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Occupational exposure to wildland firefighting and its effects on systemic DNA damage

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ABSTRACT

Background: Portugal is among the European Union countries more devastated by forest fires. Wildland firefighters are at the forefront of this battle, facing exposure to a wide range of harmful pollutants. Epidemiological studies have highlighted a potential link between occupational firefighting exposure and several diseases, including cancer. To date, very few studies have explored the biological mechanisms associated with such exposure. The present longitudinal study aims to assess changes in early effect biomarkers following wildland firefighters' occupational exposure to a real wildfire event.

Methods: Paired blood samples from 59 healthy Portuguese wildland firefighters were collected at two different time points: before wildfire season and after a fire event during wildfire season. Sociodemographic variables (e.g., age, sex) and work-related factors (e.g., years of service) were assessed via a self-reported questionnaire. Levels of early effect biomarkers, such as primary DNA damage and oxidative DNA damage (oxidised purines) were assessed via comet assay. DNA double-strand breaks (DSBs) were evaluated by phosphorylated H2AX (γH2AX). Moreover, hydroxylated polycyclic aromatic hydrocarbon metabolites (OHPAHs) and metal(loid)s were quantified in urine samples. The influence of urinary OHPAHs, urinary metal(loid)s, and other exposure-related factors (e.g., firefighting duration) on changes (Δ) in early effect biomarkers (post-vs. baseline levels) was investigated.

Results: Firefighting activities led to a significant increase in both primary DNA damage and oxidative DNA damage by 22 % (95 % CI: 1.11–1.35; $p < 0.05$) and 23 % (95 % CI: 1.04–1.45; $p < 0.05$), respectively. Results from linear regression revealed that per each unit increase of urinary 2-hydroxyfluorene (2-OHFlu) (μmol/mol creatinine), the risk of △ oxidative DNA damage increased by 20 % [FR: 1.20 (1.09–1.32); $p < 0.01$]. Additionally, each unit increase in urinary cesium (Cs) (μg/L) resulted in a significant 4 % increase in Δ primary DNA damage [FR: 1.04 (1.01–1.06); $p < 0.05$] and a 3 % increase in Δ oxidative DNA damage [FR: 1.03 (1.01–1.05); $p < 0.05$]. Post-exposure levels of γH2AX were significantly correlated with urinary 2-OHFlu levels assessed after

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firefighting ($r = 0.30$; $p < 0.05$). Furthermore, exposure duration and reported breathing difficulties during firefighting were significantly associated with increased levels of primary DNA damage.
Conclusion: Results obtained provide insights into the potential human health effects of wildland firefighting occupational exposure at the genetic and molecular levels, offering new and important mechanistic data. These findings are crucial for implementing health and safety measures, recommendations, and best practices to mitigate occupational risks and protect the health of wildland firefighters.

1. Introduction

In recent years, wildfires have occurred with increasing frequency and unprecedented intensity around the globe (Abatzoglou and Williams, 2016; Bernstein et al., 2019; Radeloff et al., 2018; Zhang et al., 2017). Firefighters are on the frontline of these events, playing a crucial role in wildfire response, and protecting population and goods. Nevertheless, the hazardous nature of their work exposes them to a broad spectrum of occupational risks, including the development of acute and long-term adverse health effects (Esteves et al., 2024). Over the years, epidemiological evidence has linked firefighting occupational exposure to an increased risk of various diseases, including cancer (Casjens et al., 2020; Glass et al., 2017; LeMasters et al., 2006; Navarro et al., 2019).

Recently, in 2022, the International Agency for Research on Cancer (IARC) classified occupational exposure as a firefighter carcinogenic to humans highlighting that such exposure has characteristics of carcinogens, including genotoxicity, induction of epigenetic alterations, oxidative stress, chronic inflammation, and receptor-mediated effects (IARC, 2023). Despite this IARC classification, research on the biological mechanisms induced by occupational exposure in firefighters remains scarce and inconclusive, especially among wildland firefighters (IARC, 2023). Most available studies are focused on firefighters' exposure to structural fires (e.g., municipal), under training/simulated conditions, or during controlled fires such as prescribed burns (IARC, 2023).

During their activities, wildland firefighters are exposed to a wide range of harmful pollutants released by the combustion of natural organic fuels (e.g., vegetation) (Esteves et al., 2022). After being absorbed and metabolised by cells, these contaminants can generate reactive oxygen species (ROS) (Danielsen et al., 2011; Leonard et al., 2007; Liu et al., 2005), which, if not neutralised by the body's antioxidant defense system, may react with proteins, lipids and nucleotides, leading to oxidative stress and genomic instability (Czerska et al., 2015; Esteves et al., 2017; Lamprecht et al., 2004). Oxidative stress is extensively described in the literature as a critical factor in the development of various pathological processes, including cancer (IARC, 2023; Poetsch, 2020; Reid et al., 2016; Senoner and Dichtl, 2019). Among the pollutants released by smoke, some are well known for their carcinogenic potential, such as, polycyclic aromatic hydrocarbons (PAHs), metal (loid)s and particulate matter (PM) (IARC, 2023). PAHs are a large class of ubiquitous pollutants formed during the incomplete combustion of organic matter and released during wildfires (Fabian et al., 2014; Hwang et al., 2022; Kim et al., 2013). It is believed that PAHs (or its metabolites) genotoxicity is primarily due to its ability to interact with DNA (Lewtas, 2007) resulting in genomic instability and alterations that can lead to the development of diseases, including cancer (Kamal et al., 2015; Lewtas, 2007). Metal(loid)s, such as lead (Pb), arsenic (As) or mercury (Hg), are also pervasive in the environment and are associated with various adverse health effects, including cardiovascular diseases, skin and respiratory problems, neurological disorders, kidney dysfunction, adverse pregnancy outcomes and cancer (Ali et al., 2023; Bharti and Sharma, 2022; Chen et al., 2023; Mitra et al., 2022). Furthermore, some metals tend to bioaccumulate, posing an additional health risk, particularly for long-term repetitive exposures (Al-Malki, 2009; Ali et al., 2023).

