Universidade de Trás-os-Montes e Alto Douro

Application of comet assay to evaluate the impact of noise in DNA damage levels among exposed workers

Dissertação de Mestrado em Biotecnologia para as Ciências da Saúde

Catarina Isabel Pereira de Sousa

Orientadora: Doutora Cristiana Maria Matos da Costa Pereira

Coorientadora: Professora Doutora Isabel O'Neill de Mascarenhas Gaivão

Vila Real, 2021

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– Versão Provisória –

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Composição do júri:

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The ideas here exposed are the author’s full responsibility.

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Resumo

O ruído está associado a vários efeitos prejudiciais não auditivos cardiovasculares, e celulares, tais como a produção de espécies reativas de oxigénio que podem causar dano oxidativo no DNA.

O presente projeto teve por objetivo a avaliação do dano oxidativo no DNA em sangue total, através do ensaio do cometa modificado com Fpg, em indivíduos ocupacionalmente expostos ao ruído. O estudo incluiu 56 trabalhadores de uma indústria metalúrgica, 44 dos quais envolvidos na produção (grupo exposto) e 12 em atividades administrativas ou semelhantes (grupo não-exposto). Os valores de exposição ao ruído foram obtidos pelo Departamento de Saúde Ambiental do Instituto Nacional de Saúde Doutor Ricardo Jorge, através de um procedimento interno acreditado, baseado na norma internacional ISO 9612:2011. Em simultâneo, foi avaliada a frequência cardiaca e de pressão vascular dos indivíduos.

As condições a utilizar no ensaio do cometa, concretamente a concentração de Fpg, foram inicialmente otimizadas com um ensaio de titração em que foram testadas diferentes concentrações de Fpg (1.16, 1.33, 2.26 and 2.6 U/mL), para deteção do dano oxidativo de células mononucleares expostas a $KBrO_3$ (0.5, 1.5, 4.5 mM). A metodologia otimizada foi utilizada para determinar os níveis de dano oxidativo no DNA em amostras de sangue total dos indivíduos incluídos no estudo.

A análise dos resultados obtidos mostrou que não existiam diferenças estatisticamente significativas entre o grupo exposto e grupo não-exposto, nos níveis de dano oxidativo no DNA (p = 0.740), frequência cardíaca (p = 0.089), pressão arterial sistólica (p = 0.553), e pressão arterial diastólica (p = 0.271). Numa segunda abordagem, a amostra foi estratificada de acordo com os níveis de exposição ao ruído (≤85, entre 85 e 87, e ≥87 dB(A); valores de ação superior e limite de exposição, de acordo com a lei portuguesa); também neste caso, as diferenças entre os grupos no que diz respeito aos níveis de dano oxidativo no DNA, frequência cardíaca, pressão arterial sistólica, e pressão arterial diastólica não foram estatisticamente significativas (p = 0.794, p = 0.494, p = 0.995, e p = 0.499, respetivamente).

Desta forma, o presente trabalho não provou haver uma relação entre a exposição ao ruído ocupacional e o aumento de dano oxidativo no DNA ou ocorrência de problemas cardiovasculares na amostra usada.

Palavras-Chave: Dano oxidativo no DNA; ensaio do cometa; Fpg; ruído.

Abstract

Noise is associated with many non-auditory harmful and cardiovascular effects such as vascular dysfunction, hypertension, leading to myocardial infarction, strokes, or heart failure and may cause oxidative DNA damage due to the release of ROS because of the stress.

The present project aimed the evaluation of oxidative DNA damage in whole blood, through the Fpg-modified comet assay technique, in individuals exposed to occupational noise. This project included a sample of 56 workers of a metallurgic factory, 44 were working on the production ("Exposed" group), and 12 were performing administrative activities, or similar ("Non-exposed" group). The noise exposure values were obtained by the Environmental Health Department of the National Health Institute Doutor Ricardo Jorge under the accredited internal procedure based on the International Standard ISO 9612:2011. Simultaneously, was evaluated the heart rate and vascular pressure of the individuals.

The comet assay conditions, specifically the Fpg concentration, were initially optimized with a titration assay where different Fpg concentrations (1.16, 1.33, 2.26 and 2.6 U/mL) where tested to allow the detection of oxidative damage on periferal blood mononuclear cells exposed to $KBrO_3$ (0.5, 1.5, 4.5 mM). The optimized method was used to assess the oxidative DNA damage in the whole blood samples of the individuals included in the study.

The analysis of the results showed that there were no statistically significant differences between the "Exposed" and the "Non-exposed" groups, regarding the oxidative DNA damage (p = 0.740), heart rate (p = 0.089), systolic blood pressure (p = 0.553), and diastolic blood pressure (p = 0.271). On a second approach, the sample was separated according to the exposure noise levels (≤85, between 85 and 87 e ≥87 dB(A); higher action and exposure limit values, acording to the portuguese law); also in this case, the differences between groups, regarding the oxidative DNA damage, heart rate, systolic and diastolic blood presures did not presented statistically significant differences (p = 0.794, p = 0.494, p = 0.995, e p = 0.499, respectively).

Thus, the present work did not show correlation between occupational noise exposure and the increase of oxidative DNA damage or occurrence of cardiovascular issues in the present sample.

Keywords: comet assay; Fpg; noise; oxidative DNA damage.

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Abbreviations List

% – percentage

%TI – percentage of tail intensity

AP site – apurinic/apyrimidinic site

BF – Buffer F

BPM – Beats per minute

dBA – A-weighted decibels

DBP – Diastolic blood pressure

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

eNOS – endothelial nitric oxide synthase

FBS – Fetal bovine serum

Fpg – formamidopyrimidine DNA glycosylase

IARC – International Agency for Research on Cancer

NADPH – nicotinamide Adenine Dinucleotide Phosphate

ºC – degree Celsius

OECD – Organisation for Economic Co-operation and Development

PBMC – peripheral blood mononuclear cells

PBS – phosphate buffered saline

ROS – reactive oxygen species

SBP – Systolic blood pressure

TE – tris-EDTA

TI – tail intensity

U/mL – units per millilitre

x g – times gravity, the unit of relative centrifugal force (RCF)

1. Introduction

Noise is known to be any sound that is unpleasant, or disturbing and causes unwanted effects through a direct or indirect pathway (Münzel *et al.*, 2017c), being a physical hazard *(Al-Arja and Awadallah*, 2020) and a non-specific stressor that affects the autonomic nervous and endocrine systems (Münzel *et al.*, 2017a).

Noise affects humans physiological functions and processes in various degrees depending on its characteristics: intensity, frequency, sound level, exposure time, nature, among others (Münzel *et al.*, 2017c; Lu *et al.*, 2018), and it may be essentially environmental and occupational.

Environmental noise (figure 1) is defined as any kind of harmful sound created during human activities, referring mainly to traffic, railways and aircraft (Al-Arja and Awadallah, 2020). On the other hand, occupational noise is defined as any kind of unwanted sound formed in a working environment (Al-Arja and Awadallah, 2020) and it is known to be the most common risk factor at workplaces, especially in the manufacturing industry since the application of machinery and industrial equipment leads to increased noise levels (Zare *et al.*, 2016), especially in mines, and in food, metal and drug industries (Bagheri Hosseinabadi *et al.*, 2019).

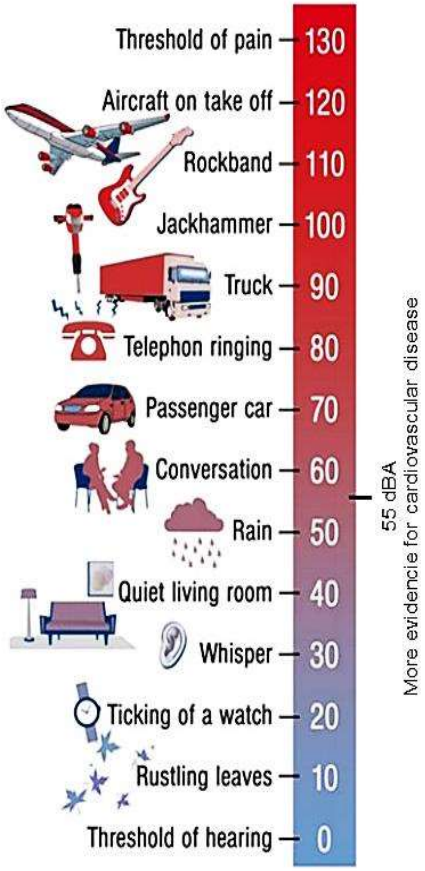

Figure 1 – Examples of several sources associated with different sound levels, on a decibel scale (dBA), representing examples of environmental noise. Adapted from Münzel et al. (2017c).

1.1. Noise impacts on health

Noise is responsible for many harmful effects (Figure 2). Besides hearing loss, general noise causes non-auditory effects such as annoyance, sleep disturbance, tinnitus and psychological stress (Münzel *et al.*, 2017c). It also leads to many cardiovascular effects such as vascular dysfunction, coronary heart disease, hypertension, atherosclerosis, and other complications such as myocardial infarction, strokes and congestive heart failure (Münzel *et al.*, 2017b; Al-Arja and Awadallah, 2020; Li *et al.*, 2019).

Environmental noise, even when in chronic low levels, can cause disturbances of activity, sleep and communication, leading to emotional responses such as stress and annoyance (Münzel *et al.*, 2017a). High levels of environmental noise have been associated with mental health diseases like depression and anxiety, that are also recognised as conditions that adverse cardiovascular functions (Münzel *et al.*, 2018).

When in a working place, occupational noise is known to be a distraction for cognitive performance, leading to decreased productivity and performance, lack of communication and

potentiating human errors thus increasing the chances of an accident (Golmohammadi *et al.*, 2020; Al-Arja and Awadallah, 2020).

Chronic noise exposure and stress by itself are already known to be a generators of cardiovascular risk factors such as increased blood pressure and dyslipidaemia, increased blood viscosity and glucose levels and activation of blood clotting factors (Münzel *et al.*, 2017a; Münzel *et al.*, 2018; Basner *et al.*, 2014).

Lu *et al.* (2018) considers annoyance to be one of the most harmful effects of occupational noise since that the physical stimulus provokes a subjective psychological response, causing different degrees of annoyance in different situations, and it can be triggered even by sound levels that wouldn't risk hearing damage.

Noise is also associated with increased levels of adrenaline that cause lack of sleep quality and increased systolic blood pressure, since the change of hormonal levels will affect several biological processes. It also increases the levels of some enzymes such endonuclease-G, that may cause DNA damage, increasing oxidative stress, and induce external and internal caspase-mediated cell death pathways and DNA damage pathways (Bagheri Hosseinabadi *et al.*, 2019). It may induce stereotypic activation of pathways such as oxidative stress, vascular dysfunction, autonomic imbalance and amplify the impact of traditional risk factors on cardiovascular diseases (Münzel *et al.*, 2017b).

The mechanisms regarding occupational or environmental noise exposure and the risk for cardiovascular diseases is not clear. Besides, it is accepted to think that the pathway from noise exposure to these clinical features implies the activation of the autonomic nervous and endocrine systems, causing the blood pressure to increase, alteration of the heart rate and the release of stress hormones, resulting in the heart diseases mentioned earlier (Li *et al.*, 2019; Basner *et al.*, 2014).

