alcohol consumption, parity, tobacco use and environmental tobacco smoke exposure. On the other hand, there was a statistically significant difference on micronuclei frequency of participants in different age groups ($p=0.034$) with younger women (< 25 years and 25-29 years) showing higher frequency of micronuclei %.

Results showed no significant association between tobacco exposure and DNA damage but suggest that age, occupation, and education can have an impact on genetic damage levels.

Keywords: H2AX phosphorylation; prenatal exposure; micronucleus; flow cytometry; genotoxicity;tobacco smoke.

List of Abbreviations

ATCC	American Type Culture Collection
BLM	Bleomycin
BSA	Bovine serum albumin
CCM	Cell culture medium
CHSJ	Centro Hospitalar de São João
CPT	Camptothecin
DMSO	Dimethyl sulfoxide
DSBs	Double strand breaks
EMA	Ethidium monoazide bromide
ETS	Environmental tobacco smoke
FBS	Fetal bovine serum
HBM	Human biomonitoring
IARC	International Agency for Research on Cancer
MMC	Mitomycin C
MMS	Methyl methanesufonate
MN	Micronuclei/Micronucleus
MTT	Thiazolyl blue tetrazolium bromide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohemagglutinin
PI	Propidium iodide

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1. Introduction

Tobacco smoking, to this day, is a frequent behavior with negative effects to human health; it is considered a major worldwide public health problem, being a leading cause of human morbidity and mortality (DeMarini, 2004).

Decades ago, smoking was mostly confined to developed countries but over the last years, the negative impacts of tobacco smoking have been aggravated worldwide as there has been a steady increase in its use also in developing countries (Palma *et al*., 2007). The large number of smokers around the world (estimated to be 1.1 billion smokers in 2025) (WHO, 2019), can be explained not only by the pharmacological addiction to nicotine, but also by social factors (Narkowicz *et al*., 2013).

Even though the number of women that smoke worldwide is much lower than men's, mainly due to social traditions, WHO (2019) reports that in 2018 there were still 244 million women that were daily smokers.

It has been reported that in 2017, around 8 million worldwide deaths could be attributable to smoking (WHO, 2019). As a growing epidemic, it is expected that by the year 2030, the number of deaths per year attributed to tobacco smoke will rise to 8 million (Acharya *et al*.,2016). Among adults, more than 1 in every 10 deaths can be related to tobacco use (Britton,2017) and it is expected that in the twenty-first century 500 million to 1 billion people will diefrom tobacco related diseases (WHO, 2008).

Tobacco smoking related diseases are responsible for causing more deaths per year than car accidents, alcohol, HIV, malaria, or drugs for example (Narkowicz *et* al., 2013; WHO, 2008). Tobacco smoke is a risk factor associated with strokes and heart attacks and it is related to 80% of all lung cancer cases worldwide (Weng *et al*., 2018), which is the cancer responsible for most deaths worldwide (Mackay, 2002). It is also documented by the International Agency for Research on Cancer (IARC) that tobacco smoke is the leading cause of cancer at other organ sites, causing more cancers than any other type of human carcinogen (DeMarini, 2004). Tobacco smoke is regarded as the biggest example of human systematic mutagen, as many studies show that tobacco smoke exposure can cause DNA damage to somatic cells, and it is also known to originate negative impacts in reproduction (Yauk *et al*., 2007).

During tobacco smoking, a smoke stream of chemicals and gases is formed, containing atleast 4000 different chemical compounds (Borgerding *et al*.,1998; Counts *et al*., 2004; Sutton *et al.*, 2017). Amid these chemicals, approximately 250 are toxic to humans, such as ethers, polycyclic aromatic hydrocarbons, phenols, ketones, nitrosamines, inorganic compounds, aromatic amines, aldehydes, etc. (Baker, 2006; Husgafvel-Pursiainen, 2004), and more than 50 hazardous compounds have been labeled as carcinogens by IARC (Hoffmann *et al*., 1997; Peterson, 2010).

When tobacco is being burned, two types of smoke stream are formed. The mainstream smoke that refers to the smoke that is expelled from the mouth end of the cigarette while puffing and the side stream smoke that is the smoke that is expelled from the burning end of the cigarette in between puffs. These two different streams of smoke form the environmental tobacco smoke (ETS) (DiGiacomo, *et al*., 2019).

ETS is a major public health concern worldwide, because this exposure is bound to happen daily, since there are more than 1.1 billion adult smokers (Avşar *et al.,* 2008; WHO, 2019). Children are one of the most affected because they have a higher intake of chemicals as they breath faster (Narkowicz *et al*., 2013). It was estimated in 2004 that almost half the children in the world (40 %) were exposed to ETS (Öberg *et al*., 2011); although most smoking parents try to reduce their children exposure to tobacco smoke, this exposure occurs mainly at their own home (Gilliland *et al*., 2001; Zhuge *et al*., 2019). Research shows that exposure to ETS predisposes children to many health problems in the future such as asthma, neurological disorders, cancer, reduced lung capacity, cardiovascular diseases and the development of an addiction to tobacco later in life (Christensen *et* al., 2017; Henderson, 2008; Osorio-Yánez *et al*., 2020).

However not only children are affected by tobacco exposure; pregnant women must also be considered a vulnerable group because it has become evident that tobacco use or exposure to ETS not only affects the women's health but also the fetus they are carrying (Almanzar *et* al., 2013; Leonardi-Bee *et al*., 2008). Even though many studies and health campaigns have shown that smoking during pregnancy negatively affects the newborn, the number of women that smoke during pregnancy is still high (Pellegrini *et al*., 2007).

In utero exposure to tobacco smoke is known to have harmful effects to the newborn

because many of the chemicals present in tobacco smoke either have low molecular weight or are water soluble which means that these xenobiotics can easily trespass the placental barrier and affect the development of the newborn (Pugmire *et al*., 2017).

Prenatal exposure to tobacco is known to be responsible for an increase on the number of cases of sudden infant death syndrome (SIDS) and a higher risk of miscarriage (Narkowicz*et al*., 2013). It is also evidenced in various studies that a prenatal exposure to ETS is responsible for increasing the chances of premature birth, and decrease in the birth weight of the fetus, which are the main causes of still birth and infant death (Dede *et al*., 2016), and of higher risk of childhood cancer and placental abruption (Jauniaux *et al.*, 2007; Rogers, 2008).

During fetus development, the nervous system of the newborn is very susceptible to the contaminants present in tobacco. It has been shown that tobacco has neuromodulatory and neurotoxic effects on the brain affecting the nervous system and cognitive functions (Polanska *et al*., 2017). Nicotine is responsible for a diminished utero-placental blood flow that decreases the amount of oxygen and nutrients reaching the fetus causing fetal hypoxia and brain growth retardation (Pintican *et al*., 2019). These complications can later in life be responsible for child behavior problems, such as attention deficit, hyperactivity disorder (ADHD) increased levels of aggressiveness, delinquency, defiance, and children performance in school (Liu *et al*., 2013).

Among children and teenagers, it has been documented cases of hearing impairment that can be explained by damage to the developing cochlea of the fetus caused by nicotine and others chemicals present in tobacco smoke (Wilunda *et al*., 2018). *In utero* tobacco exposure is also responsible for a heightened risk of other health problems later in life. There is an increased risk of metabolic syndrome, obesity and overweightness that can be explained by hormonal imbalances caused by tobacco (Scott-Goodwin *et al*., 2016). There is also an increased prevalence of wheezing in children that were exposed to pre and postnatal ETS when compared to children not exposed to tobacco smoke, and if the children continue to be exposed during their childhood there is an augmented risk of developing and worsening pre-existing asthma (Cheraghi *et al*., 2009).

As mentioned above, exposure to tobacco smoke is known to prompt a series of events in the multistage process of carcinogenesis. The genotoxic effects of tobacco are of extreme significance as they frequently ensue in the early stages of

carcinogenesis (Besaratinia *et al*., 2013).

Many of the xenobiotics in tobacco smoke serve as substrates for metabolizing enzymes such as the glutathione S-transferases or the cytochromes P450, which transform the chemicals into more water soluble and less toxic forms that can be readily excreted (Hercht,2012; Mansoori *et al*., 2015). However, during this detoxification process, electrophilic compounds, such as carbocations or epoxides, can be produced and interact with nucleophilic sites in DNA forming DNA adducts (Takahashi *et al*., 2010). Tobacco smoke isalso a major contributor for the cleavage of the both DNA strands, causing the formation ofdouble-strand breaks (DSBs), that can be explained by an increase of reactive oxygen species (ROS) and decrease of antioxidants that causes oxidative stress (Werbrouck *et al*.,2008).

DNA adducts can be responsible for initiating carcinogenesis because, if not repaired, they are a source of misinformation during DNA replication, potentializing mutations. Targeting mutation in key cancer related genes that encode proteins that control cell cycles and growth can lead to tumorigenesis (Besaratinia *et al*., 2008; Jin *et al*., 2017). The *tp53* tumor supressor gene is implicated in various processes, such as DNA repair, apoptosis, metabolism, or cell-cycle arrest, however it has been evidenced that this gene is affected by the hazardous chemicals of tobacco smoke (Deligkaris *et al*., 2019). The mutant form of this gene exerts oncogenic functions such as cell-cycle progression and cell migration that actively contribute to tumor progression (Mogi *et al*., 2011).

The various chemicals in tobacco are also known to cause damage to chromosomes that lead to the separation of a small nucleus from the main one during cellular division, the micronuclei (Pradeep *et al*., 2014). Studies have also shown that there is an increase in the incidence of chromosomal aberrations and sister chromatid exchanges in peripheral blood lymphocytes in adults in association to tobacco (de la Chica *et al*., 2005; Minina *et al*., 2017).

However, data regarding the genotoxicity of tobacco in pregnant women and their fetuses is scarcer. Using the comet assay analysis as a biomarker for tobacco genotoxicity, de Assis*et al*. (2009), Tsui *et al*. (2008) and Wu *et al*. (2007) determined that mothers who smoked or were exposed to ETS had higher levels of DNA damage compared to mothers that were not exposed to tobacco smoke, and that ETS exposure is indicative of fetal growth alterations.

There is also evidence that there is an increment of chromosomal instability and structural chromosomal abnormalities in amniocytes from fetuses carried by smokers when comparedto non-smokers (de la Chica *et al*., 2005).

To protect human health and obtain information about the toxic substances humans are exposed to, various methods of human biomonitoring (HBM) can be used. HBM consists of the assessment of doses to which the body is exposed in biological samples like hair, placenta, urine, blood, sweat, saliva etc. and in the monitorization of its biological and biochemical effects (Angerer *et al*., 2007).

To assess exposure to tobacco smoke, compounds that are present in tobacco can be quantified in different biological matrices, being designated biomarkers of exposure. Usually, nicotine and its metabolite, cotinine are the most common used biomarkers because of its specificity and relatively simple detection (Narkowicz *et al*., 2013).