Human biomonitoring (HBM) is an essential tool in exposure assessment for identifying vulnerable populations, assessing combined exposures, and tracking the effectiveness of interventions to mitigate health risks posed by the exposure to hazardous substances either in the environment, work settings or other sources (Costa and Esteves, 2023; Esteves et al., 2022). This approach involves the characterisation of exposure to chemicals by measuring their levels or metabolites in biological samples such as blood, urine, hair, saliva, or exhaled air (Costa and Esteves, 2023). HBM provides direct evidence of exposure by quantifying chemicals present in the body - biomarkers of internal dose - rather than relying solely on estimates based on environmental measurements (external dose); it also accounts for exposure through multiple routes while considering individual characteristics (e.g., age, sex) (Costa and Esteves, 2023). Exposure biomarkers provide information on the internal dose, but not on the effects resulting in its interactions with biological molecules biomarkers. In contrast, biomarkers of effect are biochemical alterations associated with pathological processes (Nordberg, 2010) and therefore, may serve as early warning signs or markers of disease (Decker et al., 2013). Genotoxicity evaluation constitutes an essential approach for assessing important occupational hazards (Costa et al., 2011). The comet assay is a valuable method for quantifying DNA strand breaks (SBs), such as single strand-breaks (SSBs) as well as other types of DNA damage, including DNA adducts, alkali-labile sites, and oxidative DNA lesions, in populations exposed to genotoxic agents (A. Collins et al., 2014). This assay is commonly used for the assessment of DNA damage mostly due to its simplicity, sensitivity and low cost (Neri et al., 2014). When coupled with a lesion-specific DNA repair enzyme (Collins, 2004), such as formamidopyrimidine DNA glycosylase (FPG), comet assay is also used to measure oxidised purines allowing for the detection of oxidative DNA damage (Møller et al., 2013). FPG specifically recognises and excises oxidised purines, including 8-oxoguanine - a well-known marker of oxidative stress linked to the development of various diseases (Cadet et al., 2012; Møller et al., 2013).

Other early indicator of genomic instability, specifically double-strand breaks (DSBs), is the phosphorylated histone H2AX (γH2AX) assay. DSBs are one of the most lethal forms of DNA damage, as they can lead to chromosomal fragmentation, rearrangements, mutations, or aneuploidy (loss of information) if not properly repaired (Cannan and Pederson, 2016; Li et al., 2023). Thus, assessing DSBs levels is crucial for understanding the biological relevance of genetic instability in diseases, particularly cancer (Li et al., 2023). In recent years, γH2AX has emerged as a key biomarker used in HBM for the evaluation of DSBs, as well as its repair of the activation of DNA damage response pathways (Nikolova et al., 2017; Valdiglesias et al., 2013). γH2AX is recognised as a robust biomarker for assessing occupational exposure to genotoxic agents and for the early detection of disease risk, including cancer (Valdiglesias et al., 2013). The analysis of γH2AX by flow cytometry is particularly advantageous for HBM studies because of its high sensitivity, specificity (Sánchez-Flores et al., 2015), and because it allows the collection of data of a large number of samples relatively fast (Laffon et al., 2021) compared to other methods (e.g., ELISA).

Very few studies have assessed DNA damage in firefighters (Abreu et al., 2017; Andersen et al., 2018a, 2018b; Oliveira et al., 2020) and, to the best of our knowledge, none have longitudinally characterised DNA damage levels (SBs and DSBs) and oxidised DNA levels in wildland firefighters enrolled in actual fire combat. Understanding wildfire fighting-related health impacts is essential for developing effective preventive measures and ensuring the safety and well-being of these essential first responders.

Our study aims to close this gap and provide insights into the biological mechanisms and potential health outcomes of occupational exposure for wildland firefighters. To achieve our goal, a group of Northern Portuguese wildland firefighters were studied at two distinct moments of their activity - before the wildfire season (Phase I, baseline levels) and during the wildfire season after combating a real wildfire event (Phase II, post-exposure levels). Paired wildland firefighters' samples were evaluated for primary DNA damage, oxidative DNA damage, and γH2AX. Their associations with urinary hydroxylated metabolites of PAHs (OHPAHs) and metal(loids) levels were assessed. The relationship between these biological endpoints and both sociodemographic and work-related variables, collected through questionnaires, was also explored.

2. Methods

2.1. Study population

The present study was conducted in accordance with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the University of Porto (reference number 92/CEUP/2020).

Fifty-nine wildland firefighters (n = 59) were recruited from five fire stations located in the northern region of Portugal, between 2021 and 2023. A pre-post study design was used, with each participant serving as their own control at two different time points: Phase I (before wildfire season, late spring) and Phase II (after combating a fire event, during wildfire season). Before their enrolment, firefighters were briefed on the study's aims, potential risks, data confidentiality, and their right to withdraw. A signed consent was required from subjects who agreed to participate. Data on sociodemographic characteristics (e.g., sex, age), medical history (e.g., chronic diseases), lifestyle (e.g., smoking habits), and occupational details (e.g., years of service) were obtained via a self-reported questionnaire applied during Phase I (before wildfire season). Additionally, after combating wildfire firefighters completed a brief survey to report details on exposure duration (in hours), wildfire event duration (in hours), and any acute symptoms experienced during fire combat.