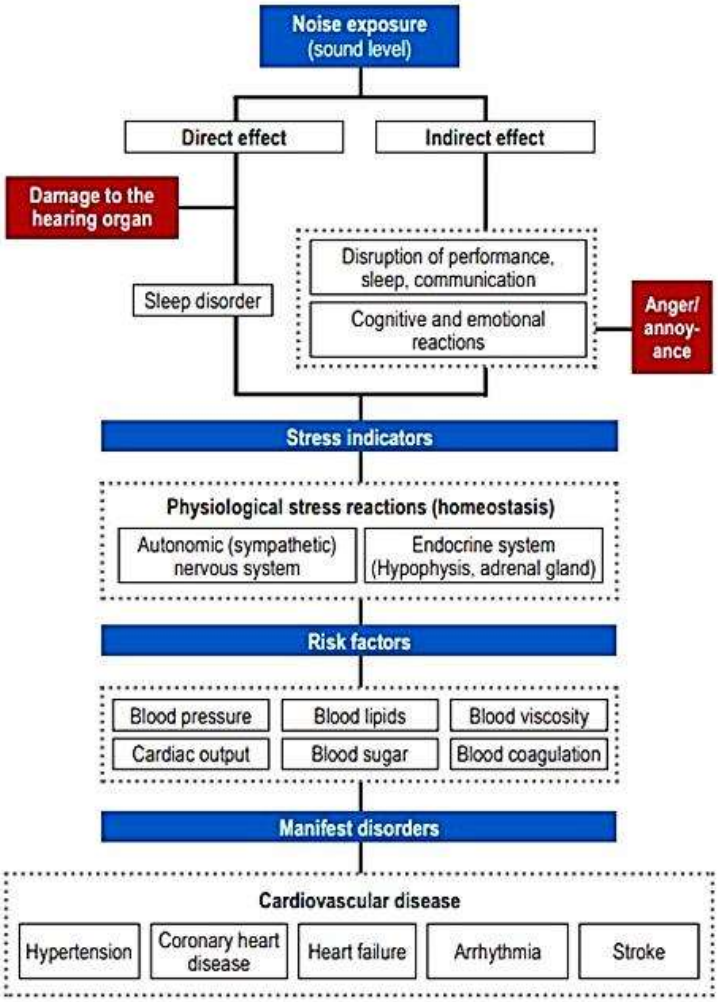

Figure 2 – The noise affect model, representing how noise exposure leads to increased development of cardiovascular risk factors, mediated by physiological and psychological stress reaction. Adapted from Hahad et al. (2019a).

1.1.1. Noise and oxidative stress

Noise exposure may lead to elevated production of reactive oxygen species (ROS), consequently causing tissue damage and lipid peroxidation, that are associated with aging, neurodegenerative diseases and cancer (Bagheri Hosseinabadi *et al.*, 2019).

Even though the mechanism behind the association between noise and cardiovascular damage and disease is not completely understood, it has been proposed that chronic noise stress may lead the perturbation of the autonomic nervous system and sympathoadrenal activation may increase levels of circulating stress hormones, as cortisol, promoting a subsequent oxidative stress-induced endothelial dysfunction, that is an early predictor for atherosclerosis,

accompanied by the release of proinflammatory mediators and activation of prothrombotic pathways, which may lead to vascular dysfunction (Münzel *et al.*, 2018; Hahad *et al.*, 2019b).

Münzel *et al.* (2018) proposed a potential mechanism underlying noise-induced cardiovascular diseases (Figure 3). Their research is based on epidemiological evidence and mechanistic insights from translational human and animal data, proposing that noise provokes a stress response, as described above, being this one characterized by the activation of the sympathetic system and increased levels of catecholamines, cortisone, angiotensin-II, among others. Angiotensin-II is an activator of the vascular and phagocytic NADPH oxidase, that can promote oxidative stress in blood. ROS forage nitric oxide and provoke endothelial nitric oxide synthase (eNOS) uncoupling due to oxidation of the eNOS cofactor tetrahydrobiopterin and eNOS S-glutathionylation, leading to increasing vascular oxidative stress. On a gene regulation base, ROS damage signalling pathways centred around phosphatidylinositol 3-kinase, converting growth factor-b1 and nuclear factor-kB, leading to activation of endothelin-1 system, increasing levels of circulating interleukin-6, and higher expression of vascular adhesion molecules, which would increase inflammation. Immune cells as neutrophils, natural killer cells and monocytes produce higher amounts of superoxide and nitric oxide leading to oxidative protein modifications, and adverse redox-regulatory effects on cellular signalling pathways. Higher glucocorticoid levels contribute to reduction of endothelial nitric oxide production, impairment of vasodilation and increased blood pressure. Constrictive pathways are activated by the increased levels of catecholamines, endothelin-1 and glucocorticoids. These vascular alterations may contribute to arterial hypertension, coronary heart disease, heart failure and metabolic disorders.

Moreover, a study by Bagheri Hosseinabadi *et al.* (2019) showed that prolonged noise exposure can perturb antioxidant defences. The increased activity of some antioxidant enzymes reflects detoxication of ROS, leading to cell damage, specially through the lipid peroxidation pathway.

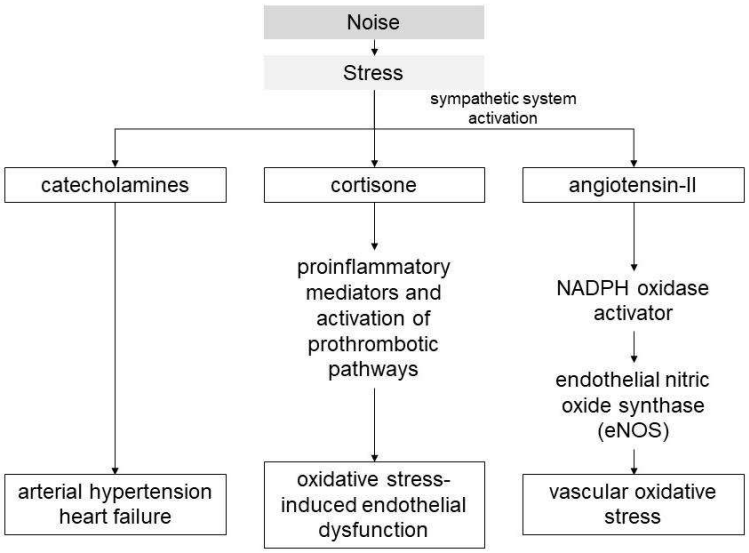

Figure 3 – A potential mechanism underlying noise-induced cardiovascular diseases, on a non-gene regulation base, proposed by Münzel et al. (2018).

1.1.2. Portuguese laws about noise

Noise is considered as one of the most common physical factors contributing to increased risk of environmental and occupational health hazards in the workplace. Additionally, noise pollution has been ranked as the second among several environmental stressors for its public health impact in a group of European countries (Al-Arja and Awadallah, 2020). In Europe, environmental noise for itself is responsible for 18000 premature deaths, 1.7 million cases of hypertension and 80000 hospitalizations every year (Hahad et al., 2019a).

In Portugal, the sectors that are subjected to the highest levels of noise are the metalworking, metallurgic, wood and furniture sectors. On the other hand, the lower levels were measured in the chemical industry (Teixeira et al., 2016).

The Portuguese law that currently rules aspects related to this issue is Decree-Law nº 278/2007, published in "Diário da República" nº 147/2007, series I of 2007-08-01. This Decree-Law presents changes on the General Regulation of Noise, the Decree-Law nº 9/2007, published in "Diário da República" nº 12/2007, series I of 2007-01-17, that already presented changes on the on the Decree-Law nº 310/2002, of December 18th and on 138/2005, of February 2nd. The law in Portugal tends to prevent noise and control noise pollution to protect human health and well-being of the population, focusing on environmental noise. Despite the Decree-Law nº 278/2007 being the most recent regulation regarding noise, it did not present alterations

regarding the occupational noise regulation, presented in the Decree-Law nº 182/2006, published in "Diário da República" nº 172/2006, series I of 2006-09-06.

The Decree-Law nº 182/2006, transposes to the national law the Directive nº 2003/10/CE, of February 6th, dictated by the European Parliament and Council, that concerns about minimum health and safety requirements regarding the exposure of workers to noise-related risks, being relevant to all activities of the private, cooperative, and social sectors, public, central, regional, and local administrations, public institutes, and self-employed individuals. This decree-law on the art. 3, refers to exposure limit values and action limit values (Table 1) and as shown the law delimits the values associated with the medium noise exposure, during the 8h/day period ($L_{exposition,\ 8h}$ dB(A)), the maximum noise exposure, being considered temporary noise peaks, and the maximum noise pressure associated.

Table 1 – Exposure limit values and action limit values according to the Decree-Law nº 182/2006.

Values in cause	**$L_{exposition,\ 8h}$ dB(A)**	**$L_{maximum}$ dB(C)**	**Noise pressure (Pa)**
Exposure limit value	87	140	200
Higher action value	85	137	140
Lower action value	80	135	112

Table 1 depicts the exposure limits, being that in under no circumstances the daily or weekly personal exposure of workers may exceed the values presented of 87 dB(A), 140 d B(C) or 200 Pa, in which determination the attenuation of hearing protectors is considered, meaning that if it was possible to measure the noise levels inside the ear canal, while wearing suitable hearing protectors, the workers' real exposure must not be equal or greater than those values. This consideration is not accounted when limiting the action values (higher and lower share values)

On the art.7 of the same Decree-Law, it is dictated that in situations where the employer can't lower the noise levels to which the employees are exposed it is mandatory that the employer provides individual hearing protectors for workers whenever the lower action values are exceeded, and they must ensure its use by workers exposed to noise values equal or exceeding the higher action values (≥85 dB(A)).

The Decree-Law nº 182/2006 also dictates that noise level measurement must be done by an accredited laboratory entitled by the Portuguese Institute of Accreditation or by Superior Occupational Health and Safety Technicians, or Health and Safety Technicians with specific training on methods and instruments for measuring noise at work, and that those instruments must be calibrated according to ISO criteria.

In an occupational environment, the measurement method and verification operations norms for measurement instruments and calibrators must be according to the norm NP EN ISO 9612:2011. According to this norm the sample of sound pressure, estimate duration of tasks, equipment and localization of the microphone are considered as sources of uncertainty during the evaluation of workers exposure to noise (JCGM, 2008).

1.2. Comet assay

The comet assay, or single cell gel electrophoresis, is a sensitive tool that was first described by Ostling and Johanson (1984), in a study where cells were ɣ-irradiated, to induce DNA breaks, lysed at a near-neutral pH, and submitted to an electrophoresis. They concluded that the "tail" of the comet seen extending towards the anode after the electrophoresis reflected the amount of DNA breaks and that the intensity of the tail's fluorescence increased with higher radiation doses.

This method is known as a way of detecting endogenous or exogenous DNA damage at individual cell level, allowing the detection of single- and double- strand breaks and if alkaline conditions are applied, also detects alkali-labile sites in the DNA, as apurinic/apyrimidinic sites or baseless sugars (Muruzabal *et al.*, 2019; Azqueta *et al.*, 2019; Koppen *et al.*, 2018; Muruzabal *et al.*, 2020), being widely used in genotoxicity testing, human biomonitoring and clinical studies (Møller *et al.*, 2020b).

As represented in the figure 4, this technique is based in cells that are embedded in agarose and this mixture is placed on a slide, the cells are lysed to remove membranes, soluble cell components and histones, by high molarity NaCl, leaving the DNA attached to a nuclear matrix, as supercoiled loops (nucleoids). Electrophoresis, usually made at a alkaline pH, draws the DNA towards the anode. Drawing only the loops containing breaks, releasing of supercoiling, being able to move significantly, forming an image that resembles the tail of a comet when stained with fluorescent dye and viewed under fluorescence microscopy (Azqueta et al., 2019), and being scored visually or using image analysis software (Muruzabal *et al.*,

2020). The relative intensity of tail fluorescence reflects the proportion of relaxed loops and therefore the frequency of breaks (Azqueta et al., 2019).