Cotinine is the most used biomarker to characterize tobacco exposure; it is a primary metabolite of nicotine that can be quantified in various biological fluids like saliva, serum, or urine and its concentration is proportional to the level of nicotine exposure (Benowitz, 1996; Sharma *et al*., 2019). Its usage as a biomarker of exposure to tobacco smoke is more advantageous than nicotine given the longer half-life (16-18 h) when compared to the only 3 h of half-life of nicotine (Sharma *et al*., 2019; Torres *et al*., 2018).

Among the most common biomarkers to detect early biological effects of genotoxic compounds, stand the chromosomal aberrations test that is used to identify substances that cause structural chromosomal aberrations in cells, and the micronuclei (MN) assay which is recognized as one of the most reliable assays for genotoxic carcinogens, being based on the formation of micronuclei in cells (Au, 2007), the comet assay to detect DNA damage and repair (Collins, 2004), and the analysis of phosphorylated histone H2AX to assess DNA DSBs.

The H2AX histone protein was reported as a specific protein from the H2A family in 1980 by West and Bonner and it constitutes 2-2.5 % of total H2A genome in mammals (Turinetto et *al*., 2015; Watters *et al*., 2009). After the formation of DSBs in eukaryotic cells caused by genotoxic compounds like the ones present in tobacco smoke, one or more of the phosphatidylinositol 3-kinase-like kinase (PIKK) family are activated and rapidly phosphorylate the H2AX at the serine 139 residue upon DNA DSBs formation

(Kopp *et* al.,2019; Sánchez-Flores *et al*., 2015).

The phosphorylation of H2AX, creates γH2AX foci that can be visible microscopically if labeled with an antibody, its formation is linearly related to the number of DSBs formed, hence being considered a very sensitive biomarker of DSBs (Banáth *et al*., 2003), up 100 times more sensitive than the comet assay in detecting DSBs (He *et al*., 2013).

The γH2AX foci have an estimated half-life of 2-7 h, being formed within minutes after DNA lesion, reaching its maximum levels after 30 min (Valdiglesias *et al*., 2013). So H2AX phosphorylation represents an early marker of DNA damage and DNA repair as this histone takes a predominant role in repairing these lesions (Bonner *et al*., 2008; Hamasaki *et al*., 2007; Watters *et al*., 2009). However, some γH2AX foci remain present in cells evenafter the DSBs repair suggesting that some DNA lesions may be permanent (Sánchez-Flores *et al*., 2015). Since DSBs can be formed during many processes that disrupt cellular stability, γH2AX detection has a wide range of applications in epidemiological studies, detector of toxic environmental agents or as a biomarker for cancer and aging (Li *et al*., 2014).

The micronucleus assay is considered a well-established biomarker of genotoxicity at a chromosomal level (Fenech, 1997). Micronuclei are formed during DNA replication, and involve a loss of DNA information on the main nucleus that is caused by aneugens that can increase chromosome miss segregation ultimately leading the cells to aneuploidy (Terradas *et al*., 2010), malformation of the mitotic fuse is a form of aneugenic action that can also form micronuclei (Iqbal *et al*., 2019), or clastogens that cause breaks that yield acentric fragments and structural chromosomal aberrations in the cells and subsequent formation ofa smaller nucleus, the micronucleus (OECD, 2016).

2. Aims

Over the last years, various projects have been studying the effects of tobacco exposure on pregnant women and their newborns, however most of these studies focus on immediate effects (e.g., perinatal outcomes) and compare only active smokers with non-smokers. Therefore, there is still a lot of misinformation regarding the potential genotoxic effects of prenatal exposure to tobacco smoking, considering both active and passive smoking.

Prior to analysis of the cord blood samples, an extensive optimization of the γH2AX and micronuclei assays have been carried out, while several methodological factors have been analyzed.

The goal of this study was to estimate genetic damage in cord blood samples of newborns of both smoking and non-smoking mothers. *In utero* exposure to tobacco smoke was previously identified through urinary cotinine concentrations, to distinguish smokers from non-smokers, and questionnaires (to characterize ETS exposure among non-smokers), genetic damage was assessed using H2AX phosphorylation and micronucleus frequency, employing flow cytometry methods.

3. Methods

3.1. Chemicals

Gibco™ phosphate buffered saline for flow cytometry (PBS) 7.4 (A1286301), fetal bovine serum (FBS) heat inactivated (10500064), sucrose 99% (57-50-1), NaCl (7647-14-5), citric acid ACROS Organics™ (77-92-9), camptothecin (7689-04-04) and phytohemagglutinin (PHA; 10576-015) were supplied by ThermoFisher Scientific (Massachusetts, USA). Propidium iodide (PI) (P4170), thiazolyl blue tetrazolium bromide (M5655-1G), bovine serum albumin (BSA) (A4503-50G), methyl methanesufonate (MMS) (129925-5G), tri-sodium citrate dihydrate (6132-04-3), nonidet P-40 (9016-45-9) and EMSURE® ethanol (100983) were acquired from Merck (Darmstadt, Germany). Penicillin-Streptomycin 10000 U/mL Penicillin, 10 mg/mL Streptomycin (P06-07100) was supplied by Pan Biotech™ (Bavaria, Germany), Amresco p-formaldehyde (J531-500G) from VWR Avantor (Pennsylvania, USA), Panreac Applichem RNAse A (A3832.0050) from Illinois Tool Works(ITW) (Illinois, USA), dimethyl sulfoxide (DMSO) (D5879) from Honeywell (New Jersey, USA), and RPMI-1640 medium (BE12-702F/U1) from Lonza (Basel, Switzerland). Premixed PBS buffer 10x, were acquired from Sigma-Aldrich (Missouri, USA). The BD Pharmigen™ Alexa Fluor® 488 Mouse anti-H2AX (pS139) (560445) was bought from BD (New Jersey, USA), the Alexa Fluor 488 phospho-histone H2A.x monoclonal antibody (CR55T33), Alexa Fluor 488, eBioscience™ (53-9865-82) was acquired from Invitrogen (California, USA) and the Alexa Fluor® 488 anti-H2A.X Phospho (Ser139) Antibody (613405) was acquired from BioLegend (California, USA). Bleomycin sulfate (9041-93-4) was bought from Cayman Chemical Company (Michigan, USA).

3.2. Method implementation and optimization

3.2.1. THP-1 cell culture

THP-1 cells, obtained from the American Type Culture Collection (ATCC), were cultured at 37ºC with 5% CO_2, at a concentration ranging from 2-4 x 10^5 to 8 x 10^5 cells/mL in RPMI-1640 medium supplemented with 10% FBS heat inactivated and 1% Penicillin-Streptomycin in T75 flasks, following ATCC indications. Cells were maintained with the addition of fresh cell medium or the replacement of medium every 2 or 3 days. Cell culture was constantly monitored under the microscope to control cell density and morphology and check microbial growth.

3.2.2. Peripheral blood collection and PBMCs isolation

Human peripheral blood mononuclear cells (PBMCs) were used in both γH2AX and micronucleus assay optimization and implementation steps. To obtain them, human peripheral blood was collected from non-smoking healthy volunteers (22 - 46 years old; the number of individuals analysed for each experiment is detailed throughout the text), by venipuncture using Vacuette® blood collection tubes coated with sodium heparin. Prior to sample collection, all subjects were informed of research aims and provided their consent, following the ethical guidelines established by the Helsinki declaration.

Immediately after blood collection, PBMCs were isolated using Lymphoprep™ following manufacturer' instructions. Lymphoprep™ was added to 15 mL falcon tubes, and then, blood samples, previously diluted in an equal volume of PBS 7.4 supplemented with 2% FBS was carefully layered on top. Right after, the tubes were centrifuged for 20 min at 800x *g* at 21ºC and PBMCs layer was carefully removed to another 15 mL falcon tube without disturbing the plasma layer and the erythrocyte and granulocyte pellet. To wash the PBMCs, PBS 7.4 was added to a total volume of 5 mL, followed by a 10-min centrifugation at 600 x*g* at 21ºC and removal of the supernatant. Subsequently, the PBMCs pellet was resuspended in PBS 7.4.

For some of the experiments, a pool of PBMCs was prepared by combining the pellets of PBMCs of different subjects, while in others, samples were processed individually. Prior to experiments or freezing, cells were counted using a Neubauer-improved counting chamber (Marienfeld GmbH & Co.KG, Germany) on a light microscope (Nikon Eclipse E400, Japan), following the manufacturer' instructions.

For freezing, 1 x 10^6 cells in 1 mL of PBS 7.4 were centrifuged for 5 min at 885 x *g* at 21ºC and resuspended in 1 mL of freezing medium (50% FBS, 40% RPMI-1640 and 10% DMSO);for slow freezing, a Mr. Frosty™ Nalgene Freezing Container was used to enable a gradual temperature drop of 1ºC per minute. After 24 h, microtubes were transferred to a box and stored at -80ºC until use.

3.2.3. Cytotoxicity assessment

Cytotoxicity assessment was performed to ensure that concentrations to be used as positive controls in γH2AX and MN assay were not cytotoxic, given that high levels of cytotoxicity may induce chromosome damage as a secondary effect of cytotoxicity and yield false positive results (OECD, 2016).

Given the high number of cells necessary for this preliminary assessment, THP-1 cell line (human monocyte-like cells) was used in this cytotoxicity assessment as a proxy for PBMCs' response (Chanput *et al.*, 2014). With this, it was possible to decrease the number of volunteers involved in these optimization experiments and the volume of blood collected to each of them to a minimum.

Cytotoxicity was assessed using the MTT assay, following a modified method based on Mossman (1983). The assay was carried out: (1) immediately after treatment for 4 h with the genotoxic compounds (as required for γH2AX assay), and (2) after exposure for 4 h to the genotoxic compounds, and incubation for an additional period of 44 h (as required for MN assay). The selection of compounds and range of concentrations to test in cytotoxicity assessment was based on previously published studies. Bleomycin (BLM), camptothecin (CPT) and methyl methanesufonate (MMS) are genotoxic agents recommended to be used as positive controls for γH2AX assay (Sánchez-Flores *et al*., 2015), and mitomycin C (MMC), alongside with MMS and BLM are indicated for MN assay as they are clastogenic agents (OECD, 2016; Rosefort *et al*., 2004; Suggitt *et al*., 2003).

Tested concentrations of BLM were 6 µg/mL, 12 µg/mL, 24 µg/mL, 48 µg/mL and 96 µg/mL; of MMC 0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL 1 µg/mL and 2 µg/mL; and of MMS 5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL and 80 µg/mL.
Firstly, THP-1 cells were plated and incubated overnight at 37ºC with 5% CO_2 with an initial concentration of 3 x 10^4 cells/well in two 96-well U-shaped bottom microplates containing 100 µL of cell culture medium (content described above) per well. In the following day, 5 µLof the tested substances were added to the wells and incubated for 4 h at 37ºC with 5% CO_2. For each condition (compound and concentration), 3 replicates were tested. Furthermore, negative controls (containing only cell culture medium) and positive controls (1% Triton X-100) were also analyzed.