2.2. Biological sampling

Biological samples, namely blood and urine, were collected in both phases. Before the wildfire season (Phase I) samples were collected during the firefighters' shift (morning period) in a clean and designated area at the fire station. For Phase II, post-fire samples were collected during the morning period, after returning to the fire station from wildfire suppression activities (combat/post-combat). The samples were collected within a similar timeframe for all participants.

2.2.1. Urine sampling collection

Participants were instructed to provide a spot urine sample in a 100 mL sterile plastic container. Urine samples were immediately coded, transported to the laboratory under cold conditions (4 °C) and stored at −20 °C for further analysis.

2.2.2. Blood sampling collection

Peripheral venous blood samples were collected via venipuncture into dipotassium ethylenediaminetetraacetic acid (EDTA)-coated tubes (Vacuette® Tube K2EDTA). After collection, blood samples were coded, maintained at 4 °C, and transported to the laboratory to be processed. Upon arrival, two aliquots with 200 μl of whole blood were immediately stored at −80 °C (slow freezing procedure) for subsequent comet assay analysis (both alkaline and modified-enzyme version). Peripheral blood mononuclear cells (PBMC) were isolated under aseptic conditions for γH2AX evaluation. Briefly, each blood sample was diluted with sterile 0.9 % NaCl at a 1:1 ratio. The diluted blood was carefully layered into pre-labelled tubes containing sterile Lymphoprep™ density gradient medium at room temperature, following manufacturer instructions. After centrifugation for 20 min at 900–1200 g the PBMC ring layer was carefully removed, transferred to another tube containing 6 mL of cold 0.9 % sterile NaCl and centrifuged at 480 g for 10 min. After discarding the supernatant, the pellet was gently resuspended in the remaining volume washed again with 6 mL of cold sterile 0.9 % NaCl and centrifuged at 480 g for 10 min. The supernatant was discarded and the resuspended PBMC were slowly cryopreserved in a solution of cold sterile 90 % fetal bovine serum (FBS) and 10 % dimethyl sulfoxide (DMSO) cryopreservation solution at −80 °C and kept stored for further analysis.

2.3. Urinalysis of OHPAHs metabolites and metal(loid)s

Urinary PAHs metabolites, namely, 1-hydroxynaphthalene (1-OHNaph), 1-hydroxyacenaphthene (1-OHAce), 2-hydroxyfluorene (2-OHFlu), 1-hydroxyphenanthrene (1-OHPhen), and 1-hydroxypyrene (1-OHPyr) were quantified by high-performance liquid chromatography (HPLC) with fluorescence detection, as described by Barros et al. (2024).

The metal(loid)s [i.e. lithium (Li), cobalt (Co), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), cadmium (Cd), antimony (Sb), cesium (Cs), barium (Ba), mercury (Hb), thallium (Tl) and lead (Pb)] were analysed by inductively coupled plasma mass spectrometry (ICP-MS) according protocol described by Paiva et al. (2024a) and Azevedo et al. (2023). Concentrations of OHPAHs and metal(loids) were normalised through urinary creatinine levels (μmol/mol creatinine).

2.4. Medium-throughput comet assay (12-gel comet assay)

2.4.1. Assay controls

Assay controls for standard alkaline comet assay and the enzyme-modified comet assay were prepared using a pooled sample of blood from four non-smoking healthy volunteers. Aliquots of untreated blood cells were used as baseline controls for the electrophoretic runs. For the enzyme-modified comet assay, blood cells were treated with 1.5 mM potassium bromate (KBrO3) – to induce DNA oxidation - in PBS (pH 7.4) for 1 h at 37 °C. After preparation, assay controls were cryopreserved, after a slow freezing process, at −80 °C for subsequent use in the comet assay, alongside participants' samples (Møller et al., 2020a).

2.4.2. Alkaline comet assay procedure

Primary DNA damage was evaluated by comet assay following Singh et al. (1988) with some minor modifications described by Esteves et al. (2020). In short, firefighters' whole blood samples and assay-controls (untreated and KBRO3-treated) were rapidly thawed on ice. Afterwards, 5 μL of blood was gently resuspended in 0.6 % (w/v) of low melting point agarose (Sigma Aldrich Company, Merck KGaA, Darmstadt, Germany) and 5 μL of this cell suspension was dropped onto a frosted glass slide pre-coated with 1 % (w/v) of normal melting point agarose (Lonza, Basel, Switzerland) forming a mini-gel. After solidification (for 5 min at 4 °C) slides were immersed in a Coplin jar containing ice-cold freshly prepared lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-base, and 250 mM NaOH, pH 10) supplemented immediately before use with 1 % (v/v) Triton X-100, for 1h, protected from light, at 4 °C. Subsequently, slides were immersed in ice-cold alkaline electrophoresis solution (200 mM Na_2EDTA, 10 M NaOH, pH 13) for 30 min, protected from light, at 4 °C, for DNA unwinding. Slides were then transferred to an electrophoresis tank (Axygen, UK) with an ice-cold electrophoresis solution. Electrophoresis followed for 20 min at approximately 1 V/cm under cold conditions (4 °C). Mini-gels were neutralised with ice-cold PBS (10 min) and washed with cold deionised H2O (10 min), dehydrated and fixed with ethanol 70 % (15 min) and 96 % (15 min) and let to dry overnight, at room temperature. For scoring, dried slides were rehydrated with Tris-EDTA (TE) buffer solution (10

mM Tris-HCl and 1 mM EDTA; pH 7.5–8.0) at room temperature for 20 min; slides were then stained with SYBR™ Gold (Invitrogen™, Waltham, MA, USA) diluted in TE buffer at room temperature for 30 min and then washed twice in distilled water and left to dry at room temperature. Microscopic analyses were performed blindly by the same reader on a fluorescence microscope (Motic BA410E) equipped with Comet Assay IV (Instem) a semi-automated image analysis software used for image capture and analysis. A total of 150 cells (75 per mini-gel) were scored for each subject (and assay controls) and the percentage of DNA in the comet tail (TDNA%) was used to evaluate the DNA damage at the cell level. The coefficient of variation for untreated assay controls was approximately 30 % (A.R. Collins et al., 2014).