Some advantages of this technique are usually described as being economically cheap, fast, relatively simple, being applicable to any eukaryotic cell or tissue sample, even frozen tissues, and requiring low material (Møller *et al.*, 2020b). The alkaline version of the comet assay was developed by *Singh et al.* (*1988*), and is mainly used for *in vitro* and *in vivo* genotoxicity testing, being extensively used in environmental and occupational human biomonitoring studies. Most of these studies apply this assay in isolated peripheral blood mononuclear cells (PBMC), since they promote a more homogenous cell population than the total leukocyte fraction, and have a longer lifetime compared with polymorphonuclear cells (Koppen *et al., 2018*).

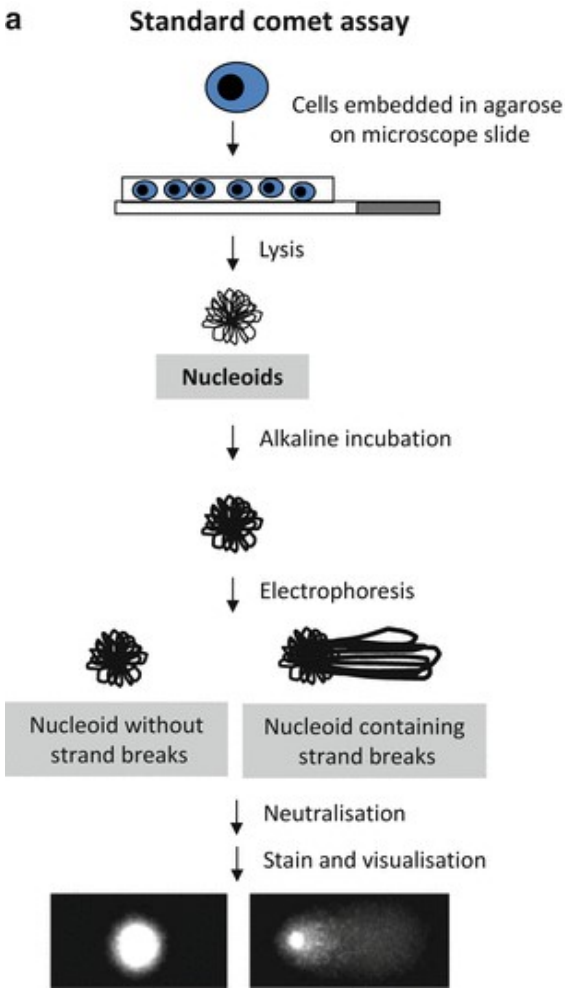

Figure 4 – Schematic representation of the standard comet assay. Adapted from Azqueta and Collins (2014).

Despite the wide use of PBMC on comet assay, the use of whole blood has many advantages, especially in human biomonitoring studies, since often large sets of samples are required to be processed and in these situations using the whole blood directly takes considerably less processing time, with the addition of required very little sample volume, as even 20 μL of whole blood is enough for its analysis, while it is required to have larger volumes when using mononuclear cells. Another advantage is that the use of whole blood reduces the risk of inducing cellular damage during the cell isolation procedures. The use of whole blood can be specially applied when available volumes of samples are restricted due to ethical considerations *(*Koppen *et al., 2018*).

When applying this technique on whole blood it is noted that polymorphonuclear leukocytes will also be treated along with the rest of the leukocytes. This polymorphonuclear leukocytes create nonhomogeneous comet tails and even though it may seem a disadvantage of this technique these may be easily identified (Figure 5), and so efficiently avoided during scoring (Al-Salmani *et al.*, 2011).

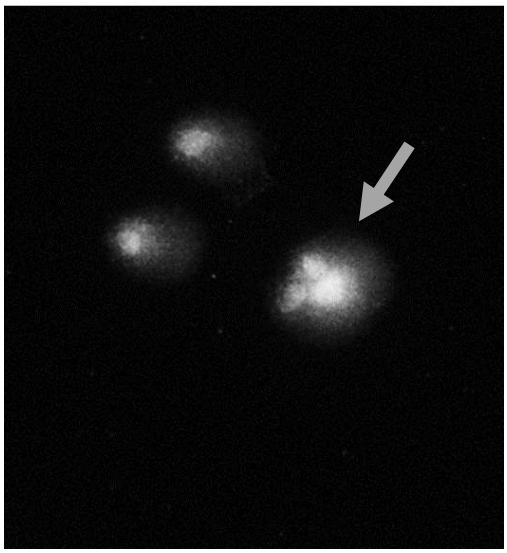

Figure 5 – Photographic representation of a polymorphonuclear cell (arrow), next to two mononuclear cells, after the comet assay and observed using the Comet IV program.

1.2.1. Enzyme modified comet assay

A considerable number of DNA-damaging agents do not directly induce strand breaks, causing other lesions such as oxidized or alkylated bases, inter- and intra-strand cross links or bulky adducts, which tend to provoke more serious consequences and these are not detected by

the standard comet assay; therefore, modifications to the existent protocol have been proposed in order to overcome this issue (Muruzabal *et al.*, 2020).

The first enzyme-modified alkaline comet assay was developed and presented in *Collins et al.* (*1993*), where they used the enzyme endonuclease III to measure the oxidized bases in human lymphocytes. Ever since 1993 several other enzymes have been used in this assay in an attempt to increase the sensivity of the standard procedure, and to measure different DNA lesions and even, more recently, methylation (Muruzabal et al., 2019).

Comparing to the alkaline comet assay, this modified protocol inserts an extra step: the incubation of the nucleoids, obtained after the lysis step, with an enzyme and enzyme reaction buffer, separately (Muruzabal *et al.*, 2019). The enzyme will induce extra breaks or AP-sites, and these are measured comparing to the data given by buffer slides (Muruzabal *et al.*, 2020), which are also used as controls of the reaction (Muruzabal et al., 2019); subtracting the score obtained from the buffer-exposed samples to the enzime-exposed samples, the "net enzyme-sensitive sites" is determined. (Muruzabal *et al.*, 2020)

This step can be used in 1 or 2 gels/slide, and the incubation of nucleoids is usually done by adding a drop of enzyme on top of the gel and covering it with a coverslip. In the case of 12 minigels/slide, a commercial metal chamber can be used to incubate these gels separately adding a certain volume in each well of the chamber, that contains one minigel. Besides these methods, when using 24, 48 or 96 gels/gelbond film, the incubation with enzyme is only possible by submersion of the slides in a bath or Coplin jar containing the enzyme, which implies the use of a greater amount of enzyme. (Muruzabal et al., 2019)

The use of enzymes in the comet assay characterises a huge advantage in all fields this technique is used, such as genotoxicity testing, human biomonitoring, ecogenotoxicology and basic research (Muruzabal et al., 2019). In order to acess the different DNA lesions many enzymes have been tested with comet assay such as endonuclease III, formamidopyrimidine DNA glycosylase (Fpg), uvrABC, T4 endonuclease V, uracil DNA glycosylase, exonuclease III, 3-methyladenine DNA dlycosylase II, 8-oxoguanine DNA-dlycosylase, endonuclease IV, among many others (Muruzabal et al., 2020).

1.2.1.1. Fpg-modified assay

Fpg-modified assay is a type of enzyme modified comet assay, where the cells are exposed to the enzyme Formamidopyrimidine DNA glycosylase (Fpg) during the comet assay procedure (Figure 6). This enzyme, widely used in human biomonitoring assays and

genotoxicity testing, is a DNA N-glycosylase from *E. coli* used to assess oxidative damage in cells, that specifically detects oxidised purines. Its main substrates *in vivo* are oxidised purines, especially 8-oxoguanine, also having the ability to excise ring-opened purines, originated from damaged adenine or guanine, creating AP-sites and also recognize alkylating damage in DNA indirectly, since during the lysis step this kind of lesions are converted into ring-opened guanines due to the alkaline treatment, usually at pH 10 (Muruzabal et al., 2020). It converts oxidised purines, specially 8-oxoguanine and ring-opened purines to breaks, and 8-oxoguanine DNA glycosylase (hOGG1) (Collins *et al.*, 2020).

It is estimated that the frequency of Fpg-sensitive sites in human lymphocytes is aproximately 0.25 per 10^9 daltons (Muruzabal et al., 2020).

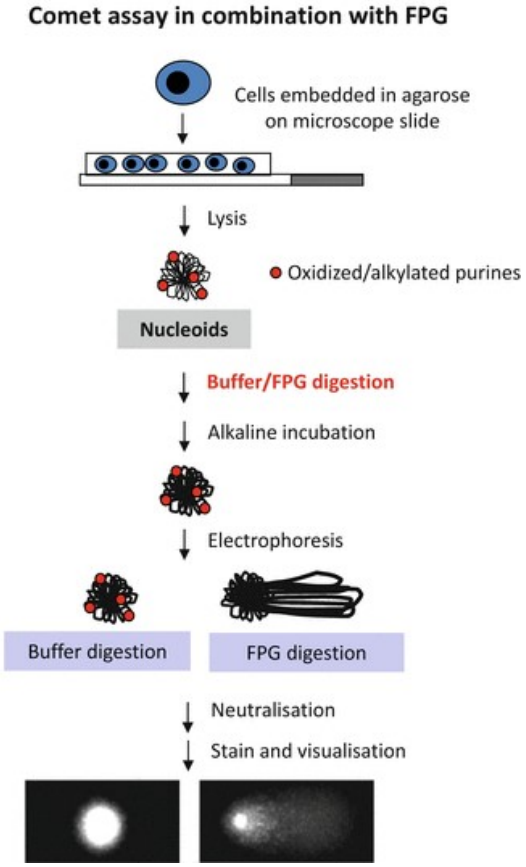

Figure 6 – Schematic representation of the Fpg-modified comet assay. Adapted from Azqueta and Collins (2014).

1.2.2. Assay controls in Fpg-modified comet assay

Assay controls are known to be samples that are included in experiments to understand if the experiment occurred as expected and if so to prove it, being obtained preferably in cryopreserved samples. For comet assay, these samples must have been exposed to a DNA

damaging agent, inducing the desired damage in the DNA, being all made in the same conditions. With its inclusion in every assay it can be used to observe inter-assay variations and to standardise the test results if this variation is too large (Møller *et al.*, 2018).

The use of assay controls for the enzyme-modified comet assay might be a standard practice when applying this technique to biomonitoring and other studies. Surprisingly very few publications actually show these results, or how these were made (Møller *et al.*, 2018; Møller *et al.*, 2020a).

Standard comet assay guideline tests for animal experiments and alkylating agents for positive controls were recommended and adopted by Organisation for Economic Co-operation and Development (OECD). In this case, cryopreserved cells that have been treated with OECD-recommended agents would be good assay controls even though some of these were classified as carcinogens by the International Agency for Research on Cancer (IARC). Probably for safety reasons, the most widely used one, even though it is a non-classified compound, is hydrogen peroxide. After tested these alkylating agents proved not to be appropriate for the enzyme-modified comet assay that detects oxidative damage on DNA (Møller *et al.*, 2020a).

So far, there is no consensus about a suitable positive assay control for Fpg-modified comet assay. Recently Møller *et al.* (2020a) carried out a project involving 10 laboratories, where $KBrO_3$ exposed THP-1 cells were used as assay/positive controls in comet assay experiments. After analysing the results, the authors concluded that their study showed that cells treated with $KBrO_3$ provide a robust assay control for Fpg-modified comet assay since it provides a high generation of Fpg-sensitive sites with little generation of DNA strand breaks. $KBrO_3$ was found to induce oxidative DNA damage due to the formation of the mutagenic 8-hydroxy-2'-deoxyguanosine (8-OHdG), that is a known biomarker of oxidative stress (Obaidi *et al.*, 2018).