After exposure to the tested compounds, microplates were centrifuged for 4 min at 250

x *g*, and the medium was aspirated. After this step, one plate was immediately tested, while in the second plate, 100 µL of cell culture medium was added to wells and cells were incubated for an additional period of 44 h at 37ºC with 5% CO_2 followed by another centrifugation of 4min at 250 x *g* and supernatant removal. Afterwards, a volume of 100 µL of thiazolyl blue tetrazolium bromide (MTT) solution (500 µg/mL) was added to each well, the pellet was homogenized and then, the microplates were incubated for 4 h at 37ºC in the dark. Subsequently, the microplates were once again centrifuged for 4 min at 250 x *g*, and the supernatant was removed. The resulting formazan crystals were dissolved using 150 µL of DMSO and transferred into a 96-well flat-shaped bottom microplate. Absorbance was read at 570 nm (with a background correction at 630 nm) using a SpectraMax ID3 microplate reader (Molecular Devices, USA).

The viability (%) of the different compounds, was calculated according to the formula presented below. The compound was deemed as cytotoxic if the value of was below 55 ± 5 % (OECD, 2016).

$$\text{Viability (\%)} = 100 - \left(\frac{\text{Abs570-Abs630 of the tested substances}}{\text{Abs570-Abs630 of the negative control}} \right) \text{x } 100$$

3.2.4. H2AX phosphorylation

Five independent experiments were performed regarding the optimization of the H2AX phosphorylation assay, in flow cytometric analysis of γH2AX, cells are fixed in p-formaldehyde and permeabilized with ethanol, and then incubated with the γH2AX antibody and with PI. To calculate the percentage of H2AX phosphorylated foci, the number of events marked with both anti-γH2AX and PI are divided with respect to the number of events marked with PI (Sánchez-Flores *et al*., 2015).

In this first stage of method optimization and implementation, different experiments have been carried out to: (1) optimize some technical aspects, namely centrifugation, fixation and staining, (2) identify adequate positive controls for the assay, (3) understand the effect of freezing on γH2AX frequencies, and (4) understand the effect of PHA stimulation on DNA damage levels and DNA damage response.

3.2.4.1. Centrifugation, fixation and staining

In the early stages of method optimization and implementation, both THP-1 and pools of PBMCs from 5 individuals were used, to ensure that enough cells would be analyzed by flow cytometry, three different protocols were implemented in which centrifugations' speed varied.

Still, to ensure the highest number of cells would be analyzed by flow cytometry, four different conditions of fixation and permeabilization of the cells were tested. After fixation with p-formaldehyde, cells were permeabilized in ethanol for 2 h at 4ºC and -20ºC or permeabilized overnight at 4ºC and -20ºC.

To determine the optimal γH2AX antibody concentration, seven different antibody conditions (100 µL) were tested, three concentrations (5:100, 4:100 and 3.5:100 which was the lowest concentration tested to confirm a previous experiment previously carried out in the laboratory) of Alexa Fluor® 488 Mouse anti-H2AX (pS139) (BD Pharmigen™ 560445), two concentrations (1:100 and 0.7:100) of eBioscience™ Alexa Fluor 488 phospho-histone H2A.x monoclonal antibody (CR55T33) (Invitrogen 53-9865-82) and one concentration (3.5:100) of Alexa Fluor® 488 anti-H2A.X Phospho (Ser139) Antibody (BioLegend 613405). All solutions were prepared in filtered cytometric phosphate-buffered solution (PBS 7.4) supplemented with 1% BSA.

The highest concentration tested from Alexa Fluor® 488 Mouse anti-H2AX (pS139) (BD Pharmigen 560445) antibody was based on the manufacturer instructions. Then, to grasp if it was possible to reduce the volume of antibody used per sample, while obtaining the same values of phosphorylated H2AX, lower concentrations of antibody than the recommended have been tested. For the remaining brands, volumes were selected for testing based on their cost.

3.2.4.2. Preparation of positive controls

A pool of PBMCs (obtained from 5 volunteers) were treated for 4 h at 37ºC with two genotoxic substances known to cause DNA DSBs, either before or after the freezing process, and before or after stimulation with PHA. BLM was used at 1 and 20 µg/mL, MMS at 6.5 and 65 µg/mL and CPT at 0.17 and 3.48 µg/mL.

PBMCs that were treated with the genotoxic substances before freezing were centrifuged for 5 min at 806 x *g* at 21ºC, the supernatant was removed, and the

cell pellet was resuspended in 1 mL of freezing medium and stored in Mr. Frosty™ Nalgene Freezing Container at -80ºC until further use.

3.2.4.3. Effect of freezing

Given that NEOGENE samples available for analysis had been previously frozen, immediately after collection, the impact of freezing on γH2AX frequencies was assessed in a PBMCs pool prepared from 5 donors, presenting baseline and high frequencies of damage.

In these experiments, 1 mL of fresh PBMCs suspensions (5 x 10^5 cells/mL; either treated or untreated) in PBS 7.4 were immediately used for γH2AX analysis after the counting step (protocol described in section 3.2.4.5). Simultaneously, an aliquot of the same suspensions was frozen according to the procedure mentioned above (section 3.2.2) and kept at -80ºC for at least 2 days.

In the day of analysis, samples were quickly thawed at 37ºC bath water prior to experiments. Then, the content of the microtubes was transferred to a 15 mL Falcon tube; PBMCs cell culture medium (PBMCs-CCM) containing RPMI-1640 medium with L-glutamine supplemented with 15% heat inactivated FBS and 0.5% Penicillin-Streptomycin was added to make up a final volume of 6 mL. The falcon tubes were centrifuged for 10 min at 400 x *g*at 4ºC and supernatant was removed by aspiration. Afterwards, the pellet was resuspended in PBS 7.4 to a total volume of 1 mL, for counting. The volume containing 5 x 10^5 cells/mL was completed to 1 mL and γH2AX analysis was initiated (section 3.2.4.5).

According to the described by Turinetto *et al.*, 2016, frozen samples have also been analyzed after a recovery period of 16 h (overnight) in PBMCs-CCM.

To understand the effects of thawing and refreezing PBMCs, some samples were quickly thawed at 37ºC in a water bath, then placed on ice for 1 h and refrozen at -80ºC as mentioned above (section 3.2.2) for analysis of γH2AX levels.

3.2.4.4. Effect of stimulation

The effect of stimulation before and after treatments on DNA damage levels was investigated in both fresh and frozen suspensions of a PBMCs pool prepared from 5

volunteers' samples to better understand DNA damage induction and response.

In all cases, stimulation was induced using 1% of PHA, that was added to cell suspensions for 24 h; incubation was carried out at 37ºC with 5% CO_2. In the case of stimulation after treatment, by the end of the exposure period, cells were centrifuged for 5 min at 806 x *g* followed by the removal of the supernatant and the addition of 1 mL of PBMCs-CCM with 1% of PHA.

3.2.4.5. γH2AX analysis

γH2AX assay was performed based on the protocols described by Sánchez-Flores *et al*. (2015), Tanaka *et al*. (2009) and Watters *et al*. (2009) with some adjustments.

A PBMCs pool (collected from 5 donors) suspensions were centrifuged at 806 x *g* at 21ºC for 5 min. Immediately after centrifugation, the supernatant was aspired, and cell pellets were fixed for 15 min at 4ºC in1 mL of cold 1% p-formaldehyde. Then, the 15 mL falcon tubes were centrifuged for 5 min at 1300 x *g* at 4ºC; after centrifugation, the 1% p-formaldehyde was removed, and cells were permeabilized with 1 mL of cold 70% ethanol and stored overnight at 4ºC.

In the next day, the cells suspension was centrifuged for 5 min at 1300 x *g* at 21ºC, and after the removal of the supernatant, cells were washed in 1 mL of filtered PBS 7.4 with 1% BSA. Right after, cells were once again centrifuged for 5 min at 1300 x *g* at 21ºC and the supernatant was aspired. Then, cells were incubated at room temperature in the dark for 15 min in 100 µL of 1:100 Alexa Fluor 488 phospho-histone H2A.x monoclonal antibody (Invitrogen 53-9865-82) diluted in filtered PBS 7.4 with 1% BSA. After this period of incubation, 1 mL of filtered PBS 7.4 1% BSA was added to wash the cells and then, cells were centrifuged again for 5 min at 1300 x *g* at 21ºC and the supernatant was removed; afterwards, cells were suspended in 500 µL of PBS 7.4 (containing 40 µg/mL of PI and 0.1mg/mL of RNAse A) for 30 min in the dark at room temperature.

The cell suspension was analyzed by flow cytometry, at medium flow, in a GUAVA EasyCyte 8HT (Luminex Corporation, USA) flow cytometer, equipped with a 488 nm excitation laser. Initially, the PBMCs population (R1) was gated in a dot plot, according to size measured with the forward scatter (FSC-Hlin) and complexity, assessed with the side scatter (SSC-Hlog). In a second dot plot gated in R1, doublets were discriminated

and excluded (R2) using Red-B width (Red-B-Wlin) and Red-B Fluorescence Area (Red-B-Alin).Finally, using a dot plot with quadrant analysis, the signal measured in Red-B Fluorescence (Red-B-Hlin) and Green-B Fluorescence (GRN-B-HLog) was assessed, to identify cells marked with PI and anti-γH2AX, respectively; in this final plot, at least 10000 events positively marked with PI were acquired.

The % of PBMCs expressing H2AX phosphorylation was calculated according to thefollowing equation:

$$\%\gamma H2AX = \frac{\text{cells positively marked with PI and anti-}\gamma H2AX\text{ fluorochromes}}{\text{cells positively marked with PI}} \times 100$$

3.2.5. Micronucleus assay

Four independent experiments of optimization of the micronucleus assay were performed to identify adequate positive controls, the effect of freezing, and the effect of stimulation, following the OCDE guidelines for the *in vitro* micronucleus assay (OECD, 2016). At the optimization stage, it was followed one of the first methods described to analyze MN by flow cytometry (herein indicated as method 1); however, based on the results obtained, a different method has been implemented (method 2), that was used to analyze samples of the NeoGene project.

3.2.5.1. Preparation of positive controls

In accordance with the OCDE guidelines for the *in vitro* micronucleus assay, cell lines must be maintained in an exponential growth phase and primary cells must be stimulated with mitogenic agents to initiate cell division. Furthermore, after exposure to the test chemicals,cells need to be incubated in fresh medium for an additional period with a duration equivalent to 1.5 – 2 normal cell cycle length (OECD, 2016).