2.4.3. Enzyme-modified comet assay (FPG)

Oxidative DNA damage was evaluated via comet assay following Teixeira-Gomes et al. (2020) and others (Costa et al., 2011). In brief, after lysis, slides were washed 3 times (5 min each) with ice-cold buffer F (40 mM HEPES, 100 mM KCl, 0.5 mM Na2EDTA, 0.2 mg/ml BSA, pH 8). After assembling the 12-Gel Comet Assay Unit™ (Severn Biotech Ltd., Worcestershire, United Kingdom), 30 μL of formamidopyrimidine DNA glycosylase enzyme (FPG) solution (8000 units/mL), used according to manufacturers' instructions, acquired from New England Biolabs®, Ipswich, MA, USA) or 30 μL buffer F were added to each well covering the mini-gel. Incubation was done for 30 min at 37 °C. The following steps were performed according to the comet assay described in previous studies (Esteves et al., 2020). The percentage of DNA in the comet tail (TDNA%) was the DNA damage parameter chosen to evaluate comet formation. Net FPG-sensitive sites, for each subject, were calculated by subtracting the TDNA% value obtained for buffer and from the value obtained for FPG. The coefficient of variation of TDNA% KBRO3-treated assay controls, for net FPG measurements, across the different experiments was 21 % (Esteves et al., 2020; Møller et al., 2020b).

2.5. H2AX phosphorylation (γH2AX)

2.5.1. Assay controls

γH2AX assay controls were prepared under aseptic conditions using a pool of PBMC from four non-smoking healthy volunteers. As described herein, lymphocytes were isolated using a sterile Lymphoprep™ density gradient, following the manufacturer's protocol. PBMC were then subjected to treatment with bleomycin (20 μL/mL) for 4 h at 37 °C to serve as a positive control for DNA DSBs induction. Untreated PBMC were used as negative controls. Aliquots containing approximately 1×10^6 cells were resuspended in 1 mL of sterile freezing medium (50 % FBS, 40 % RPMI-1640 and 10 % DMSO) and slowly cryopreserved at −80 °C until further analysis.

2.5.2. γH2AX analysis

The γH2AX analysis was performed based on the protocols described by Sánchez-Flores et al. (2015), Tanaka et al. (2009) and Watters et al. (2009). In brief, cryopreserved PBMC samples were thawed at a 37 °C water bath for approximately 60 s (Barcelo et al., 2018). Following thawing, cells were resuspended in 5 mL of culture medium (RPMI-1640 with L-Glutamine, supplemented with 15 % heat-inactivated fetal bovine serum and 1 % Penicillin-Streptomycin solution). The cell suspensions were centrifuged at 810 g for 10 min, and the supernatant was discarded. Cells were fixed with 1 mL of cold, filtered 1 % p-formaldehyde for 15 min at 4 °C, and followed by centrifugation at 1300*g* for 5 min (4 °C). The supernatant was removed, and cells were permeabilised with 1 mL of cold 70 % ethanol (−20 °C) and stored overnight at 4 °C. On the following day, cells suspensions were centrifuged at 1300 g for 5 min at 21 °C. After centrifugation, the supernatant was aspired, and the cell pellet was washed with filtered phosphate-buffered solution (PBS) (pH 7.4) with 1 % BSA. Cells were again centrifuged for 5 min at 1300 g at 21 °C. Subsequently, cells were incubated with 100 μL of 1:100 Alexa Fluor™ 488 anti-phospho-histone H2A.X (Ser139) monoclonal antibody (Invitrogen 53-9865-82) diluted in filtered PBS 7.4 with 1 % bovine serum albumin (BSA) for 15 min at room temperature in the dark. After this period of incubation, cells were washed with 1 mL of filtered PBS (pH 7.4) containing 1 % BSA and then centrifuged for 5 min at 1300 g at 21 °C. The supernatant was removed, and cells were resuspended in 250 μL of PBS (pH 7.4) containing 0.1 mg/mL of RNAse A and 40 μg/mL of propidium iodide (PI) and incubated for 30 min in the dark at room temperature.

The analysis by flow cytometry was performed in a GUAVA EasyCyte 8HT (Luminex Corporation, USA) flow cytometer, equipped with a 488 nm excitation laser. Data were analysed using Guava® easyCyte™ software. The PBMC population was gated based on side scatter (SSC) for complexity and forward scatter (FSC) for size. Approximately 20,000 event gates were acquired for each sample. The analysis assessed the signals in Red-B Fluorescence (Red-B-Hlin) and Green-B Fluorescence (GRN-B-HLog) to identify cells marked with propidium iodide (PI) and anti-γH2AX, respectively. Each sample was analysed in duplicate, and the result for each sample was calculated as the mean of both duplicates. To obtain the percentage of γH2AX, the following equation was used: "number of PBMC positively marked with antibody γH2AX and PI/total number of PBMC positively marked with PI" x 100.