Either way, positive controls don't exist in biomonitoring studies since a group of people cannot be exposed deliberately to a DNA damaging agent (Møller *et al.*, 2018; Møller *et al.*, 2020a).

2. Objectives

The aim of the present study is to evaluate the impact of noise in the DNA of human individuals occupationally exposed to noise, measuring oxidative DNA damage levels in samples provided by the National Health Institute Dr. Ricardo Jorge from a group of workers of a metallurgic industry.

In order to achieve this goal, the following tasks were performed:

- Optimisation of the enzyme-modified comet assay technique, by performing the enzyme titration to achieve optimal performance, and production of assay controls;
- Assessment of the oxidative DNA damage levels in the study population, via enzyme-modified comet assay;
- Statistical analysis to understand if in this specific population the oxidative DNA damage significantly differs between exposed and non-exposed individuals.

3. Materials and methods

3.1. Sample characterisation

The present study is part of the project "Contribution of noise exposure on development of cardiovascular disease – an epigenetic approach" [project 2018DSA1551, Environmental Health Department of the National Health Institute Doutor Ricardo Jorge].

This study involved 56 volunteer participants, workers of a metallurgic factory, from which 44 individuals were working on the production and 12 were performing administrative activities or similar functions, being 35 male individuals, 18 female individuals and 3 were unknown/not answered.

To be included in the study the participants had to be over 18 and under 65 years old, and, additionally, for the exposed group the included participants had to be working in the activity for over 12 months, on dayshifts.

All procedures performed were approved by the Ethics Committee of the National Health Institute Dr. Ricardo Jorge.

Each participant was requested to fulfil 3 questionnaires on sociodemographic data, cardiovascular risk assessment and lifetime-noise exposure, along with a written informed consent, allowing the use of the data obrained during this project.

The blood samples used in this work were drawn in tubes with 5 mL of EDTA and were stored at -20ºC.

Every laboratorial procedure of this project was fulfilled on the National Health Institute Doutor Ricardo Jorge, on the Environmental Health Department, which was also responsible for the field noise measurements under the accredited internal procedure based on the International Standard ISO 9612:2011 (Acoustics – Determination of occupational noise exposure – Engineering method). Measurements were made with class sound level meters from Bruel & Kjaer™ (2250) with a 1/3-octave filter, measuring A-weighting and C-weighting frequencies, and loudness, noise rating, and tone. Registry of sources of noise, source functioning (when applicable), weather conditions, location of measurement, microphone orientation, time and length of measurement, were also performed as described in the standard procedures. Workers identified as multi-tasking, and thus, exposed to different sources and levels of noise during their activities, were monitored with portable dosimeters - CESVA™

DC112 - which measure the equivalent level with A and C frequency-weightings, and simultaneously carry out a real time frequency analysis, with frequency-weighting and by octave bands from 63 Hz to 8 kHz, and also Peak Level with C frequency weighting [LCpeak]. In both strategies, measurements were made to characterize the work-day exposure.

3.2. Fpg modified comet assay

In order to optimize the Fpg modified comet assay, it was necessary two prior steps, namely the production of assay controls and the Fpg titration.

During the procedure it was also tested and optimized the dilution factor of the cells, in either conditions yet to be described, in different amounts of low melting point agarose.

3.2.1. Comet assay controls

3.2.1.1. Isolation of peripheral blood mononuclear cells (PBMC)

In order to obtain the assay controls for oxidative damage, 5 random blood samples, obtained by venipuncture, with aproximately 6 mL each, were drawn to heparin sodium tubes. This samples where transferred to a 15 mL tube and diluted by 50% using a solucion with phosphate buffered saline (PBS) (Lonza Group AG, SWZ) plus 2% fetal bovine serum (FBS) (Biowest, FR). Each mix with 50% blood was separated in two 15 mL new tubes, and to these 3 mL of Lymphoprep were added, at room temperature, carefully. This tubes where centrifuged during 20 min at 800 x g at room temperature.

The PBMC, presented in the “cloud” between the plasma and the erythrocytes, were transfered to another tube, using a pasteur pippete, and to these 5 mL of PBS was added. This new 15 mL tubes where then centrifuged during 10 min at 200 x g.

The supernatant was removed, and the pellet was ressuspended in the PBS remaining and then every sample was transferred to the same 15 mL tube, forming a PBMC pool. The tubes that once contained the pellet were washed with 1 mL PBS each, and this PBS was also aditionated to the mononuclear cells pool.

The mononuclear cells presented in the pool where counted used a Neubauer chamber, and then the amount required of $12.5x10^6$ was transferred to two tubes, that where centrifuged during 10 min at 200 x g. The supernatant was removed and to each tube was aditionated 11 mL of lymphocits culture medium, and joined in a 50 mL tube.

3.2.1.2. $KBrO_3$ treatment

Since the purpose was inducing oxidative damage in the DNA, the next step involved treatments with $KBrO_3$. This procedure was based on a Møller *et al.* (2020a) work, before it was published.

The PBMC pool obtained was distributed to 5 new 15 mL tubes, obtaining aproximatelly 4.5 mL in each tube. It was required to do a 45 mM $KBrO_3$ stock solution, dissolved in 3 mL RPMI 1640 without L-Glutamine (Lonza Group AG, SWZ), and a different treatment was applied to each tube:

- Control 1 and 2 – 0 mM $KBrO_3$;
- Control 3 – 0.5 mM $KBrO_3$;
- Control 4 – 1.5 mM $KBrO_3$;
- Control 5 – 4.5 mM $KBrO_3$.

These tubes were incubated at 37ºC during 1 h, except “control 1”. The ”control 1” was not incubated at all since, when comparing controls 1 and 2, would be possible to understand if the incubation step would induce celular damage, which didn’t.

After the incubation all tubes were centrifuged during 5 min at 250 x g. The supernatant was removed and the pellet was ressuspended in 5 mL PBS, centrifuged again in the same conditions as before, once again the supernatant was removed and the pellet was ressuspended in 5.5 mL of freezing medium with 50% FBS, in order to reduce oxidative stress, 40% RPMI and 10% Dimethyl Sulfoxide (DMSO). These controls were distributed, 500 µL in each microtube, and imediatlly stored at -80ºC.

3.2.2. Fpg titration

In order to optimize the efficiency of the Fpg activity (New England Biolabs, USA) during the comet assay procedure a titration assay was performed testing different enzime concentrations (1.16 U/mL, 1.33 U/mL, 2.26 U/mL and 2.6 U/mL), tested in incubations of 20 min, at 37ºC. This titration was made with the control samples described above and using the comet assay procedure that will be explained next.

3.3. Comet assay protocol

The comet assay protocol was based on Azqueta *et al.* (2013).

The slides used during the assay were prepared beforehand. In order to be used in the comet assay, and to avoid losing samples, one of the sides of the slides was coated with a layer of 1% normal melting point agarose (Lonza Group AG, SWZ), prepared in distilled water. The slides were submerged in this solution while hot, one of the sides was cleaned up and the slide was left to dry horizontly at room temperature.

The comet assay technique was performed in the 12 minigels system using the medium-throughput 12-gel comet assay unit (Severn Biotech Ltd®, Kidderminster, UK). In each slide 6 samples were loaded: 2 minigels (with replicates) of a negative and a positive assay control, and four samples (in duplicates) from the project. Understanding negative control as "control 1 or 2" and positive control as "control 3, 4 or 5", that, respectively, weren't and were incubated with $KBrO_3$. For each set of 6 samples 3 equal slides were prepared: one for the lysis control, where the standard alkaline comet assay was performed, one for the enzyme's buffer incubation and one for the enzyme incubation. In each of this situations 100 nucleoids were scored from each sample and the net of Fpg-sensitive sites analysed given by calculating the medium of the percentage of tail intensity (%TI) and then subtracting the Fpg incubation results, to the buffer F incubation results (Fpg-BF).

3.3.1. Comet assay protocol applied to the project samples

In this study, whole blood samples were used for the comet assay technique. 5 µL of whole blood were added to 200 µL of 0,6% low melting point agarose (Sigma-Aldrich, USA) previously prepared with PBS (Lonza Group AG, SWZ), and kept at 37ºC. From this mixture, 2 minigels of 5 µL each were placed on the coated slide. After placing all the minigels needed the slides were left, so the agarose would solidify, at 4ºC during 10 min.

After that the slides were placed vertically on a Coplin jar, containing the lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-base, adjusted at pH 10 with NaOH, stored at 4ºC), to which 1% Triton X-100 (Merck, DE) was added, and kept at 4ºC for at least 1h, so the lysis of the cells could happen and only the nucleoids would remain.

After the lysis step the slides that need incubation were removed from the Coplin Jar, and the third slide remained in the lysis solution until the end of the incubation step. Those two slides where washed two times with distilled water at 4ºC and three times with buffer F - this solution containing 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL BSA, adjusted at pH 8 with KOH, was stored at -20ºC in 50 mL tubes, that beforehand were defrosted and diluted in 450 mL of distilled water at 4ºC.

Two microtubes containing 600 µL of buffer F each were kept on ice, and to one of those 10 µL of Fpg was added (New England Biolabs, USA), thus obtaining the solutions for buffer and enzyme incubations, respectively.

The medium throughput 12-gel comet assay unit supports were set, having a metal base, stored at 4ºC in the base where the slides were placed with the samples on top of them and placing an acrylic and a plastic piece on top of the slide. This last pieces had the shape of the wells, in order to keep the buffer or the enzimatic solution in that defined area, where the minigel containing the sample would also stand. To each of these 12 wells 30 µL of buffer F were added in one slide, and 30 µL of Fpg in the other slide. These 2 slides where incubated during 30 min, at 37ºC, in a wet environment.

After the incubation the supports were disassembled and these two slides, plus the slide left on the lysis solution, were placed horizontly on a electrophoresis tank and to the tank the electrophoresis solution (300 mM NaOH, 1 mM EDTA, dissolved in distilled water at 4ºC, pH>13) was added. Before the starting the electrophoresis, the samples were kept in the cooled eletrophoresis tank filled with the solution during 20 min, to promote the unwinding of the DNA.

After the unwinding step, the electrophosis tank, connected to a converter previously setted to run the electrophoresis at 18 V and 300 mA (0.7 v/cm), was turned for 20 min, at 4ºC.

When the electrophoresis was over, all the 3 slides were placed in a coplin jar and washed with PBS, then with cold distilled water during 10 min each, at 4ºC, and then washed with etanol at 70% and after 96%, during 15 min each, at room temperature.

The slides were placed horizontly in a dark place, to dry at room temperature.

After drying, usually overnight, the samples were stained using a solution of 25 mL buffer TE, stored at 4ºC, and 20 µL of previously diluted SYBR® Gold (Invitrogen, Thermo Fisher Scientific, USA) , stored at -20ºC. The slides were placed in this staining solution during 20 min in agitation and in the dark. Then, the staining solution was removed, and replaced by distilled water, where the slides stood in agitation and in the dark, during 10 more min, in order to wash the gels.

The samples were analysed in a fluorescence microscope (Motic BA410 ELITE) attached to an epifluorescence illuminator (FilterSet: Exciter D480/30x, Emitter D535/40 nm, Dichroic 505DCLP), with 100x magnification and the Comet Assay IV^{TM} software (Perceptive Instruments, UK). With the aid of this software 50 comets per gel/ 100 comets per sample were scored and from the readings given by this software the tail intensity (%TI) was the parameter

chosen to obtain information on the oxidative damage levels of each sample. Using the average of %TI of 100 nucleis scored from each samples exposed to Fpg and subtracting the average of the %TI of the 100 nucleis scored from the same samples incubated with buffer F the oxidative DNA damage levels was calculated (FPG-BFF=%TI).