For this, THP-1 cells (3 x 10^5 cells/well), with a cell cycle of 24 h (Tsuchiya *et al*., 1980) were plated overnight, after which cells were treated for 4 h at 37ºC with 3 genotoxic compounds known to lead to the formation of micronuclei. BLM was used at 12 and 24 µg/mL, MMS at 20 and 40 µg/mL and MMC at 0.5 and 1 µg/mL. After exposure, cells were centrifuged for 5 min at 806 x *g* at 21ºC, the supernatant was removed, and the cell pellet was resuspended 1 mL THP-1- cell culture medium (CCM) and incubated for 44 h to permit cell division. After this incubation, micronuclei assay was initiated (section 3.2.5.4).

In the case of PBMCs, considering the genotoxicity assessed in THP-1 cells and the MTT assay results, only MMC and MMS were tested, alongside negative controls. In brief, 6 x 10^5 cells/mL in 1 mL were stimulated with 1% of PHA for 44 h, treated for 4 h at 37ºC with MMC (0.25 and 0.5 µg/mL), and MMS (20 and 40 µg/mL). After 4 h of exposure, cells were centrifuged for 5 min at 806 x *g* at 21ºC, the supernatant was removed, and the cell pellet was resuspended 1 mL of PBMCs-CCM in the presence of 1% of PHA and incubated for an additional period of 24 h. By the end of the incubation, cells were immediately analyzed or frozen as indicated above (section 3.2.2).

3.2.5.2. Effect of freezing

Given that NEOGENE samples available for analysis had been previously frozen, immediately after collection, the impact of freezing on MN frequencies was assessed in PBMCs samples of 5 individuals, presenting baseline and high frequencies of damage.

For fresh cells, 1 mL of PBMCs suspension (3 x 10^5 cells/mL) in PBMCs-CCM was used for micronuclei analysis after counting. Frozen PBMCs were quickly thawed at 37ºC bath water. After that, the volume containing 3 x 10^5 cells/mL was determined, and PBS 7.4 was added to make up a volume of 1 mL to start micronuclei analysis.

Some PBMCs samples were also thawed and refrozen as described in section 3.2.4.3, to understand the effect of multiple freeze-thaw cycles.

After thawing, some samples were incubated in PBMCs-CCM for a recovery period of 16 h (overnight) as described by Turinetto *et al.*, 2016.

3.2.5.3. Effect of stimulation

Even though *in vivo* MN assay by flow cytometry does not involve cell stimulation prior to analysis (OECD, 2016), it is widely recognized that cell stimulation offers the cell, the opportunity to express all DNA damage accumulated *in vivo* (Speit *et al*., 2012). Therefore, the effect of PHA stimulation in both fresh and frozen PBMCs (untreated and treated), from 5 different individuals has been analyzed.

The micronucleus assay was rapidly started after treatment for unstimulated cells while

the stimulated cells were centrifuged for 5 min at 885 x *g* at 21ºC and resuspended in 1 mL of PBMCs-CCM in the presence of 1% of PHA and incubated for 24 h prior to analysis.

3.2.5.4. Micronuclei Analysis (method 1)

This method for determination of micronuclei followed the methods described by Nüsse *et al*. (1994) and Roman *et al*. (1998) with some modifications.

First, PBMCs suspensions were centrifuged for 6 min at 290 x *g* at 21ºC, followed by the removal of the supernatant and then, to destroy the cell membranes and cytoplasm, cell pellet was vortexed in 500 µL of solution I (composed by 584 mg/L NaCl, 1 g/L Sodium Citrate, 0.3 mL/L Nonidet P-40 prepared in ultrapure water). Suspensions were kept at room temperature in the dark for 30 min. After incubation with solution I, 10 µL of PI (50 mg/mL) and 2.5 µL of RNAse A (0.05 mg/mL) were added, and the cell suspensions were kept in the dark at room temperature for 30 min one more time. Then, to remove the cytoplasm still attached to the nuclei and micronuclei, 500 µL of solution II (composed by 15 g/L of citric acid, 0.25 M of sucrose in ultrapure water) was added to the cell samples, vortexed and kept in the dark at room temperature for 15 min. Immediately before flow cytometry analysis,cell suspensions were filtered through a 50 µm nylon mesh cell strainer.

The flow cytometry analysis of the nuclei and micronuclei suspension was performed in a GUAVA EasyCyte 8HT (Luminex Corporation, USA) flow cytometer at medium flow. First, the PBMC population was identified according to size with forward scatter (FSC-Hlin) and complexity with side scatter (SSC-Hlog); using this first dot plot, voltages were adjusted to move the nucleus population to the fourth decade of SSC-HLog, that were gated in R1.

In order to discriminate and exclude doublets, a second plot (dot), gated in R1, Red-B Fluorescence (Red-B-HLog) with forward scatter (FSC-HLog) was initially used, but with no success. In the final protocol, doublet discrimination and exclusion (R2) were successfully achieved using side scatter width (SSC-Wlin) and side scatter height (SSC-Hlin). In a third plot (dot), gated in R2, nuclei and micronuclei populations were differentiated from nonspecific debris particles, based on Red-B-Fluorescence (Red-B-HLog) and Forward Scatter (FSC-HLog); in this final plot, a minimum of 10000 events in the nuclei region were acquired.

The % of micronuclei formed was calculated by:

$$\%MN = \frac{\text{total events acquired in micronuclei region}}{\text{total events in nuclei region}} \times 100$$

3.2.5.5. Micronuclei Analysis (method 2)

This method was based on the methodology described in Avlasevich *et al*. (2006) and Bryce *et al*. (2007).

After counting, PBMCs were centrifuged for 5 min at 600 x *g*; after the removal of the supernatant, 300 µL of ethidium monoazide bromide (EMA) solution (compounded by 5.1 µL and 294.9 µL of PBS with FBS 2%) was added to each tube to resuspend the cells' pellets. The 15 mL falcon tubes were then submerged 2 cm in crushed ice and placed 10 cm under a fluorescent light bulb (650 W) for 30 min.

After the EMA photoactivation, 3 mL of PBS +2 % FBS were added, and cells were centrifuged once again for 5 min at 600 x *g,* and 250 µL of solution I (that consisted of 584mg/L NaCl, 1 g/L Sodium Citrate, 0.3 mL/L Nonidet P-40 prepared in ultrapure water), containing 20 µM Helix NP™ Green and 0.05 mg/mL RNAse) was added to destroy the cell membranes and cytoplasm. Cell suspensions were kept in the dark at room temperature for 1 h. Then, to remove the cytoplasm still attached to the nuclei and micronuclei, 250 µL of solution II (composited by 15 g/L of citric acid, 0.25 M of sucrose in ultrapure water) containing 20 µM Helix NP™ Green was added to the samples that were kept in the dark at room temperature for another 15 min. Just before cytometry analysis, cell suspensions were filtered through a 50 µm nylon mesh cell strainer.

The flow cytometry analysis of the nuclei and micronuclei suspension was performed in a GUAVA EasyCyte 8HT (Luminex Corporation, USA) flow cytometer at medium flow. First, the PBMC population was identified according to size with forward scatter (FSC-Hlin) and complexity with side scatter (SSC-Hlog); using this first dot plot, voltages were adjusted to move the nucleus population to the fourth decade of SSC-HLog, that was gated in R1.

In order to discriminate and exclude doublets, a second plot (dot), gated in R1, analyzing forward scatter in height (FSC-HLin) and area (FSC-ALin) was used. In a

third plot (dot), gated in R2, green fluorescence voltage was adjusted so that G1 nuclei were positioned in the third decade; a region was created to include both nuclei and sub 2n chromatin. The fourth plot, Red-B-HLog with Green-B-HLog allowed the identification and exclusion of dead/dying cells. Finally, nuclei and micronuclei populations were differentiated from nonspecific debris particles, based on Green-B-Fluorescence (Red-B-HLog) and Forward Scatter (FSC-HLog); in this final plot, a minimum of 10000 events in the nuclei region were acquired.

The % of micronuclei formed was calculated as for method 1.

3.2.6. Statistical analysis

Statistical analysis was performed in the Windows statistical package SPSS (version 26.0). When data was normally distributed (according to the Kolmogorov-Smirnov goodness of fittest) parametric tests were considered adequate for statistical analysis; differences between groups were tested using the paired samples T test and correlations were tested using the Pearson's correlation test. When data significantly departed from normality (Kolmogorov- Smirnov goodness of fit test) non-parametric tests were considered adequate for the statistical analysis. Differences between groups were tested using Kruskal-Wallis test, associations between variables were analyzed by Wilcoxon signed rank test. Experimental data were expressed as mean ± standard error of the mean and a P-value of 0.05 was considered significant.

4. Genotoxicity associated to prenatal exposure to tobacco smoke

4.1. Study population and questionnaire

The study here presented was carried out in the framework of the NeoGene project (reference FAPESP/19914/2014), a cross-sectional birth study that aims to investigate the genetic and epigenetic effects of prenatal tobacco use and ETS exposure, considering otherpossible co-exposures. The NeoGene project has been previously approved by the Ethics Committee of Centro Hospitalar de São João (CHSJ) (reference nr. 326/16) and the National Commission for Data Protection (2726/2017).

The NeoGene project recruited a total of 838 pregnant women seeking prenatal care in CHSJ, located in the Porto metropolitan area, Portugal, from April 2017 to July 2018. Inclusion criteria comprised women that spoke portuguese and had singleton pregnancy. Women with infectious diseases, mental and physically disabilities were excluded as well as pregnancies with foetal congenital malformations and genetic disorders. Before enrolment, each participant received detailed information about the project objectives and risks and benefits of their participation. All those that agreed to participate provided writteninformed consent.

Participants providing samples for the study were invited to answer a questionnaire in an individual face-to-face interview within 72 hours after delivery, during the hospital stay. Data on demographic and socioeconomic characteristics (e.g., age, years of formal education, occupation), and lifestyles (including tobacco use, ETS exposure, and alcohol intake) were collected. Age was categorized according to Molina-García *et al.,* 2019, and parity as described by Casas *et al.,* 2015; the remaining socio-demographic variables were treated as previously described in Madureira et al., 2020. Tobacco use was defined based on the urinary cotinine concentration (as presented below); ETS exposure was established basedon the reported in the questionnaire. Participants were asked to report the duration and frequency of exposure to ETS at home, work, and leisure places in the 3 months prior to the pregnancy, and in the 1st, 2nd and 3rd trimesters. For statistical analyses, responses were dichotomized as “Yes” and “No” and participants were further classified as exposed to ETS and not exposed to ETS, respectively.

4.2. Sample collection and processing

Sample collection (both urine and cord blood) was possible for 578 participants. For some blood samples, it was not possible to isolate PBMCs used in this study, and therefore 545 samples have been analyzed in this work.

Cord blood was collected in sodium heparin tubes immediately after delivery by the medical and nursing staff of CHSJ; maternal urine was also collected around the time of birth. All samples were coded and transported to the laboratory for processing.

Immediately after arrival to the laboratory, PBMCs were isolated as above described in section 3.2.2. Cell suspensions were frozen with low freezing rate up to -80ºC; freezing medium was composed by 90% FBS and 10% DMSO. Urine samples were aliquoted and frozen at -80ºC until further testing.