2.6. Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 29.0 for Windows. Descriptive statistics were used to summarise the data, with continuous variables reported as means ± standard deviations (SD), and categorical variables presented as frequencies and percentages. The normality of the data was assessed using the Kolmogorov-Smirnov test and visual analysis of graphical dispersion plots. Distribution departed from normality and therefore nonparametric tests were considered for statistical analysis. Univariate analysis was conducted to investigate possible associations between genotoxicity biomarkers i.e. primary DNA damage, oxidative DNA damage, and γH2AX, and exposure-related factors i.e., urinary metal(loid)s, urinary OHPAHs, and self-questionnaire retrieved variables (e.g., exposure duration (hours), acute symptomatology experienced during firefighting, wildfire duration). The effect of age, sex and smoking habits was also investigated. Participants were classified as non-current smokers if they had never smoked or had quit smoking for more than one year. Those who were currently smoking or had quit smoking for one year or less, were classified as current smokers. Statistical differences among independent groups were tested using the Man-Whitney *U* Test (two groups) and Kruskal-Wallis test (more than two groups). Bonferroni adjustment was used for multiple comparisons. The Wilcoxon test was employed to assess the differences in biomarkers between paired samples collected before and during the wildfire season. Correlations between continuous variables were analysed by Spearman's rank correlation test. Frequency ratios (FR) with 95 % CI were calculated to assess the relative change in the studied endpoints (i.e., primary DNA damage, oxidative DNA damage and γH2AX) between pre- and post-exposure.

Effect biomarkers that exhibited statistically significant differences after wildfire exposure were further analysed. Linear regression analysis was conducted to identify statistically significant associations between exposure factors and variations in effect biomarker levels across the two study phases (Pre-exposure – Phase I; Post-exposure – Phase II). To assess the relative change in biomarker levels, we first log-transformed the dependent variables for both phases (Phase I – Pre-exposure; Phase II – Post-exposure) and then calculated the difference in the log-transformed values [log(Post-exposure) - log(Pre-exposure)] to obtain the variation in respective biomarker [Δ = log(Post-exposure) - log(Pre-exposure)]. This step allowed the calculation of the FR by exponentiating the beta coefficients (e^{β}) from the linear regression analysis. A statistical significance level of 0.05 was considered.

3. Results

The general characteristics of the study population (n = 59) are summarised in Table 1. The wildland firefighters had a mean age of 35.5 ± 9.0 years (range: 20.0–55.0) and were predominantly males (78 %). In terms of smoking habits, 32 (54.2 %) were current smokers and 27 (45.8 %) were non-current smokers, with all former smokers (n = 12) reporting that they had quit for more than one year (data not shown). Also, on average, our group, had 15.9 ± 8.9 years of service as wildland firefighters, ranging from 3 up to 34 years.

Data retrieved from the short survey on Phase II, showed that most of the reported wildland fires (49.2 %) lasted for 12 h or less, 23.7 % were between 48 and 72 h, 20.3 % persisted for more than 72 h, and a smaller proportion (6.8 %) lasted between 12 and 48 h.

When categorising exposure duration to active wildfire as reported by wildland firefighters: 40.7 % reported being exposed during fire-combat activities for 1 h to 3 h, 30.5 % for 3–12 h, and 28.8 % for more than 12 h. As for the symptoms, among others, the most reported symptomatology was eye and respiratory irritation (22.0 %), difficulty breathing (20.3 %), and headaches (11.9 %) (Table 1).

Regarding effect biomarkers, our data showed that participation in wildland firefighting significantly increased the levels of primary DNA damage and oxidative DNA damage by 22 % (95 % CI: 1.11–1.35; p = 0.03) and 23 % (95 % CI: 1.04–1.45; p = 0.04), respectively (Fig. 1). No statistically significant differences in γH2AX (%) levels were observed after fire intervention (FR = 0.91, 95 % CI: 0.83–1.22; p > 0.05).

Univariate analysis showed significant differences between Phase I and Phase II levels of urinary markers (1-OHNaph+1-OHAce, 1-OHPy, Ni and As and Cs), as well as γH2AX and comet assay endpoints (Table S1 and Table S2, respectively - supplementary material). No significant effects from self-reported variables (age, sex, smoking) or other studied covariates (i.e., BMI, smoking habits, physical exercise, diet and years of service as a firefighter) were observed on the studied endpoints in Phase I (Table S3 - supplementary material). Fig. 2 shows the significant positive correlations found between post-exposure levels of genotoxicity biomarkers and exposure biomarkers, respectively; post-exposure levels of γH2AX (%) were significantly correlated with urinary 2-OHFlu levels assessed after fire-combat (r = 0.30; p = 0.03) (Fig. 2a). Post-exposure levels of oxidative DNA damage were sightly significantly correlated with post-exposure levels of urinary 2-OHFlu (r = 0.26 p = 0.05) (Fig. 2b) and significantly correlated with urinary Cs (r = 0.42; p = 0.01) (Fig. 2c). Moreover, a significant positive correlation was found between levels of primary DNA damage and oxidative DNA damage measured after firefighting exposure (r = 0.34; p = 0.01), confirming the association between these two endpoints (results not shown).

Table 1
General characteristics of the study population (n = 59).

Study participant characteristics		Wildland Firefighters (n = 59)
Sociodemographic	Age (years) [a]	35.5 ± 9.0 (20.0–55.0)
	Sex	
	Male, n (%)	46 (78.0 %)
	Female, n (%)	13 (22.0 %)
Lifestyle-related	Smoking habits	
	Non-current smoker, n (%)	27 (45.8 %)
	Current smoker, n (%)	32 (54.2 %)
Occupational-related	Years of service [a]	15.9 ± 8.9 (3.0–34.0)
Active wildfire exposure	Fire duration	
	≤ 12 h, n (%)	29 (49.2 %)
	> 12–48 h, n (%)	4 (6.8 %)
	> 48–72 h, n (%)	14 (23.7 %)
	> 72 h, n (%)	12 (20.3 %)
	Exposure duration (hours)	
	1–3 h, n (%)	24 (40.7 %)
	>3–12 h, n (%)	18 (30.5 %)
	> 12 h, n (%)	17 (28.8 %)
	Reported symptoms [b]	
	Eyes & respiratory irritation, n (%)	13 (22.0 %)
	Breath difficulty, n (%)	12 (20.3 %)
	Headaches, n (%)	7 (11.9 %)

[a] Mean ± Standard deviation (min-max).
[b] Symptomatology during fire suppression activities.