3.3.2. Comet assay protocol applied to the control samples

In the case of the control samples, the comet assay was performed using samples of mononuclear blood cells to the concentration of 4000 cells per gel of 5 µL.

This samples were defrosted and transferred to a 15 mL tube, where a defrosting solution (50% FBS, 40% RPMI and 10% glucose) was added. This solution containing the sample, was centrifuged at 176 x g during 10 min. The supernatant was removed, and the pellet was ressuspended in the cell washing solution (10% FBS and 90% RPMI) and again centrifuged in the same conditions as before. The supernatant was removed and the pellet was ressuspended in 1 mL of PBS and transferred to a microtube.

In order do count the cells present in each microtube, 10 µL of the sample with 5 µL of Trypan Blue (Invitrogen Corporation, Thermo Fisher Scientific, USA were mixed, and of this 15 µL of final volume, 10 µL were placed in a Neubauer chamber and 16 squares of it were scored in a optical microscope. The volume of the sample containing 6000 cells was dilluted in a microtube containing 500 µL of PBS and centrifuged at 8000 x g. The supernatant was removed and the pellet was ressuspended in 70 µL of low melting point agarose preparared the same way as in point 3.3.1. The minigels and the steps after placing the minigels in the slides were performed the same way as described in point 3.3.1.

3.4. Data analysis

The statistical analysis of the obtained data was performed using two different approaches, in which the individuals were separated in different groups.

On a first approach the 56 individuals were separated based on their funcion on the factory: the 44 volunteers that worked on the production were included as a “exposed group” and the 12 volunteers that performed administrative activities or similar were included as a “non-exposed group”.

On this first approach the statisitical difference between the data associated to those two groups described was tested based on the parameter significance, associated with the p-value ($p<0.05$). This procedure was applied on the oxidative DNA damage data, obtained from the comet assay, regarding the exposure status and heart rate data, measured on the day the blood samples were colected.

On a second approach the 56 volunteers were grouped based on the parameters of the Decree-Law nº 182/2006 regarding the exposure limit values, as described on the Table 1. These individuals were now separated in three different groups: 12 individuals were associated with the "lower share exposure group", represented as ≤85 dB(A); 33 individuals formed the "exposure limit exposure group", represented as ≥87 dB(A); and 11 individuals that were exposed to values in between, represented as >85;<87, formed a third group.

Associated with this second approach the statistical differences ($p<0.05$) between the three groups described were tested, and their heart rate, DNA damage, systolic and diastolic blood pressures.

The statistical analysis and graphic representations were obtained using the IBM® SPSS® version 26, and DATA*tab* softwares.

The sample characterization was made through an univariated analysis.

To assess the variance equality of the samples, was performed a *Levene* test of variance equality.

In the case of the approach 1 (two groups), to assess if the mean of two groups was statistically different it was realized a T-test for independent samples. Regarding approach 2 (three groups), the assessment of the statistical significance between groups was performed using one-way analysis of variance (ANOVA). In both cases values were considered statistically different when p-value was below 0.05.

The values are presented with mean expression ± standard error of the mean.

Regarding the $KBrO_3$ controls it was made a R^2 correlation test between the Fpg-sensitive sites. Associated with the Fpg titration, it was applied the same test but associating the values obtained (%TI) in the different $KBrO_3$ controls, when exposed to different Fpg concentrations, associated with a linear regression line.

4. Results and discussion

4.1. Sample characterization

The sample used was characterized through a univariated analysis, being constituted of 56 workers of a metallurgy factory. On this sample, the results were acessed using two different approachs: on approach 1 the sample was divided in two groups regarding their job inside de factory, being the exposed group related to production operators and the non-exposed group the ones performing administrative activities; on approach 2 the same sample was divided in three groups depending on their exposure levels (under 85, between 85 and 87 and above 87 dB(A)), acording exposure and action limit values expressed on the Decree-Law nº 182/2006 explained on the topic 1.1.2 of the present work.

According to the 1st approach (Table 2) the exposed group constituted of 44 (78.6%) indivuals, and the non-exposed group constituted of 12 (21.4%) individuals. From the exposed group, 33 (75%) indivuals were males and 11 (25%) individuals were females; the non-exposed group was represented by 5 (41.7%) males and 7 (58.3%) females. The individuals from the exposed group were working on that metallurgy factory for 9 ± 7,4 years, had 37.6 ± 8.3 years old wand were exposed to 89.73 ± 4.67 dB(A) on their 8h/day job. The non-exposed group were working in the same place for 5.75 ± 5.9 years, had 33.1 ± 7.7 years old and were exposed to 36.61 ± 29.18 dB(A) on the same period than the exposed group.

Table 2 – Approach 1 - Sample characterization, analysing sex, age, years on the job and noise exposure of the individuals.

	Exposed Group	**Non-exposed Group**	***p***
Total (%)	44 (78.6%)	12 (21.4%)	
Male/Female (%)	33 (75%) / 11 (25%)	5 (41.7%) / 7 (58.3%)	0.03
Age	37.6 ± 8.3	33.1 ± 7.7	0.11
Years on the job	9 ± 7.4	5.75 ± 5.9	
Noise exposure (dB(A))	89.73 ± 2.15	36.61 ± 29.18	

On the 2nd approach (Table 3) there was 14 (25%) individuals exposed to noise exposures equal or under 85 dB(A), being these 7 (50%) male and 7 (50%) female, exposed to values between 85 and 87 dB(A) there was 9 (16%) individuals, where 7 (78%) were male and

2 (22%) female and 33 (59%) individuals where exposed to values equal or above 87 dB(A), being those 24 (73%) male and 9 (27%) female.

The group of individuals exposed to ≤ 85 had 34.1 ± 8.-6 years old and worked on that factory for 6.7 ± 6.7 years, being exposed to noise of 42.3 ± 31.4 dB(A), the group exposed to exposure levels between 85 and 87 dB(A) had 35.3 ± 9.1 years old and had 9.4 ± 8.7 years on the job, being exposed to 86.1 ± 0.2 dB(A). The individuals exposed to ≥ 87 dB(A) were 38 ± 8.2 years old, being working there for 8.8 ± 7.3 years and were under the exposure of 91.7 ± 1 dB(A).

Table 3 – Approach 2 **-** Sample characterization while separated as groups of exposure levels.

	≤ 85	**> 85 < 87**	**≥ 87**	***p***
Total (%)	14 (25%)	9 (16%)	33 (59%)	
Male/Female (%)	7 (50%) / 7 (50%)	7 (78%) / 2 (22%)	24 (73%) / 9 (27%)	0.38
Age	34.1 ± 8.6	35.3 ± 9.1	38 ± 8.2	0.47
Years on the job	6.7 ± 6.7	9.4 ± 8.7	8.8 ± 7.3	
Noise exposure (dB(A))	42.3 ± 31.4	86.1 ± 0.2	91.7 ± 1	

It is important to notice that the noise values where obtained in the room, meaning that even though 33 individuals where exposed to values equal or above 87 dB(A), it does not necessarily mean that any law was broken since they were using auditive protection as the law requires.

This data was collected by the time the blood samples were drawn, and was collected in questionaires refered in the topic 3.1 of the present work.

4.2. $KBrO_3$ controls

In order to obtain reaction positive controls for the enzyme activity in the Fpg-modified comet assay, oxidative damage was induced in the PBMC, and exposures of 0.5 mM, 1.5 mM and 4.5 mM of $KBrO_3$ were made, as well as 2 controls without exposure to this compound, to which one of those was incubated at 37ºC, for 1h, as well as the exposure controls.

These controls where kept at -80ºC, and defrosted and tested using the Fpg-modified comet assay procedure, incubated in the Fpg (1.33 U/mL) and 100 nucleis/sample scored using the Comet IV software. In Figure 7, examples of cell nucleis are shown taken from the Comet IV software, being all of those pictures taken from the same assay. It is possible to realize that the tail size is different depending on the concentration of the $KBrO_3$, showing that the exposure was successful since the enzyme recognized more sensitive sites when exposed to higher concentrations of $KBrO_3$.

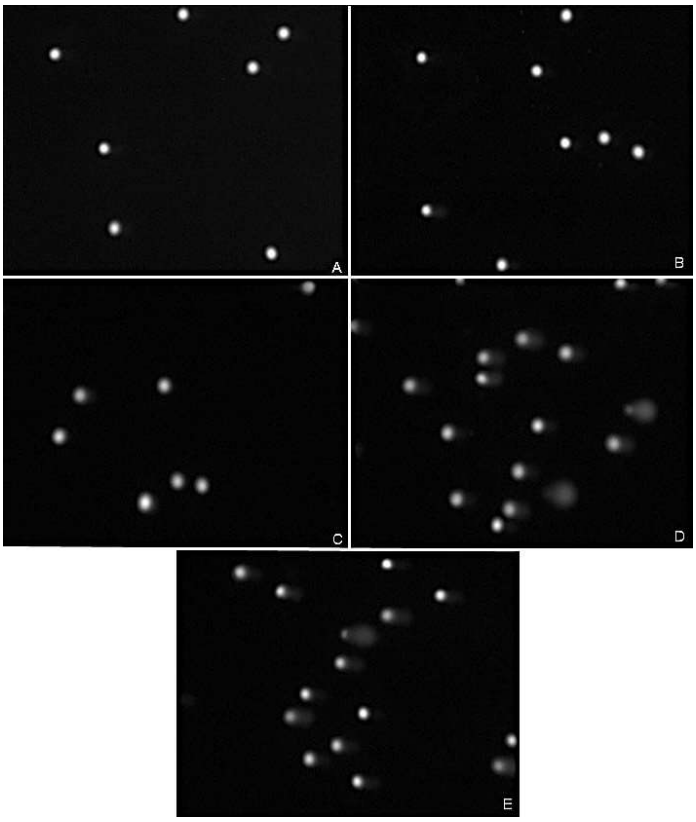

Figure 7 – Images of the enzymatic incubation of $KBrO_3$ controls, after the comet assay and toked from Comet IV program in 100x magnification.
A- Without exposition or incubation; B- Without exposition, with incubation; C- Incubated with 0.5 mM $KBrO_3$; D- Incubation with 1.5 mM $KBrO_3$; E- Incubation with 4.5 mM $KBrO_3$.

To understand if this was a good method, during the statistical treatment of this assay the results of Fpg-BF, that provides information of the net Fpg-sensitive sites, of the different controls showed a correlation (R^2) of 0.89, showing a good correlation between the results. Besides that, the figure 8 shows the lysis images of the same assay from where the pictures of the figure 7 were taken. The standard deviation of the results regarding the lysis of the different samples was 0.14% tail intensity (TI). It is possible to observe on the figure 8, the exposure of this compound doesn't increase the strand breaks while inducing oxidative damage.