4.3. Tobacco exposure assessment (cotinine in urine)

A solid phase competitive ELISA kit (KA0930, Abnova Corporation, Taipei City, Taiwan) with a sensitivity of 1 ng/mL was used to quantify cotinine in urine. Briefly, centrifuged samples and cotinine enzyme conjugate were added to wells coated with anti-cotinine antibody, and then incubated for 60 minutes in the dark. Unbound cotinine and cotinine enzyme conjugate were washed off 3 times with wash buffer. Upon the addition of the substrate, absorbance was measured at 450 nm (Cambrex ELx808 microplate reader) and cotinine concentrations were determined by interpolation in a five-parameter logistic curve. Samples with concentrations above 100 ng/ml were analyzed with three different dilution factors and concentrations calculated using the mean value of these determinations. A cut-off of 74 ng/mL (established on the obtained data; Silva *et* al., 2022) was used to categorize participants in smokers and non-smokers.

4.4. Genotoxicity assessment

As mentioned in section 3.2.4.3, samples were quickly thawed at 37ºC bath water prior to analysis. Then, samples were transferred to a 15 mL tubes containing 2 mL of PBMCs-CCM; microtubes was washed twice with 1 mL of PBMCs-CCM. The falcon

tubes were centrifuged for 10 min at 400 x *g* at 4ºC and supernatant was removed by aspiration. Afterwards, the pellet was resuspended in PBS 7.4 to a total volume of 1 mL, for counting.

The volume containing 5 x 10^5 cells/mL was completed to 1 mL with PBS and γH2AX analysis was initiated (as indicated in section 3.2.4.5). For micronucleus assay, the volume containing 3 x 10^5 cells/mL was also completed to 1 mL with PBS and the protocol initiated as described in section 3.2.5.5.

Study samples were analyzed in parallel with positive control samples.

For γH2AX analysis, 65 aliquots of positive controls were prepared using a pool of 1 x 10^6 PBMCs (obtained as described in section 3.2.2) that were resuspended in 1 mL of PBMCs-CCM and treated for 4 h at 37ºC with 20 µg/mL of bleomycin. After the 4 h treatment, cells were centrifuged for 5 min at 806 x *g* at 21ºC, the supernatant was removed, and cell pellet was resuspended in 1 mL of freezing medium. Cell suspensions were frozen at a gradual slow rate up to -80ºC. At the time of use, positive controls were thawed and immediately used as described in section 3.2.4.5.

For MN analysis, 25 aliquots of positive controls were prepared using a pool of 6 x 10^5 PBMCs (obtained as described in section 3.2.2) that were resuspended in 1 mL PBMCs-CCM and stimulated with 1% PHA for 48 h at 37ºC with 5% CO_2, so that cells would initiate cell division. Then, cells were treated with 0.25 µg/mL of mitomycin C for 4 hr. After exposure, cells were stimulated with 1% PHA for 24 h at 37ºC with 5% CO_2, then cells were centrifuged at 806 x *g* at 21ºC for 5 minutes and supernatant was removed, and cell pellet was resuspended in 1 mL of freezing medium and stored at -80ºC; samples were frozen at a low rate up to -80ºC. At the time of use, positive controls were thawed, and analysis was promptly carried out as described in section 3.2.5.5.

4.5. Statistical analysis

Statistical analysis was performed using SPSS for Windows statistical package (version 26.0). Distribution of the response variables departed significantly from normality (Kolmogorov–Smirnov goodness of fit test) and therefore non-parametric tests were considered adequate for the statistical analysis of these data. Extreme values (outliers) of γH2AX and MN analysis were identified and removed from analysis

(final number of 499 participants). Differences among groups were tested using Chi-squared test, Kruskal-Wallis test and Mann–Whitney U-test. The associations between two variables were analyzed by Spearman's correlation. Experimental data were expressed as mean ± standard error of the mean and a P-value of 0.05 was considered significant.

5. Results

5.1. Method implementation and optimization

5.1.1. Citotoxicity assessment

The MTT cytotoxicity assay was performed with the aim of determining at which concentrations the 3 different compounds (BLM, MMC and MMS) presented cytotoxicity below 55 (± 5%) and could not be used in the γH2AX or micronucleus assay.

To that ending, THP-1 cells were either exposed for 4 h to various concentrations of BLM, MMC and MMS (as required for the γH2AX assay) or exposed for 4 h to various concentrations of BLM, MMC and MMS followed by a recovery period of 44 h (as required for the MN assay), results obtained are shown in figure 1 and 2, respectively.

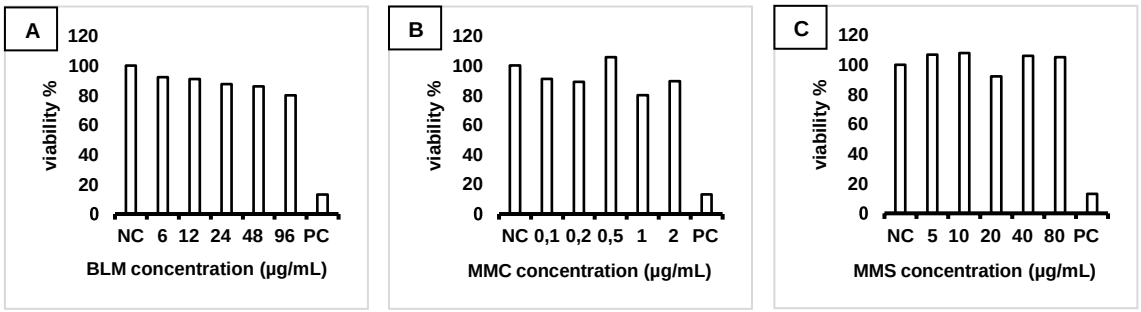

Figure 1. Results of the MTT cytotoxicity assay of THP-1 cells exposed for 4 hours to 3 genotoxic compounds: BLM, MMC, MMS; 1% Triton x-100 used as positive control and non-treated cells as negative control (NC).

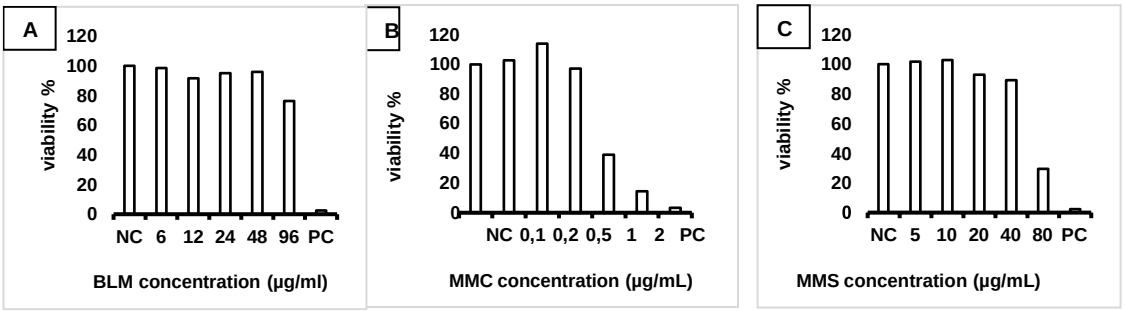

Figure 2. Results of the MTT cytotoxicity assay of THP-1 cells exposed for 4 h to 3 genotoxic compounds: BLM, MMC, MMS followed by a recovery period of 44 h; 1% Triton x-100 used as positive control (PC) and non-treated cells as negative control (NC).

As shown in figure 1, none of the concentrations tested of the three genotoxic compounds were found to be cytotoxic, even though there seems to exist a dose-dependent decrease in viability after exposure to BLM (figure 1A). In the case of MMC and MMS (figure 1B and1C, respectively), small differences in viability (%) were observed but with no evident pattern. In figure 2A, it is observed that none of the BLM concentrations were cytotoxic, although the highest concentration tested (96 µg/mL) decreased cell viability to 76.2%. The two highest concentrations of MMC tested (1 and 2 µg/mL) showed to be cytotoxic (figure 2B). The highest concentration of MMS (80 µg/mL) tested was also deemed as cytotoxic (figure 2C).

5.1.2. H2AX phosphorylation

5.1.2.1. Determination of optimal γH2AX antibody concentration

Figure 3 shows the results obtained for the determination of optimal γH2AX antibody concentration. In the figure 3A, the recommended volume of antibody (5 µL/sample) from BD and two other volumes were tested, all the 3 volumes tested showed similar results of γH2AX % in negative controls and cells exposed to BLM, so further experiments were carried on using the lowest volume of antibody (3.5 µL).

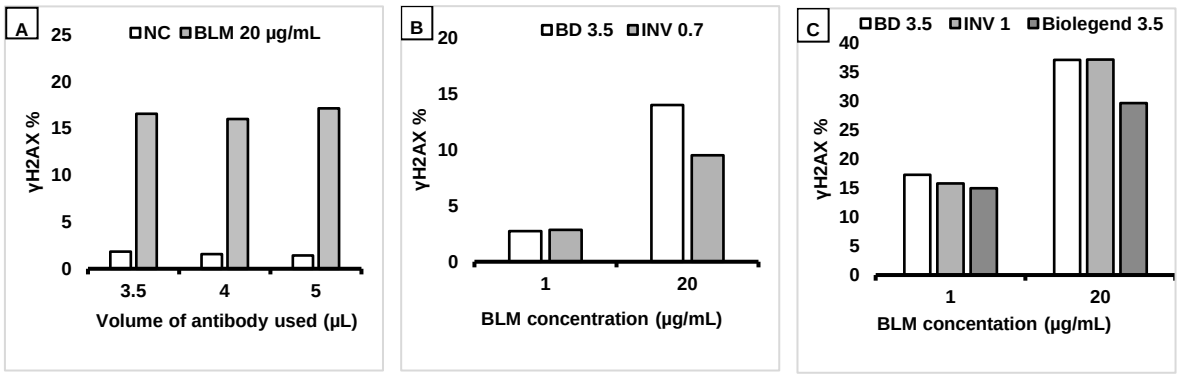

Figure 3. Determination of the optimal γH2AX antibody volume of different brands (INV- Invitrogen; BIOL- Biolegend) used per sample.

Later, 0.7 µL/sample of Invitrogen antibody was tested and compared with the BD antibody (3.5 µL/sample) as shown in figure 3B. The γH2AX % was similar between the two antibodies on the lower concentration of BLM tested (1 µg/mL), however, for the highest concentration of BLM tested (20 µg/mL), the γH2AX % was much lower when compared with the antibody from BD.

For the final antibody test, an additional brand of antibody was included (BioLegend).