When exploring the influence of exposure biomarkers on the variation of effect biomarkers [△ = log(Post-exposure) - log(Pre-exposure), FR], our data showed that per each unit increase of urinary Cs (μg/L), the risk of △ primary DNA damage and △ oxidative DNA damage increased by 4 % [FR = 1.04 (1.01–1.06); p < 0.05] and 3 % [FR = 1.03 (1.01–1.05); p < 0.05], respectively (Table S4 supplementary material). In addition, our data showed that per each unit increase of 1-OHFlu (μmol/mol creatinine), there was a 20 % higher risk of △ oxidative DNA damage [FR = 1.20 (1.09–1.32); p < 0.01] (Table S4 supplementary material). No association between other urinary OHPAHs or metal (loid)s, on △ primary DNA damage and △ oxidative DNA damage was observed in this study. The described results on △ effect biomarkers were maintained when adjusted for sex, age, and smoking status (Tables S5–S7, supplementary material).

Significant associations were found between post-exposure levels of genotoxicity biomarkers and the prevalence of symptomatology reported by firefighters during wildfire combat (Phase II). Wildland firefighters who reported breathing difficulties during firefighting (20.3 %) had significantly higher levels of primary DNA damage than those who did not (23.31 ± 2.62 vs. 16.01 ± 0.98, respectively; p = 0.01) (Fig. 3), with this association remaining after adjusting for smoking habits (data not shown). Although oxidative DNA damage was also higher in firefighters who reported breathing difficulties during firefighting activities (16.87 ± 1.61 vs. 13.14 ± 1.17, p = 0.02), this significance disappeared after controlling for smoking habits; a similar trend was observed for γH2AX levels (1.42 ± 0.41 vs. 1.02 ± 0.19), though the difference was not statistically significant (data not shown).

Firefighters reporting participating in a fire event lasting for more than 72 h presented higher △ oxidative DNA damage than those reporting a fire event lasting for less than 12 h (0.71 ± 0.14 vs 0.31 ± 0.15, respectively; p = 0.02) (data not shown).

Regarding the duration of fire combat, wildland firefighters who reported being in the field between 3 and 12 h exhibited significantly increased levels of primary DNA damage compared to those engaged less than 3 h (21.07 ± 2.37 vs. 14.30 ± 1.11; p = 0.01) (Fig. 4).

4. Discussion

With the global upsurge in wildland fires, preventing occupational health effects on wildland firefighters has become an urgent and critical priority of public concern (Stephens et al., 2013; Westerling et al., 2006). Despite firefighting being classified as carcinogenic to humans, very few studies have investigated the biological impacts of occupational exposure on wildland firefighters (IARC, 2023), particularly the effects induced by exposure during real wildfires. Our study aimed to evaluate the genotoxic effects experienced by wildland firefighters at different stages of occupational exposure, before (Phase I) and during wildland fire season (Phase II). We observed a statistically significant increase in both primary DNA damage and oxidative DNA damage levels after firefighting activities, compared to baseline levels (Phase I). Based on our knowledge, this is the first study longitudinally assessing primary DNA damage and oxidative DNA damage among wildland firefighters exposed to real wildfire events. Other studies carried out in real-combat scenarios had a cross-sectional design (Abreu et al., 2017; Oliveira et al., 2020). Abreu et al. (2017) reported increased levels of both primary and