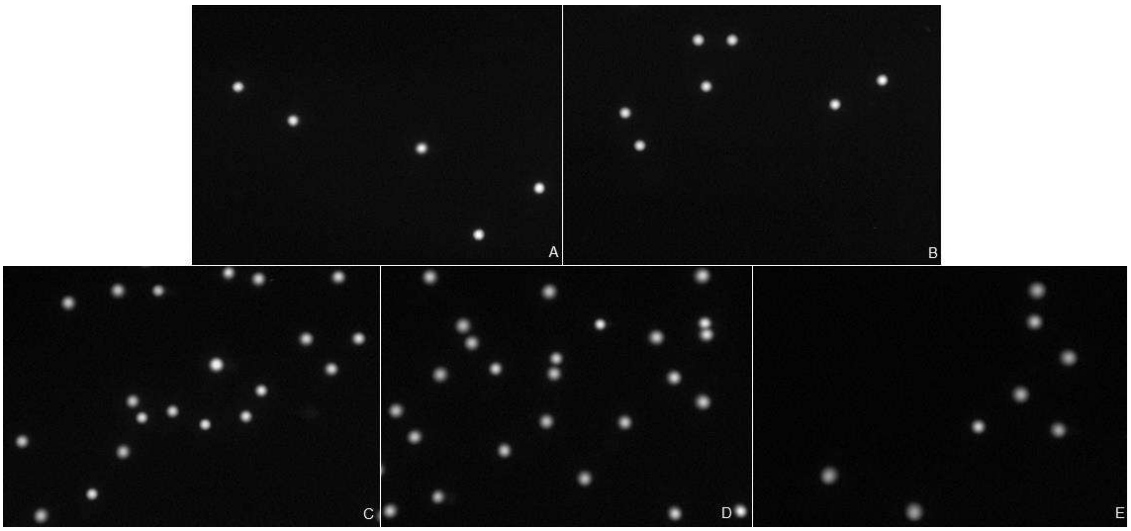

Figure 8 - Images of the lysis of the KBrO3 controls, after the comet assay procedure, toked from the comet IV program in 100x magnification.
A- Without exposition or incubation; B- Without exposition, with incubation; C- Incubated with 0.5 mM $KBrO_3$; D- Incubation with 1.5 mM $KBrO_3$; E- Incubation with 4.5 mM $KBrO_3$.

As it is possible to observe, these cells suffered different amounts of induced oxidative damage, and this method can be used to prepare positive controls for the Fpg-modified comet assay while showing a concentration-response relationship, as predict by Møller *et al.* (2020a) on an assay where the authors exposed THP-1 cells to 0, 0.5, 1.5 and 2.5 mM, at 37ºC for 1h.

Other studies, such as Bausinger and Speit (2014) also showed that potassium bomate exposure did not induce strand breaks, detected by comet assay, on DNA in PBMC and T cells colected from fresh whole blood and exposed during 2h at 37ºC, at concentrations up to 10 mM, but when the comet assay was performed with combitation with Fpg there was seen a clear and concentration-related effect in both. Another study by Bausinger and Speit (2016) exposed PMBC and whole blood to various chemical mutagens including $KBrO_3$ at the concentrations 5 and 10 mM (2h at 37ºC), where besides the expected conclusions, they also concluded that this kind of exposure also works on whole blood, also showing low strand breaks when performed alkaline comet assay, but when in combination with Fpg it was seen clear effects both in whole blood and PBMC. In both studies the Fpg concentration used is unknown and even though in none of them the $KBrO_3$ was used as an assay control the results presented showed to be similars to the ones presented in this study.

4.3. Fpg titration

To optimize the Fpg-modified comet assay, and the efficiency of the enzyme Fpg during the procedure, was realized a titration. Different concentrations of 1.16 U/mL, 1.33 U/mL, 2.26 U/mL and 2.6 U/mL, were tested during 20 min of incubation. This titation was realized in using $KBrO_3$ control samples, after understanding the efficacy of such. It was used controls without exposure to $KBrO_3$, 0.5 mM, a 1.5 mM and a 4.5 mM of $KBrO_3$.

In order to understand which concentration of the enzyme presented the best performance under the assay conditions, it was proceded to realize a linear trend line between the control samples (mean %TI Fpg-BF) incubated in different concentrations of Fpg, associated with each R^2 value (Figure 9).

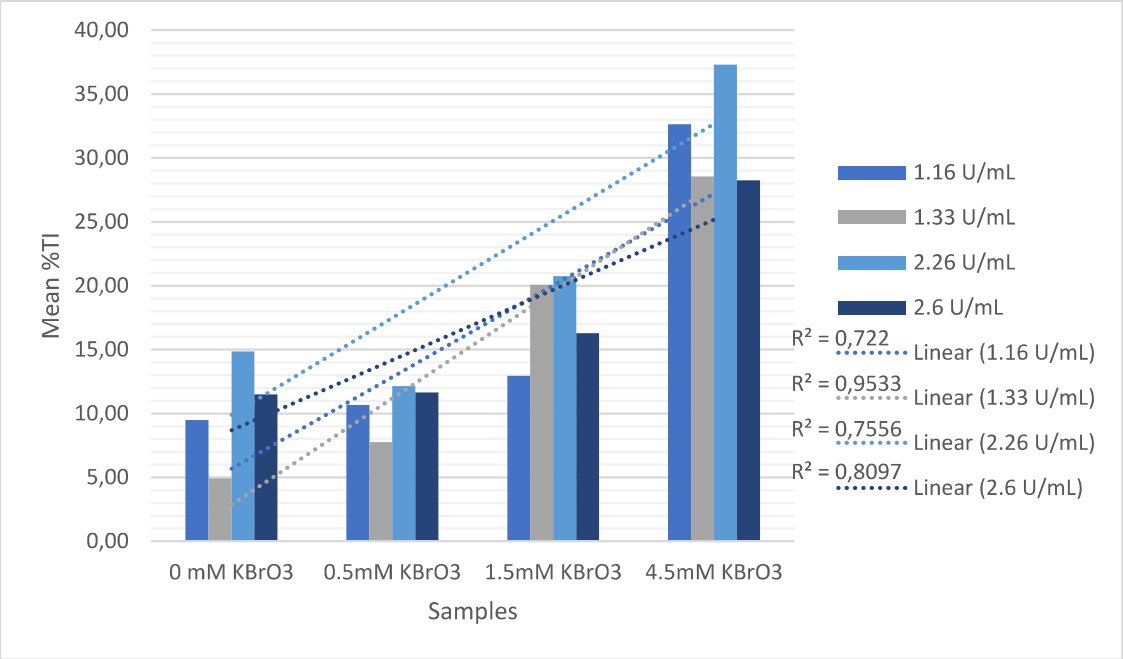

Figure 9 – Graphic representation of a titration, with linear tread lines and the representation of R^2 of each. Is represented the mean %TI of a comet assay applied to 4 different concentrations of $KBrO_3$ exposures, being incubated in 4 different Fpg concentrations.

In Figure 9 we can observe that the samples incubated with lower Fpg concentratios 1.16 U/mL and 1.33 U/mL had, respectively, a R^2 of 0.722 and 0.9533, under the higher Fpg concentrarions of 2.26 U/mL and 2.6 U/mL, they presented the R^2 of, respectively, 0.7556 and 0.8097.

It was very dificult to understand what enzimatic concentrations is the most used since most literature don't specify it or say that the enzime was an offer. Muruzabal (2019) made a

titration, using NorGenoTech (Oslo, Norway) FPG and, in the 12 minigels/slide format, used the concentrations of 1/10000, 1/30000, 1/100000, 1/300000 and 1/1000000, and they concluded that the higher concentration was the one that gave better results. Using this information, and assessin the manufacturer website it was possible to understand that 1/10000 meant 1.16 U/mL.

Therefore it was tested the concentration used on that paper (1.16 U/mL), the concentration used on the laboratory where I interned (1.33 U/mL) and the respective approximate doubles. As it is observable, the enzime concentration that presented the best correlation was 1.33 U/mL, with a R^2 value closer to 1.

4.4. Comet assay

4.4.1. Oxidative DNA damage vs Noise Exposure

Following “Approach 1” where two groups were defined as “Exposed” and “Non-exposed” we obtained a non-significant difference for the oxidative DNA damage levels between groups.

Regarding the oxidative DNA damage levels (Table 4, Figure 10), obtained with comet assay, it can be observed that the “Exposed” group presented 15.26 ± 5.09 %TI, with values between 0.07 and 26.93 %TI. The “Non-exposed” group presented 14.74 ± 3.84 %TI, with values between 8.36 and 20.81% TI.

The “Exposed” group exhibited higher levels of oxidative DNA damage (TI%) (M = 15.26, SD = 5.09) than the “Non-exposed” group (M = 14.74, SD = 3.84). The *Levene* test of variance equality was performed and shown that variance equality could be assumed (p=0.341).

Table 4 – Descriptive statistics for oxidative DNA damage levels between “Exposed” and “Non-exposed” groups

		Group	
		Exposed	Non-exposed
Oxidative DNA damage (%TI)	Frequency	44	12
	Mean	15.26	14.74
	Std. Deviation	5.09	3.84
	Minimum	0.07	8.36
	Maximum	26.93	20.81

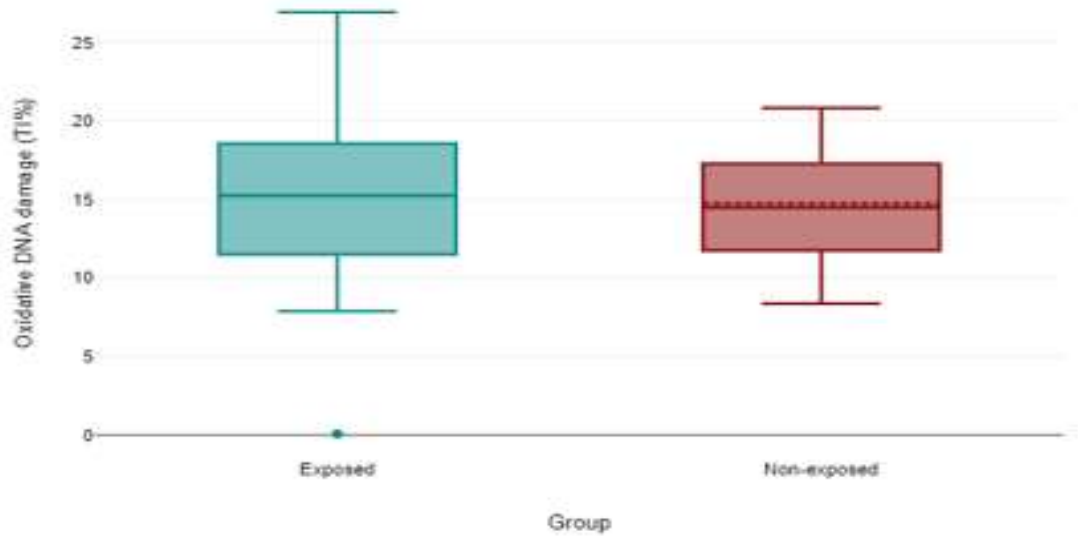

Figure 10 – Oxidative DNA damage levels (%TI) in Exposed and Non-exposed groups (boxplot with mean, interquartile, and standard deviation)

A t-test (Table 5) for independent samples (equal variances assumed) showed that the difference between the groups was not statistically significant, t (54) = 0.33, p = 0.740, and a 95% confidence Interval [-2.65, 3.71].

Table 5 – T-Test for independent samples summary associated with oxidative DNA damage values.

		t	df	*p-value* (2-tailed)
Oxidative DNA damage (TI%)	Equal variances	0.33	54	**0.740**
	Unequal variances	0.39	22.76	0.699

As it can be observed on the Table 6 and the Figure 11, knowing that Class 0 represents exposure levels equal or under 85 dB(A), Class 1 exposure levels between 85 and 87 dB(A) and Class 2 exposure levels equal or over 87 dB(A) it is possible to observe that, regarding the oxidative DNA damage, the Class 0 presented 14.51 ± 5.11 %TI, the Class 1 presented 15.97 ± 3.8 %TI and the Class 2 presented 15.16 ± 5.12 %TI.

When following "Approach 2" a similar scenario was obtained. There are differences between the three classes of noise defined regarding the oxidative DNA damage, yet these are not significant.