In this experiment, frozen PBMCs were exposed to the same two concentrations of BLM. In figure 3C, results obtained showed that the γH2AX % from the volume chosen of the BioLegend antibody were considerably lower when compared to the Invitrogen antibody specially in the highest concentration of BLM tested. Again, the γH2AX % obtained using the Invitrogen antibody (1 μL/sample) was found to be very similar to the one obtained using BD antibody.Given that the Invitrogen antibody was the most cost efficient of all the antibodies, this was employed in all further experiments.

5.1.2.2. Effect of freezing

Table 1 presents the impact of freezing on the levels of phosphorylated H2AX in PBMCs, by presenting the mean values of γH2AX % in the different groups of samples (fresh, frozen and frozen twice). Statistical analysis shows that there is a statistically significant increment($p<0.001$) of γH2AX % in frozen cells when compared to fresh cells, a statistically significant increase ($p=0.005$) of γH2AX % between fresh cells and cells that were frozen twice, and a statistically significant increase ($p=0.005$) of γH2AX % when cells are thawed and frozen again, as presented in table 2. A statistically significant positive high correlation was found between % γH2AX in fresh and frozen ($r=0.818$; $p<0.001$), fresh and frozen twice ($r=0.782$; $p=0.008$) and between frozen and frozen twice ($r=0.818$; $p=0.004$).

Table 1. Effect of freezing on γH2AX % in PBMCs

Condition	N	γH2AX %[1]	Minimum %	Maximum %
Fresh	48	6.93 ± 1.63	0.12	41.69
Frozen	48	22.35 ± 3.70	1.19	81.95
Frozen twice	10	54.63 ± 10.78	16.81	89.18

[1] mean ± standard error of the mean

Table 2. Wilcoxon signed-ranked test (effect of freezing on phosphorylated H2AX %)

	Negative ranks			Positive ranks			Positive ranks		
Condition	n	Mean rank	Sum of ranks	n	Mean rank	Sum of ranks	Ties	Z	p-value
Fresh-Frozen	5	17.20	86	43	25.35	1090	0	-5.149[a]	<0.001
Fresh-Frozen twice	0	0	0	10	5.5	55	0	-2.803[a]	0.005
Frozen-Frozen twice	0	0	0	10	5.5	55	0	-2.803[a]	0.005

The results evidenced in figure 4 show that there are significant differences in the % γH2AX, depending on the type of sample conditioning carried out after the thawing of a frozen sample. It is possible to see that when non-treated PBMCs (negative controls) have a period of 16h of recovery or 24h of stimulation with PHA after thawing, the levels of phosphorylated H2AX significantly increase ($p<0.001$), however when PBMCs are previously treated with 1or 20 BLM μg/mL, the period of recovery or stimulation after thawing enables the cell to significantly reduce DNA damage ($p<0.005$ and $p<0.001$, respectively).

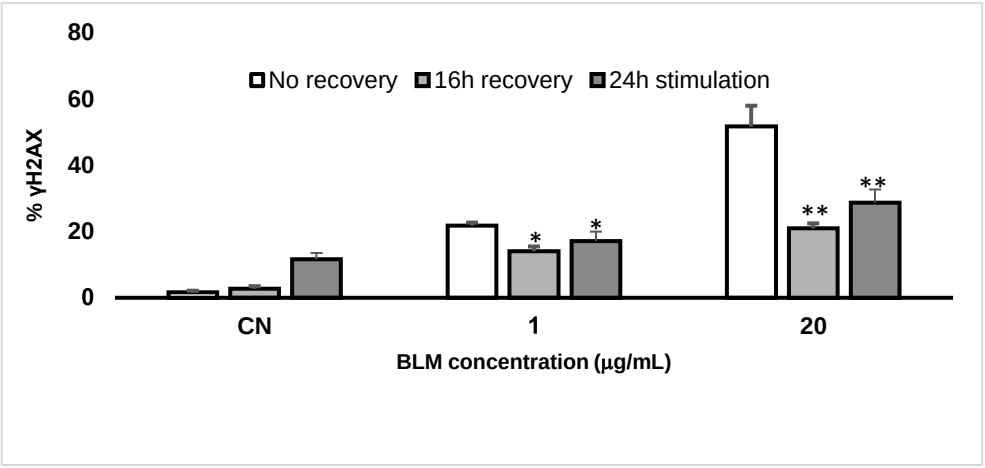

Figure 4. Effect of different types of sample conditioning after thawing on % γH2AX with standard error; *p<0.05 and **p<0.001, significant difference regarding the "no recovery" condition (Kruskal-Wallis test).

5.1.2.3. Effect of stimulation

The effect of stimulation before treatment with various genotoxic compounds in in the levels of phosphorylated H2AX was analyzed in both unstimulated PBMCs and stimulated PBMCs. This experiment was carried out in fresh (figure 5) and frozen (figure 6) PBMCs.

Results show that both in fresh and frozen PBMCs, there is an increase of DNA

damage in all treatments when cells are stimulated prior to treatment, when compared to unstimulated cells, except for the frozen PBMCs treated with 20 μg/mL of BLM.

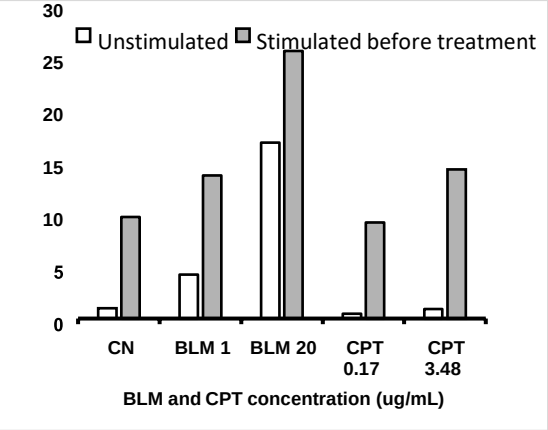

Figure 5. Effect of PHA stimulation before treatment in DNA damage in fresh PBMCs

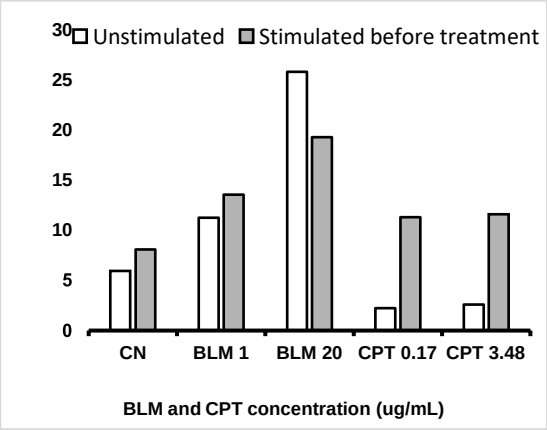

Figure 6. Effect of PHA stimulation in frozen PBMCs regarding DNA damage

To analyze the effect of stimulation on cell's response to DNA damage, PBMCs were stimulated after being treated with bleomycin. As it is shown if figure 7, fresh PBMCs, when stimulated, presented a higher level of phosphorylated H2AX, when compared to unstimulated cells. However, in frozen PBMCs as shown in figure 8, the exact opposite happens, frozen PBMCs stimulated after treatment show a decrease of DNA damage comparatively to unstimulated frozen PBMCs.

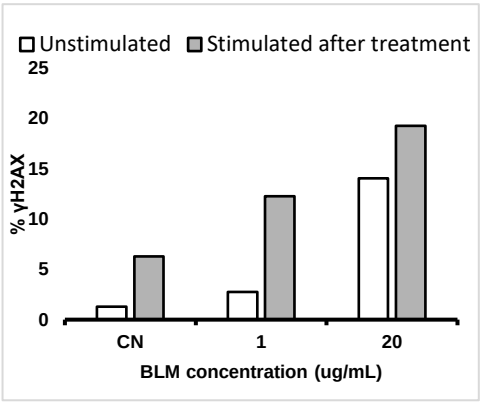

Figure 7. Effect of PHA stimulation before treatment in DNA damage in frozen PBMC

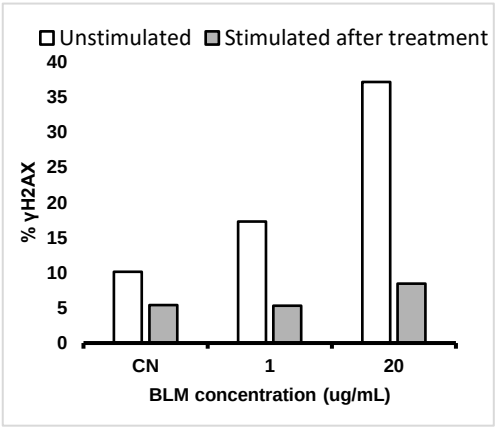

Figure 8. Effect of PHA stimulation in frozen PBMCs regarding DNA damage

5.1.3. MN assay

5.1.3.1. Positive controls

Figure 9 shows the results of both fresh and frozen PBMCs exposed to MMC and MMS. It shows that there is an increase of MN % in frozen cells when compared to fresh cells in all the conditions.

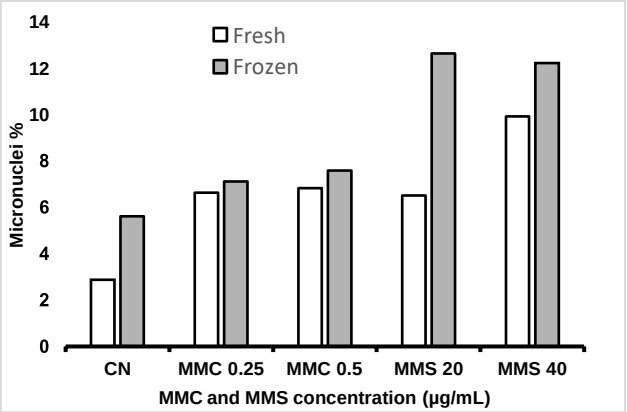

Figure 7. Results of micronuclei % in fresh and frozen PBMCs treated with MMC and MMS.

5.1.3.2. Effect of freezing

Table 3 displays the values of micronuclei % and respective standard error in PBMCs exposed to three different conditions: fresh PBMCs, frozen PBMCs and PBMCs that were thawed and refrozen. The table also shows the minimum and maximum value of each condition. The results obtained show that there is a decrease of micronucleated cells between the fresh and frozen conditions, and a small decrease of micronucleated cells (p=0.159) between the frozen and the 2x frozen conditions.

Table 4 shows that the decrease of micronucleated cells between the fresh and frozen conditions is statistically significant, p=0.001.

In the table 5, it is possible to see that there is a statistically significant positive high correlation between the fresh and frozen cells.

Figure 10 shows that there are significant differences between the conditions analyzed. After cells being frozen, cells that are immediately analyzed display lower levels of micronucleated cells when compared to cells that were either incubated in PBMCs-CCM for 16 h or cells that were incubated with PHA for 24h.