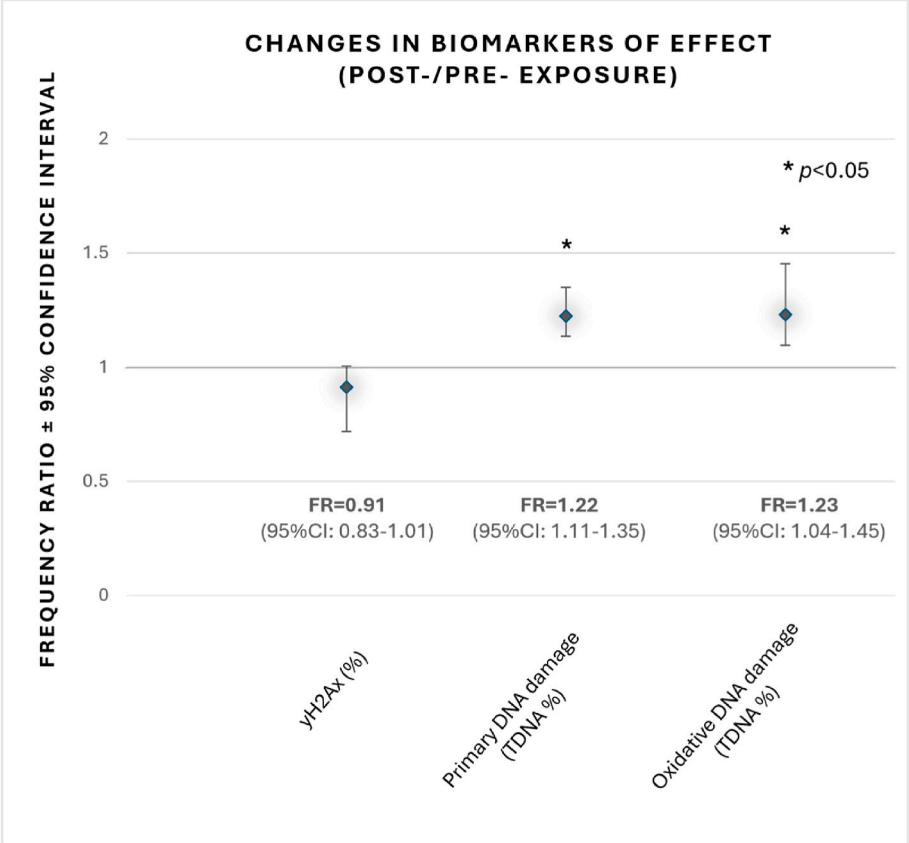

Fig. 1. Frequency ratios (FR) and respective 95 % confidence interval, showing changes in effect biomarkers after firefighting occupational exposure (post-/pre-exposure). *Statistical significance is indicated by FR values significantly different from 1 (no effect), with p-values <0.05.

oxidative DNA damage (measured by comet assay) in a group of Portuguese wildland firefighters (n = 60) compared to controls from the general population (n = 63). However, only primary DNA damage reached statistical significance, being 76 % higher than controls (Abreu et al., 2017). Another study conducted by Oliveira et al. (2020) found significantly higher levels of oxidative DNA damage in a group of non-smoking wildland firefighters (n = 48), directly involved in firefighting activities within 48h before sample collection, compared to firefighters with no recent exposure (n = 93); results for primary DNA damage were inconclusive (Oliveira et al., 2020).

Consistent with our findings some authors have also observed increased levels of other oxidative stress markers associated with wildland firefighting exposure (Gaughan et al., 2014; Wu et al., 2020). Gaughan et al. (2014), for example, found significantly elevated levels of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, in U.S. male firefighters recently exposed to wildland firefighting (n = 20) compared to those with no recent exposure (n = 18). In addition, Wu et al. (2020) found higher levels of 8-isoprostane, a marker of lipid peroxidation, in the exhaled breath condensate of U.S. wildland firefighters (n = 12) during prescribed burns, when compared to levels assessed in non-burn days.

Our data also aligns with reports from studies enrolling structural firefighters (Andersen et al., 2018a, 2018b). Andersen et al. (2018a) investigated the association between firefighting and levels of DNA damage (primary and oxidative DNA damage) in a cross-over study involving 53 firefighter conscripts from Denmark undergoing firefighter structural training. The authors found that firefighting activities significantly increased the levels of oxidative DNA damage in PBMC by 8.0 % (95 % CI: 0.02–15.9), but no association was observed for primary DNA damage (Andersen et al., 2018a). However, a significant increase in primary DNA damage levels was found after exercises involving wood pallets fuel, compared to exercises using mixed fuels (i.e., wood, foam mattresses, and electrical cords) (Andersen et al., 2018a). In a subsequent study also conducted by Andersen et al. (2018b) authors followed a group of 22 male structural professional firefighters (Copenhagen, Denmark) before and after a 24-h shift. No association was found between work-shift exposure and primary and oxidised DNA damage levels. It is important to note, that firefighters used a self-contained breathing apparatus (SCBA) which protects structural firefighters from inhaling contaminants (Andersen et al., 2018b). Insufficient smoke-exposure gradient and sampling-time challenges were some of the other limitations presented by Andersen et al. (2018b) that could have contributed to the observed results. It is important to note that, generally the use of SCBA is not feasible in a wildland firefighting context, either because of its weight or the limited duration of air supply (Esteves et al., 2022). Inhalation of wood smoke has been demonstrated to adversely influence human health (Augusto et al., 2020). In a controlled intervention trial, Ferguson et al. (2016) investigated the effects of wood smoke inhalation by exposing a group of participants from general population (n = 10) to wood smoke for 1h and found significantly higher levels of 8-isoprostane (marker of oxidative stress) in the exhaled breath condensate compared to control conditions (filtered air) ($p < 0.05$) (Ferguson et al., 2016).

Regarding our analysis of γH2AX, no significant differences were observed following wildland firefighting exposure. To date, no studies have assessed this endpoint to monitor occupational exposure in wildland firefighters. However, Jasra et al. (2022) reported a significant accumulation of γH2AX foci in lymphoblasts (GM03798) exposed *in vitro* to aerosolised dust originated from the World Trade Centre fires (WTC-PM), characterised to contain high concentrations of PAHs. Given the exposure of first responders, mostly firefighters, to high levels of WTC-PM, the authors suggested that the dust exposure might have