Table 6 – Descriptive statistics for oxidative DNA damage levels between "Noise class" groups. Class 0 represents exposure levels equal or under 85 dB(A), Class 1 exposure levels between 85 and 87 dB(A), and Class 2 exposure levels equal or over 87 dB(A).

Class	N	Mean	Std. Deviation
0	14	14.6	4.92
1	9	15.97	3.8
2	33	15.16	5.12
Total	56	15.14	4.87

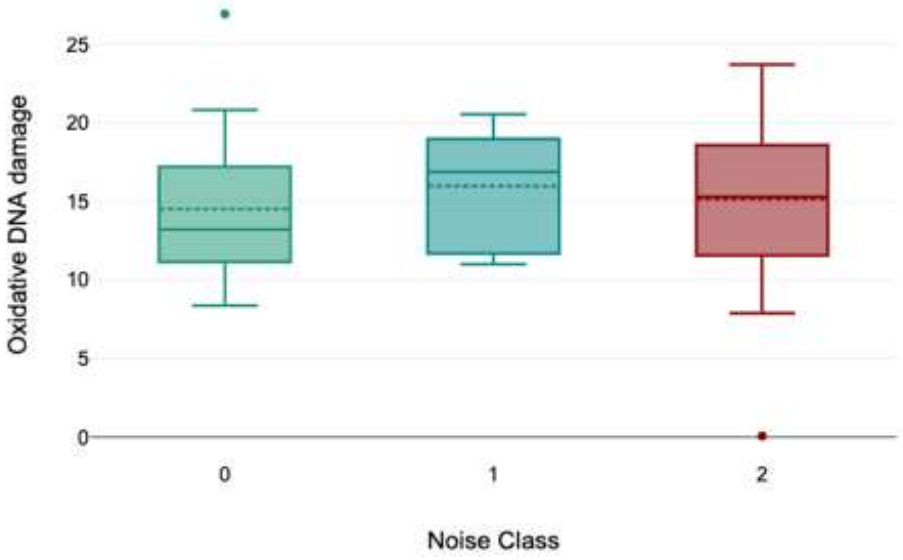

Figure 11 – Noise Class groups' oxidative DNA damage levels (%TI) in boxplot with mean, interquartile, and standard deviation.

A one-factor analysis of variance (ANOVA) was performed showing that there is no significant influence between the categorical variable "Noise Class" and the variable Oxidative DNA damage F = 0.23, p = 0.794 (Table 7).

Table 7 – ANOVA test summary associated with the noise classes and the oxidative DNA damage.

	Sum of Squares	**df**	**Mean Squares**	**F**	***p-value***	**Critical F-Value**
Between Groups	11.31	2	5.66	0.23	**0.794**	3.18
Within Groups	1268.38	52	24.39			
Total	1279.7	54				

Even though these results were not the expected ones it is known that very few studies have been performed on the correlation between the effect of noise exposure on human DNA damage (Bagheri Hosseinabadi *et al.*, 2019). Cases similar to the one exposed on the present study have already been showed in different reports, such as Hemmingsen *et al.* (2015), that on a study on traffic noise (48 or 75 dB(A)) effects on human DNA damage applying the comet assay, did not found statistically significant differences between Fpg-sensitive sites, in PBMC,

from samples taken before and after the traffic noise exposure, but still the authors concluded that noise may cause oxidative DNA damage.

A study performed by Nawaz and Hasnain (2013) in humans exposed to different levels of noise exposure, showed that the levels of 8-hydroxy deoxyguanosine, that was used as an oxidative DNA damage biomarker, on the serum, as well as the levels of cortisol were significantly higher in groups exposed to noise, showing that noise exposure can lead to stress and promote oxidative DNA damage.

On the other hand, some studies performed on animals showed that noise induces DNA damage. A studied performed by Kvandova *et al.* (2020) where it was associated the exposure of environmental noise on Ogg1 $^{-/-}$ mice exposed to repetitive playbacks of 2 h long noise patterns of aircraft noise events with a maximum sound pressure of 85 dB(A) and an average of 72 dB(A). The authors concluded that noise-exposed mice suffered an oxidative burst since showed had higher ROS levels derived from activated granulocytes in whole blood, that showed that chronic noise exposure causes oxidative DNA damage. This study also presented other oxidative damage markers as 4-hydroxynonena, that also showed elevated levels on exposed mice, and the authors associated with a possible inhibition of base excision repair enzymes, leading to cumulated DNA damage.

A study by Ceylan *et al.* (2016) exposed 3 exposure groups of new-born rats (3-6 days old) of 40, 70 and 110 dB(A) for periods of 30 min a day, for 7 days. The authors showed that the group exposed to 110 dB(A) showed statistically higher values of 8-hydroxy deoxyguanosine presented on the serum, per comparison with the control group (40 dB(A)) and the lower exposure group, concluding that DNA damage occurred in new-born rats.

A study realized by Koppen *et al.* (2018), where they compared DNA strand breaks and Fpg-sensitive sites in frozen whole blood and frozen isolated PBMC showed that there was no statistical significant differences observed between the net Fpg-sensitive sites of the assays performed in whole blood and in PBMC, showing that the results wouldn't be statistically different if this procedure had been done in PBMC.

4.5. Cardiovascular function parameters

4.5.1. Heart rate vs Noise exposure

Following "Approach 1" and regarding the heart rate is possible to observe that the "Exposed" group presented 74.7 ± 13.39 bpm and the "Non-exposed" group 66.8 ± 10.82 bpm (Table 8, Figure 12).

Table 8 – Descriptive parameters for "Heart rate" between "Exposed" and "Non-exposed" groups.

		N	Mean	Std. Deviation	Std. Error Mean
Heart Rate (bpm)	Exposed	43	74.7	13.39	2.04
	Non-exposed	10	66.8	10.82	3.42

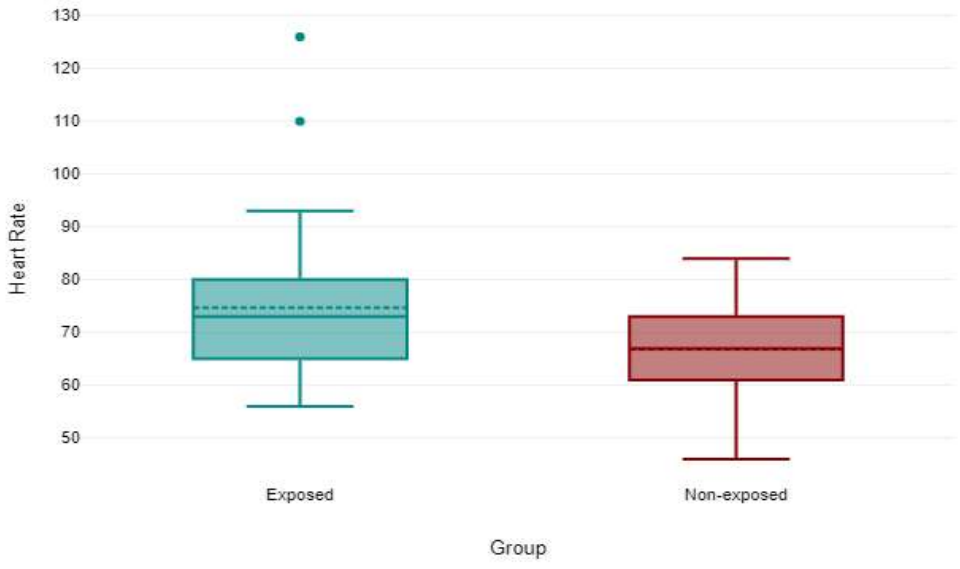

Figure 12 – Heart rate (bpm) among 'Exposed' and 'Non-exposed' groups (boxplot with mean, interquartile, and standard deviation).

The "Exposed" group showed higher values for the variable Heart Rate (M = 74.7, SD = 13.39) than the "Non-exposed" group (M = 66.8, SD = 10.82), yet, the t-test for independent samples (Table 9) showed that this difference was not statistically significant, p = 0.089, 95% confidence Interval [-1.26, 17.05], considering that the *Levene* test showed that equality of variances could be assumed (p = 0.727).

Table 9 – T-Test for independent samples summary associated with heart rate values.

		t	df	*p-value (2-tailed)*
Heart Rate	Equal variances	1.73	51	**0.089**
	Unequal variances	1.98	16.12	0.065

Following "Approach 2", as showed on the Table 10 and Figure 13, can be observed that the sample exposed to values equal or under 85 dB(A) presented 70.18 ± 21.36 bpm, the group exposed to values between 85 and 87 dB(A) 70.67 ± 9.1 bpm and the ones exposed to values equal or above 87 dB(A) 74.97 ± 10.79 bpm. Overall, the workers of the metallurgic factory presented values of 73.21 ± 13.35 bpm.

Table 10 – Descriptive statistics for Heart rate between "Noise class" groups. Class 0 represents exposure levels under 85 dB(A), Class 1 exposure levels between 85 and 87 dB(A), and Class 2 exposure levels over 87 dB(A).

Class	N	Mean (bpm)	Std. Deviation
0	11	70.18	21.36
1	9	70.67	9.1
2	32	74.97	10.79
Total	52	73.21	13.35

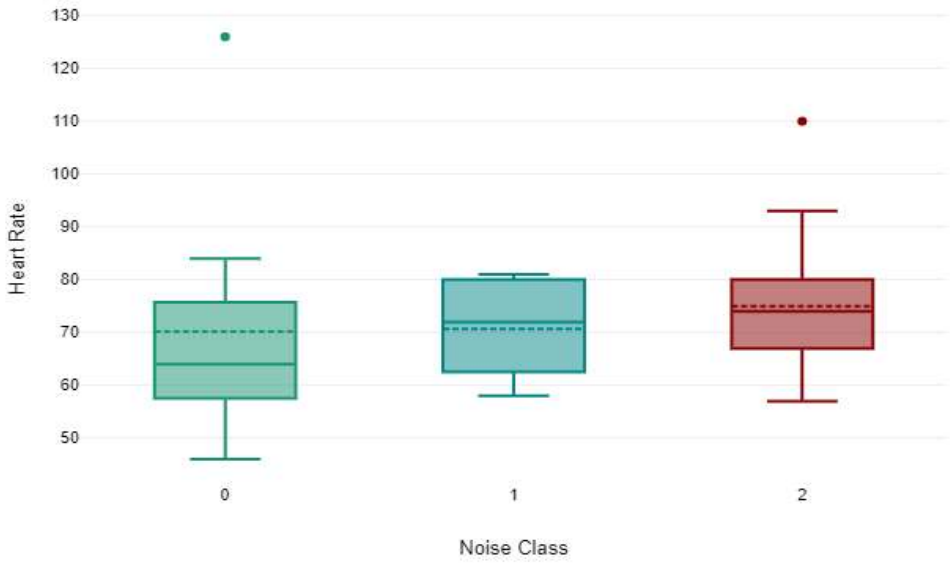

Figure 13 – Noise Class groups heart rate values in bpm (boxplot with mean, interquartile, and standard deviation).

The one-factor analysis of variance (ANOVA) shows that there is no significant influence between "Noise Class" and the "Heart Rate" value (p = 0.494), as shown on Table 11.

Table 11 – ANOVA test summary associated with the noise classes and the heart rate values.

	Sum of Squares	**df**	**Mean Squares**	**F**	***p-value***	**Critical F-Value**
Between Groups	258.07	2	129.03	0.72	**0.494**	3.19
Within Groups	8832.61	49	180.26			
Total	9090.67	51				

Even though it was not seen any statistical difference between the groups, either in approach 1 or 2, it is important to notice that the subjects presented normal values of heart rates, being there known to be values of 60-100 bpm for normal adult humans (Rexlin *et al.*, 2020).