Table 3. Effect of freezing on micronuclei %

Condition	N	Micronuclei %[1]	Minimum %	Maximum %
Fresh	11	8.15 ± 2.94	2.83	11.32
Frozen	11	4.88 ± 2.72	1.95	3.18
2x Frozen	5	2.98 ± 0.57	2.27	3.80

[1] mean ± standard error

Table 4. Paired samples t-test (effect of freezing on micronuclei %)

Condition	p-value
Fresh-Frozen	0.001*
Frozen-2x Frozen	0.159

*p<0.05

Table 5. Pearson's correlation test (effect of freezing on micronuclei %)

Condition	Pearson's ρ	p-value
Fresh-Frozen	0.625	0.040*
Frozen-2x Frozen	0.108	0.862

*p<0.05

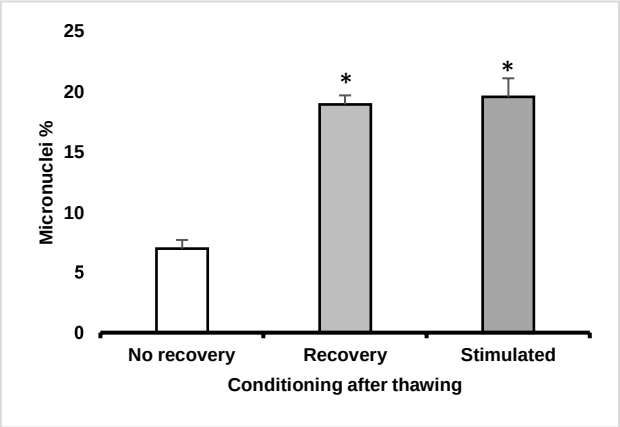

Figure 8. Effect of different types of conditioning after thawing on micronuclei % with standard error; *p=0.003, significant difference regarding the "no recovery" condition (Kruskal-Wallis test).

5.1.3.3. Effect of stimulation

Table 6 displays the % of micronucleated cells in unstimulated and stimulated fresh PBMCs with its respective standard error and minimum and maximum values. It shows that stimulated cells have higher levels of micronucleated cells compared to unstimulated cells, however, that increment is not statistically significant as shown in table 7, p=0.097.

In table 8 it is possible to see that there it exists a positive high correlation between the two conditions, however this correlation is not statistically significant.

Table 6. Effect of stimulation in fresh PBMCs' micronuclei %

Condition	N	Micronuclei %[1]	Minimum %	Maximum %
Unstimulated	5	6.49 ± 3.21	2.83	11.32
Stimulated	5	9.04 ± 2.14	6.20	11.84

[1] mean ± standard error

Table 7. Paired samples t-test (effect of stimulation in fresh PBMCs' micronuclei %)

Condition	p-value
Unstimulated-Stimulated	0.097

*p<0.05

Table 8. Pearson's correlation test (Effect of stimulation in fresh PBMCs' Micronuclei %)

Condition	Pearson's ρ	p-value
Unstimulated-Stimulated	0.576	0.310

*p<0.05

Table 9 shows the percentages of micronucleated in both unstimulated and stimulated frozen PBMCs. It also displays the standard error and minimum and maximum values.

In frozen PBMCs there is a significant increase of micronucleated cells when cells are stimulated, p<0.001 as shown in table 10.

Table 11 shows a positive high correlation between the results obtained in unstimulated and stimulated PBMCs.

Table 9. Effect of stimulation in frozen PBMCs on micronuclei %

Condition	N	Micronuclei %[1]	Minimum %	Maximum %
Unstimulated	11	4.88 ± 2.72	1.95	9.34
Stimulated	11	18.98 ± 4.48	11.71	25.32

[1] mean ± standard error

Table 10. Paired samples t-test (effect of stimulation in frozen PBMCs on micronuclei %)

Condition	p-value
Unstimulated-Stimulated	0.000*

*p<0.05

Table 11. Pearson's correlation test (effect of stimulation in frozen PBMCs onmicronuclei %)

Condition	Pearson's ρ	p-value
Unstimulated-Stimulated	0.518	0.102

*p<0.05

5.2. Genotoxicity associated to prenatal exposure to tobacco smoke

The study population of this project consisted of 499 participants. Cotinine levels were analyzed in the participants urine where a cut-off of 74 ng/mL was established (from the obtained data) in order to distinguish the non-smokers from the smokers.

Table 12 displays the study population characteristics according to the cotinine validated smoking status, 83 were considered smokers and 416 non-smokers. Most participants in this study that are smokers have portuguese nationality (98.8%), the table shows that women with 2 or more children have a higher percentage of smokers and that non-smokers self-reported that they are exposed to ETS (66.9%).

From all the variables analyzed, age (p=0.011), education level (p<0.001) and occupation (p=0.002) were the only variables that had a statistically significant difference between the group of smokers and non-smokers. The table shows that there is a higher percentage of younger women (<25 years) that are smokers than non-smokers, it is also evidenced that unemployed/housewives and women with lower educational level are more likely to smoke during pregnancy.

Table 13 shows the correlations between the three biomarkers used in this study. The results show that there is a positive low correlation between the biomarkers, that is statistically significant.

Table 14 displays the frequencies of phosphorylated H2AX and micronucleated

cells according to the population characterization.

Regarding γH2AX levels, no significant differences were observed in any of the categories analyzed. Younger women presented slightly higher levels of phosphorylated H2AX, Portuguese women had lower levels of γH2AX, among smokers, pregnant women that ceased smoking during pregnancy evidenced lower levels of phosphorylated H2AX compared to women that did not stop smoking.

Regarding MN %, there is a statistically significant difference regarding the ages of the participants ($p=0.034$) younger women (< 25 years and 25-29 years) showed higher frequency of micronuclei %, also women with higher levels of education had lower levels of micronuclei %, unemployed women showed higher frequency of micronucleated cells and that smokers (cotinine-validated) have higher frequencies of micronuclei %.

Table 12. Study population characteristics according to cotinine validated smoking status

	Total (n=499)	Smokers (n=83)	Non-Smokers (n=416)	p-value
Age	31 ± 6 (18-46)	30 ± 6 (18-46)	31 ±5 (18-44)	0.011*
< 25 years	59 (11.7%)	17 (20.5%)	42 (10.1%)	0.047*
25-29 years	125 (25.1%)	21 (25.3%)	104 (25.0%)	
30-34 years	175 (35.1%)	27 (32.5%)	148 (35.6%)	
≥ 35 years	140 (28.1%)	18 (21.7%)	122 (29.3%)	
Nationality				
Portuguese	471 (94.4 %)	82 (98.8%)	389 (93.5%)	0,056
Other	28 (5.6 %)	1 (1.2%)	27 (6.5%)	
Area of residence**				
Sub-urban/rural area	27 (5.4%)	3 (3.6%)	24 (5.8%)	0,423
Urban area	470 (94.6%)	80 (96.4%)	390 (94.2%)	
Education Level				
≤ 9 years	130 (26.0%)	41 (49.4%)	89 (21.4%)	<0.001*
10-12 years	184 (36.9%)	31 (37.3%)	153 (36.8%)	
≥ 13 years	185 (37.1%)	11 (13.3%)	174 (41.8%)	
Occupation				
Unemployed/Housewives	101 (20.2%)	28 (33.7%)	73 (17.5%)	0.002*
Students	11 (2.2%)	2 (2.4%)	9 (2.2%)	
Employed - Manual Job	177 (35.5%)	31 (37.4%)	146 (35.1%)	
Employed - Non-manual Job	210 (42.1%)	22 (26.5%)	188 (45.2%)	
Alcohol consumption				
No	432 (86.6%)	71 (85.5%)	361 (86.8%)	0,763
Yes	67 (13.4%)	12 (14.5%)	55 (13.2%)	
Parity				
0	279 (55.9%)	39 (47.0%)	240 (57.7%)	0,051
1	178 (35.7%)	32 (38.5%)	147 (35.1%)	
≥ 2	42 (8.4%)	12 (14.5%)	30 (7.2%)	
Tobacco dose				
Cigarettes/day		6.4 ± 5.1 (0.0-20.0)		NA
ETS (self-reported) **				
Exposed			277 (66.9%)	NA
Non-exposed			137 (33.1%)	

*p<0.05; **2 missing values

Table 13. Spearman's correlation test among studied biomarkers

Biomarker	Spearman's ρ
γH2AX %-MN %	0,129*
γH2AX %-Cotinine (ng/mL)	0.089*
MN %-Cotinine (ng/mL)	0.110*

*p<0.05

Table 14. Frequency of γHAX and MN according to population characteristics

	N	γH2AX %	p-value	N	MN %	p-value
Total	499	3.98 ± 0.13		333	8.42 ± 0.61	
Age						
< 25 years	59	4.72 ± 0.46	0,217	36	9.81 ± 2.14	0.034*
25-29 years	125	4.23 ± 0.32		87	10.83 ± 1.43	
30-34 years	175	3.89 ± 0.19		108	7.40 ± 1.14	
≥ 35 years	140	3.56 ± 0.17		102	6.94 ± 0.67	
Nationality						
Portuguese	471	3.96 ± 0.13	0,119	313	8.54 ± 0.65	0,712
Other	28	4.41 ± 0.43		20	6.63 ± 1.13	
Area of residence						
Sub-urban/rural area	27	3.90 ± 0.49	0,973	18	8.62 ± 1.15	0,65
Urban area	470	3.98 ± 0.13		315	8.40 ± 0.63	
Education Level						
≤ 9 years	130	3.93 ± 0.25	0,854	89	9.31 ± 1.21	0,088
10-12 years	184	4.07 ± 0.21		124	9.17 ± 1.16	
≥ 13 years	184	3.94 ± 0.20		120	6.98 ± 0.82	
Occupation						
Unemployed/Housewives	101	4.34 ± 0.35	0,429	63	11.01 ± 1.70	0,075
Students	11	4.47 ± 0.92		7	3.21 ± 1.11	
Employed – Manual Job	177	3.82 ± 0.22		127	8.64 ± 1.09	
Employed – Non-manual Job	210	3.93 ± 0.16		136	7.27 ± 0.76	
Alcohol consumption						
No	432	3.98 ± 0.14	0,904	296	8.67 ± 0.68	0,238
Yes	67	4.04 ± 0.34		37	6.36 ± 1.15	
Parity						
0	279	4.10 ± 0.18	0,265	186	8.84 ± 0.91	0,993
1	178	3.76 ± 0.19		119	8.18 ± 0.94	
≥ 2	42	4.16 ± 0.35		28	6.62 ± 1.11	
Smoking Status (cotinine-validated)						
Smokers	83	4.07 ± 0.29	0,374	54	10.85 ± 2.20	0,084
Non-Smokers	416	3.97 ± 0.14		279	8.01 ± 0.64	
Cessation (among smokers)						
No cessation	75	4.06 ± 0.31	0,208	48	1.71 ± 0.49	0,321
Cessation 1st trimester	48	3.40 ± 0.28		32	1.90 ± 0.43	
Cessation 2nd trimester	7	3.76 ± 0.47		3	18.56 ± 4.06	
Cessation 3rd trimester	2	1.48 ± 0.01		2	1.18 ± 3.53	
ETS exposure (self-reported) among non-smokers)						
Non-exposed	137	3.97 ± 0.21	0,623	78	7.81 ± 1.11	0,991
Exposed	277	3.94 ± 0.18		201	8.08 ± 0.78	

*p<0.05

6. Discussion

Throughout life, humans are constantly exposed to chemicals that cause harm, the recognition that exposures could produce damage to the DNA was a major landmark for prevention and risk assessment (Baccarelli and Bolatti, 2009).

Tobacco smoke is a major environmental pollutant, considered the biggest example of a systematic mutagen (Yauk *et al*., 2007) that englobes a large amount of chemicals from which approximately 250 are known to be toxic to humans (Baker, 2006; Husgafvel-Pursiainen, 2004). Even though it is known that tobacco smoking is responsible for a lot of diseases that can lead to death, such as cancer, strokes, or chronic obstructive pulmonary disease (de la Chica *et* al., 2005; Feil and Fraga, 2012; Weng *et al*., 2018).

Despite all these complications, tobacco smoking is still very prevalent in today's society, inevitably affecting the smoker but also surrounding people that inhale ETS (Avsar *et al*., 2008; WHO, 2019). The dangers of tobacco smoke or ETS is especially high regarding pregnant women because the tobacco smoke will impact the pregnant woman as well as the newborn (Almanzar *et al*., 2013).

Intra uterine development of the newborn is known to be a period of high susceptibility to chemical compound, as it is marked by the rapid development of organs and tissues, physiological immaturity and greater absorption and retention of toxic substances (Whyatt and Perera, 1995) as well as the deficiency in the metabolic condition of the individual, which is why it can be affected by exposure levels that do not affect adults (Perera, 2008). Several studies suggest that fetus' gene expression can be linked with maternal exposures,thus there may be an adaptive response of the fetus to changes in the uterus that may prove permanent and influence its health later in life (Fucic et al., 2008).

This study pretended to estimate the impact of a maternal exposure to tobacco smoke in umbilical cord blood samples of newborns. Genetic damage was assessed using H2AX phosphorylation and micronucleus frequency assays, which were later compared to urinary cotinine concentrations and questionnaires.

In recent years, there has been an increase of studies that used the flow cytometry γH2AX assay as biomarker of genotoxicity, as it is an assay that has shown to be a robust assayable to detect DNA-damaging agents (Kopp *et al*., 2019), with high

throughput, making it torapidly quantify the levels of phosphorylated H2AX in many samples (Smart *et al*., 2011).

It is a very sensitive method that can detect the existence of DNA DSBs, which are very hazardous since a single unrepaired DSB can cause cell death and ultimately lead to chromosomal rearrangements if not repaired (Sánchez-Flores *et* al., 2015; Yamamoto *et al*., 2011). Furthermore, as γH2AX is an early event of DNA damage response it appears 30 minutes after the induction of DSBs (Valdiglesias *et al*., 2011), the persistence of γH2AX, even after DNA repair can be a signal of lethal DNA damage or genomic instability (Podhorecka *et al*., 2010). Due to the practicality and sensitivity of this technique, this assay can be used in a large array of studies, as it was found to be useful in DNA damage biomonitoring studies, in detecting early stages of cancer (Porcedda *et al*., 2006). However, due to the lack of protocol standardization, the results obtained using this assay are very different between investigation groups making its institution as a routine biomarker in populational studies harder (Sánchez-Flores *et al*., 2015).

So, with that in mind, a meticulous process of optimization and implementation was carried out, to determine how PBMCs respond to genotoxic compounds known to cause DSBs, to cell stimulation with PHA, and freezing and thawing regarding the levels of phosphorylated H2AX analyzed by flow cytometry, which is a technique with high yield, reproducible whereit is possible to analyze a large amount of samples, however this technique is less specific compared to the more traditional technique, that uses the microscope (Brzozowska *et* al., 2012; Watters *et al*., 2009).

During the optimization process various conditions were tested to understand in what way they would affect the levels of γH2AX. Our data has shown that during storage, freezing and thawing process, cells are susceptible to DNA damage which drastically increases the basal levels of γH2AX. This result is supported by the work of Sánchez-Flores, 2015 in which it was also possible to see that the freezing process raised basal levels of γH2AX. To the best of our knowledge, no studies tested cell recovery process after thawing process. In negative controls, stimulation of 24h with PHA, allowed the cell to start its cell cycle, resulting in higher basal levels of γH2AX caused by the damage of the freezing process. Treated cells conditioned in PBMCs-CCM or simulated with PHA were able respond to the damage and reduce its levels of γH2AX. Regarding cell stimulation with PHA, fresh and frozen cells stimulated with PHA after treatment with genotoxic compounds have shown significant increase in γH2AX levels

except in the treatment with the highest concentration of bleomycin in frozen cells, this decrease can be explained by cell death of the most damaged cells resulting in a lower level of γH2AX (Huang *et al*., 2003). Fresh cells stimulated with PHA after treatment evidenced higher levels of γH2AX, frozen cells were able to react to the damage caused by the freezing process and reduce γH2AX levels in negative control and treated cells. From our data we can conclude that stimulation prior to cell treatment activates cell cycle that leads to DNA damage to the cells, however in frozen cells, stimulation after treatment enables a cell response to DNA damage.

With the results obtained in the γH2AX assay, we can conclude that when γH2AX levels are measured at a single point, this result provides us the current DNA damage caused by DSBs in the cell, but when the γH2AX levels are read at different moments a decrease in γH2AX levels can be correlated with the repair of DSBs (Bourton et al., 2011; Brzozowska et al., 2012).

Regarding human population studies, since many times it is impossible to analyze samples right after collection, sample cryopreservation is a good option; the implementation of cell stimulation with PHA should be decided during study design according to the goal of the study and mechanism of the individuals.

The micronucleus assay has been categorized as a reliable alternative to the chromosome aberration test (Bryce *et* al., 2013; Corvi *et al*., 2008), with a superior ability to detect aneugens and the fact that can be executed with less technical expertise (Avlasevich *et* al.,2006; Fenech *et al*., 2011). The flow cytometry MN assay is a method that has been gaining more traction over the years due to its high yield, practicality, and possibility to analyze a large number of samples in less time (Bryce *et al*., 2008).

In this study, during the optimization process of this method, several variables were tested to see how they would influence the micronuclei %. Our data showed that the freezing and thawing process does not hinder cell's ability to respond to PHA stimulation and that this process did not increase the basal levels of micronuclei %, as previously described by Zijno *et al*., 2007, moreover, in fact, in our study the micronuclei % decayed after freezing process, this decrease can be explained by the cell death of the most damaged cell duringthis process.

The effect of PHA stimulation in both fresh and frozen cells was analyzed being determined that cell stimulation with PHA increases the micronuclei %, an expected result since cell stimulation "gives an opportunity" to initialize its cell cycle and express

the DNA accumulated *in vivo* (Speit *et al*., 2012).

After freezing, cells were analyzed by flow cytometry immediately after thawing process, after a period of recovery in cell medium or after cell stimulation with PHA, being evidenced that a recovery period or stimulation of the cells significantly increases micronuclei %, this data suggests that this type of cells is not capable to reduce its basal levels of damage after being thawed. This study further confirms that PBMCs can be cryopreserved, stored and used for micronuclei analysis.

It was determined through the levels of cotinine in the urine of the participants (n=499), 416 were considered non-smokers and 83 were considered smokers. Three biomarkers were used to analyze the data, cotinine levels, γH2AX levels and micronuclei %; correlations between these biomarkers were low but significant, that, in one hand, shows that there is no clear association between tobacco exposure and DNA damage levels, and on the other, confirms that the γH2AX and micronuclei assays detect different types of DNA damage biomarkers.

The results in this study regarding γH2AX levels did not show significant differences in all the categories analyzed. Younger women showed higher levels of γH2AX, this value correlates to the fact that <25 age group has a higher % of smokers. Unemployed women,which also is the group with higher % of smokers, showed higher levels of γH2AX comparatively to employed women. γH2AX levels were also higher in smokers comparatively to non-smokers. Cessation of smoking during pregnancy also seemed to reduce γH2AX levels.

The γH2AX assay is a technique that can be used in various types of investigations since it is a good technique to detect DSBs formation in cells (Kopp *et al*., 2019), however it seems to exist no data that directly correlates γH2AX levels and tobacco usage.

The results in this study regarding micronuclei frequency showed that younger pregnant women had higher micronuclei % comparatively to older pregnant women. This result does not follow the scientific consensus that ageing gradually increases the levels of micronuclei (Fenech and Bonassi, 2011; Zijno *et al*., 2007), nonetheless this result can be explained because between the four different age groups, in the under 25 years old, there is a higher percentage of smokers within the group, according to the cotinine levels, which elevates the frequency of micronuclei. Data shows that there is a non-significant correlation between higher levels education and lower micronuclei

%, in accordance with Ceretti *et al*., 2020 work. Unemployed women were the group that showed higher levels of micronuclei % although no statistical significance was verified, Murgia *et al*., 2008 study showed that occupation had no influence in micronuclei %, data also showed that smokers, cotinine-validated, showed higher % of micronuclei (p=0.084), however, published data showed non-significant decrease of micronuclei % in current and former smokers comparatively to non-smokers (Fenech and Bonassi, 2011; Fenech *et al*., 2011; Hitoshi *et al.*, 2003).

7. Conclusion

In a first stage, this study allowed us to clarify what happens to DNA damage levels, namely γH2AX levels and micronuclei frequency, in cells that undergo cryopreservation and stimulation .

It was possible to conclude that, amongst the different genotoxic compounds tested, bleomycin was the best genotoxic compound to generate DSBs, as detected by the γH2AX assay. Regarding formation of micronuclei, mitomycin C was found to be the genotoxic responsible for a greater increase in the frequency of micronuclei in cells.

Regarding H2AX analysis, data showed that when cells are submitted to stimulation with PHA before treatment, the levels of genetic damage increase; on the other hand, when cells are stimulated with PHA after being treated with bleomycin, there is a decrease in the levels of γH2AX.

Regarding the impact of the freezing process, results showed that cells are damaged during the freezing process resulting in an increase of phosphorylated H2AX; the opposite is observed for the micronuclei frequency that decreases after one freezing cycle. Our data also evidenced that, after freezing, cells with higher levels of damage can recover after a period of recovery or stimulation with PHA.

The determination of γH2AX levels and % micronuclei in the NeoGene cord blood samples suggested that age, education, and occupation may have some importance on DNA damage levels, but no significant association between tobacco exposure and genetic damage was observed.

The results obtained in this study provide a scientific basis for the adoption of a better study design and sample handling in future biomonitoring studies using PBMCs. Due to the lack of studies with γH2AX and prenatal exposure to tobacco smoke, more studies are needed on this topic to confirm results here presented.

8. References

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