A study realized by Bagheri Hosseinabadi et al. (2019) referred above also demonstrated similar results to the ones presented in this study, when it comes to the association of noise exposure with heart rate. The authors concluded that even though noise exposure represents an important risk factor for increasing both systolic and diastolic blood pressure, they realized that in their sample where was not shown a statistical difference in the heart rate of the groups exposed and not exposed to noise.

In a study of Zamanian *et al.* (2013), 50 steel factory workers exposed to occupational noise were subjected to a control noise exposure of 40 dB(A) and then exposed to 85-105 dB(A) and their heart rate was measured in both conditions in 3 different days. Their results showed that there was not a statistical difference between the results of when they were under the different noise exposures.

Even though various studies showed similar results to the ones showed in the present work, Veljovic et al. (2019) performed a study that included 30 workers on machine press at a factory, that were exposed to levels of noise of 65-110 dB(A) in their workplace. After a monitorization of 3 years, where their heart rate information was taken at every 6 months, the authors concluded that noise had increased heart rate, as well as blood pressure, over time, even though it stood at normal heart rate levels.

Moreover, Singhal et al. (2009) presented a study that was conducted on 114 workers from various lock factories in Aligarh, when comparing their heart rates with the control group (n=30) it was noticed that the heart rate of the exposed group were statistically higher than the control group.

4.5.2. Blood Pressure vs Noise exposure

Regarding the blood pressure it was measured the systolic and diastolic blood pressures. Following "Approach 1" (Table 12, Figure 14), the systolic blood pressure (SBP) on the "Exposed" and "Non-exposed" groups was respectively 128.42 ± 13.47 mm Hg and 125.7 ± 10.29 mm Hg. Regarding the diastolic blood pressure (DBP) was 78.28 ± 11.02 mm Hg on the "Exposed" group and 78.3 ± 6.5 mm Hg on the "Non-exposed" group.

Table 12 - Descriptive parameters for "Blood pressure" between "Exposed" and "Non-exposed" groups.

		N	Mean	Std. Deviation	Std. Error Mean
Systolic Blood Pressure	Exposed	43	128.42	13.47	2.05
	Non-exposed	10	125.7	10.29	3.25
Diastolic Blood Pressure	Exposed	43	78.28	11.02	1.68
	Non-exposed	10	78.3	6.5	2.06

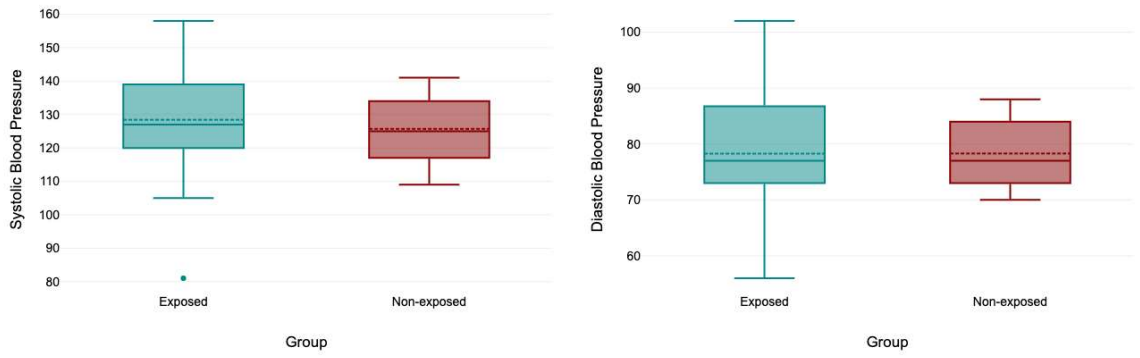

Figure 14 – Blood pressure data (mm Hg) in "Exposed" and "Non-exposed" groups (boxplot with mean, interquartile, and standard deviation), being the systolic blood pressure on the left and diastolic blood pressure on the right.

The analysis of the blood pressure was made comparing the values of the SBP and DBP between groups. When referring to the analysis of SBP and using the 'approach 1' we found that the "Exposed" group had higher values of SBP (M = 128.42, SD = 13.47) than the "Non-exposed" group (M = 125.7, SD = 10.29) and the t-test for independent samples (equal variances assumed), presented on the Table 13, showed that the difference was not statistically significant (p = 0.553, 95% confidence Interval [-6.43, 11.87]). In the same way, the analysis of the DBP the "Exposed" group shows lower values (M = 78.28, SD = 11.02) than the "Non-exposed" group (M = 78.3, SD = 6.5) but statistically non-significant (p = 0.995, 95% confidence Interval [-7.34, 7.3]).

Table 13 – T-Test for independent samples summary associates with systolic and diastolic blood pressure values.

		t	***df***	***p-value (2-tailed)***
Systolic Blood Pressure	Equal variances	0.6	51	0.553
	Unequal variances	0.71	17.03	0.489
Diastolic Blood Pressure	Equal variances	-0.01	51	0.995
	Unequal variances	-0.01	22.88	0.994

Following "Approach 2" (Table 14, Figure 15), the SBP of the group exposed to values equal or under 85 dB(A) (Class 0) had 122.18 ± 8.53 mm Hg, the group exposed to values between 85 and 87 dB(A) (Class 1) had 128.67 ± 11.85 mm Hg and the group exposed to values equal or higher 87 dB(A) (Class 2) had 129.5 ± 14.27. Regarding the DBP "Class 0" had 77.82 ± 8.67 mm Hg, "Class 1" 74.89 ± 10.51 mm Hg and "Class 2" 79.5 ± 10.9 mm Hg. Overall, the sample presented the SBP of 127.81 ± 12.99 and DBP of 78.35 ± 10.36.

Table 14 – Descriptive statistics for blood pressure (mm Hg), separated in SBP and DBP, between "Noise class" groups. Class 0 represents exposure levels under 85 dB(A), Class 1 exposure levels between 85 and 87 dB(A), and Class 2 exposure levels over 87 dB(A).

		Systolic Blood Pressure (SBP)		***Diastolic Blood Pressure (DBP)***	
Class	N	Mean	Std. Deviation	Mean	Std. Deviation
0	11	122.18	8.53	77.82	8.67
1	9	128.67	11.85	74.89	10.51
2	32	129.5	14.27	79.5	10.9
Total	52	127.81	12.99	78.35	10.36

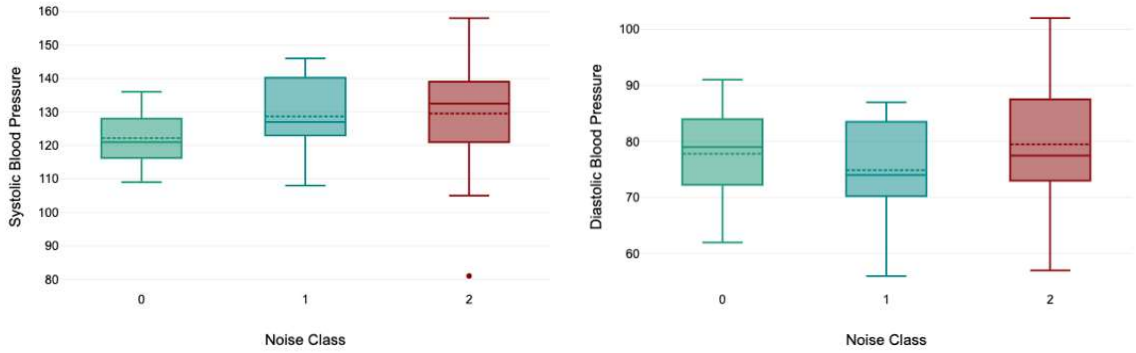

Figure 15 – Noise Class groups blood pressure values (mm Hg) separated in SBP and DBP, respectively (boxplot with mean, interquartile, and standard deviation).

The analysis regarding the blood pressure was made comparing the values of SBP and DBP of the different noise classes. As it can be observed, associated with the SBP the pressured where higher in the groups of higher exposure, on the other hand, regarding the DBP the lower value was presented on the Class 1. The one-factor analysis of variance (ANOVA), presented on the Tables 15 and 16, shows that there is no significant influence between "Noise Class" and the "SBP" or the "DBP", presenting a p-value of respectively 0.271 and 0.499.

Table 15 – ANOVA test summary regarding the SBP.

SBP	***Sum of Squares***	***df***	***Mean Squares***	***F***	***p-value***	***Critical F-Value***
Between Groups	446.44	2	223.22	1.34	0.271	3.19
Within Groups	8161.64	49	166.56			
Total	8608.08	51				

Table 16 – ANOVA test summary regarding the DBP.

DBP	***Sum of Squares***	***df***	***Mean Squares***	***F***	***p-value***	***Critical F-Value***
Between Groups	153.24	2	76.62	0.71	0.499	3.19
Within Groups	5320.53	49	108.58			
Total	5473.77	51				

Regarding the blood pressure parameters it is important to notice that the SBP and DPB considered optimal and normal are values <120-130 and <80-85, respectively (Nassiri et al., 2019).

A study realized by Zamanian et al. (2013), with the study conditions referred above, in wish there weren't found to occur different systolic or diastolic blood pressure values in the individuals when they were exposure to sounds of 40 dB(A) comparing with the groups of 85, 95 and 105 dB(A) exposures.

Rizi and Dehghan (2013) performed a study where they selected 90 factory workers exposed to occupational noise of 95.2 ± 10.1 dBA. In that study the authors considered that the relationship between the blood pressure (both SBP and DBP) and the noise level was not significant.

On the other hand, a study realized by Bagheri Hosseinabadi *et al.* (2019), where the food-factory workers were separated in a control group (n=123) and a exposed group (n=109) the authors considered that, regarding blood pressure, there was a direct relation between the increase of noise exposure and the increase of SBP and DBP since those values where significantly higher in the group exposed to 96 dB(A) than the group exposed to 80 dB(A). In that work it was also referred that noise exposure, age, and work experience were predictors of systolic blood pressure, while noise exposure and age were predictors of diastolic pressure.

Yaghoubi *et al.* (2018) realized a study regarding occupational noise exposure where it was compared a control group (n=26), exposed to 60-70 dB(A), and two exposure groups of 26 individuals each (75–85 dB(A) and 85-95 dB(A)), all of them being workers on an automotive factory. During this study, the blood pressure parameters were measured before work and during lunch break, for 10 days. It was possible to observe that before work hours there was not observer a significative difference between the control groups and the exposed groups, on the other hand it was possible to verify significant differences between the control and the exposed groups.

5. Conclusion

Many studies alert to the problematic of non-auditory issues caused by noise exposure, such as cardiovascular diseases, sleep disorders, annoyance, and DNA oxidative damage. The results obtained in the present work did not show any statistically difference in oxidative DNA damage, heart rate, systolic and diastolic blood pressure between studied groups, regardless of the type of stratification (exposed and non-exposed individuals; ≤85; between 85 and 87; ≥87 dB(A)). Nevertheless, higher oxidative DNA damage levels, heart rate and systolic blood pressure levels were observed among the exposed groups in both approaches of analysis.

The present work is hindered by the small sample size that does not allow getting any definite conclusions in either way, and to the unknown effect of the use of hearing protection in the parameters under study, since it is not clear if the non-auditory effects are dependent on the audible stimulus.

To better understand this problematic, it is important to analyse bigger samples, and analyse different dB(A) levels and exposure durations, and study populations differently affected by noise such as those that do not use auditive protection while exposed.

In the future, is important to understand if the non-auditory effects are or not dependent on audible stimulus, and if the hearing protection also works on these effects, since most literature regarding non-auditory effects noise uses samples of unprotected individuals.

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