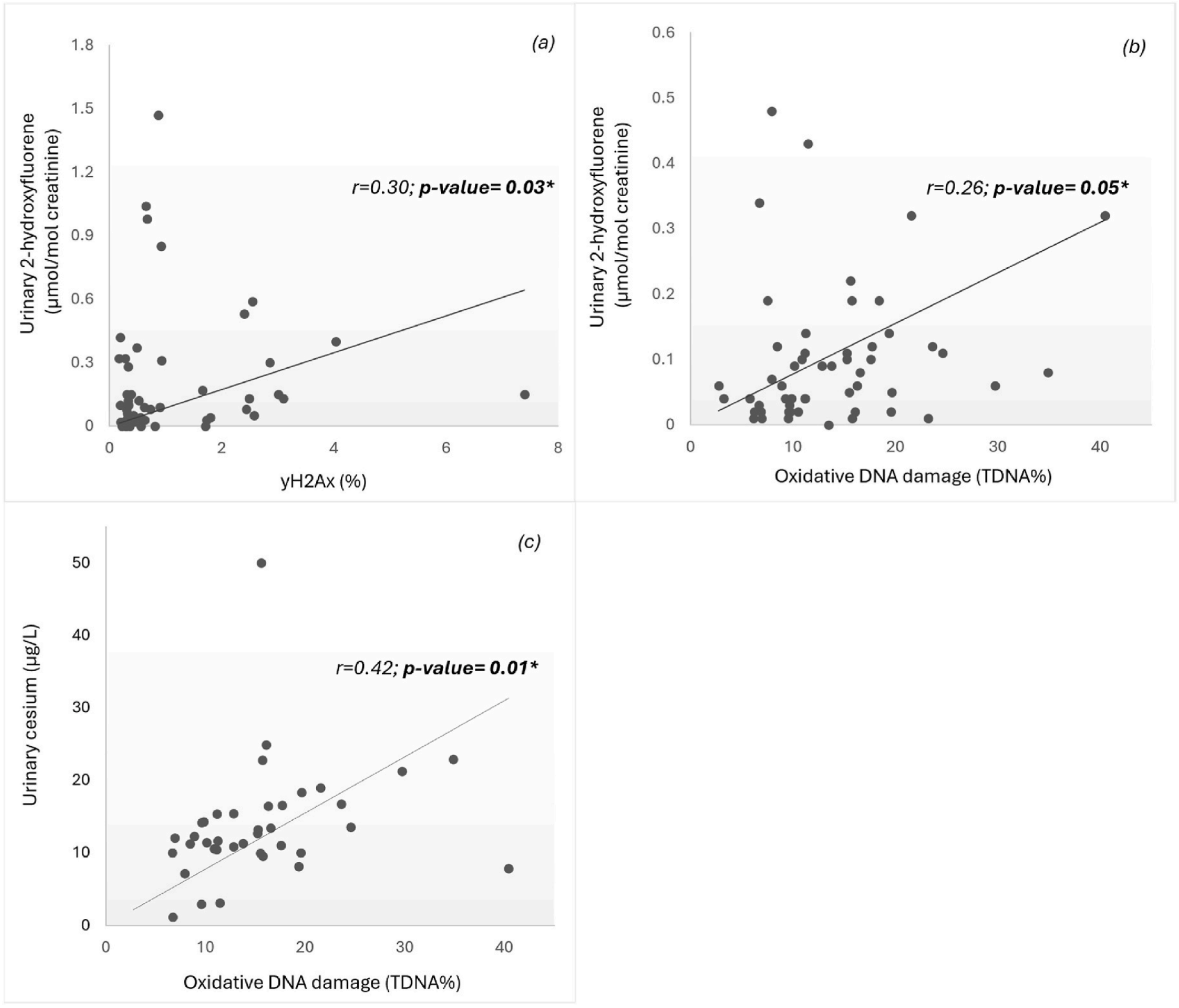

Fig. 2. – Scatter plots illustrating statistically significant correlations between post-exposure levels of exposure and effect biomarkers. *Indicate the statistical significance of FR, with p-values ≤0.05.

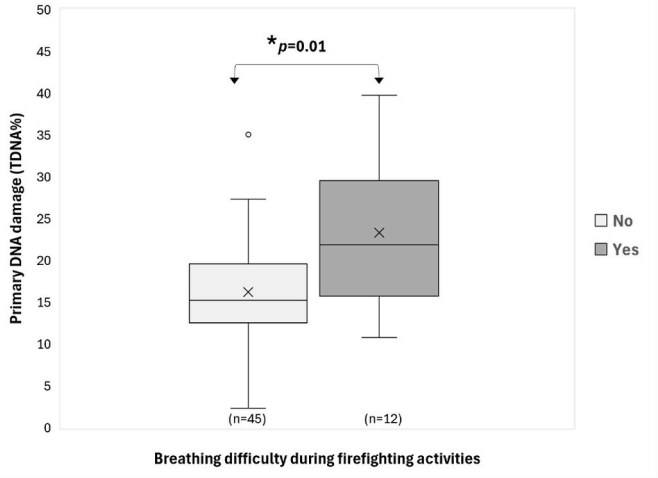

Fig. 3. – Post-exposure levels of primary DNA damage levels (TDNA%) in firefighters with and without breathing difficulties during firefighting activities. *Statistically significant difference $p < 0.05$. Statistical significance maintained when adjusted for smoking.

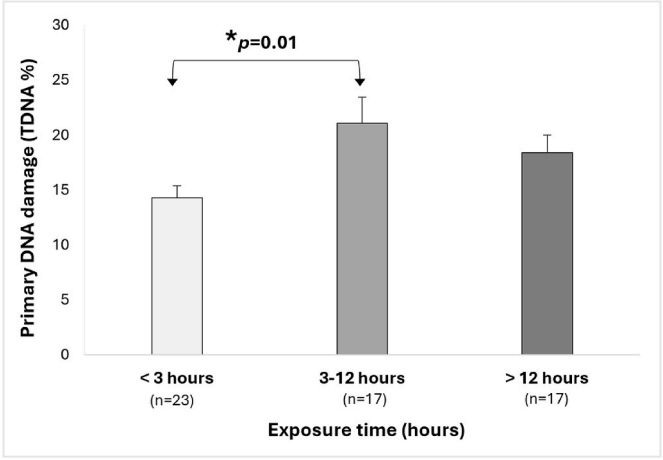

Fig. 4. Post-exposure levels of primary DNA damage (TDNA %) according to the number of hours spent in firefighting activities. * Statistically significant difference $p < 0.05$.

induced γH2AX formation. The phosphorylation of H2AX, forming γH2AX, is an early event in DNA damage response. It occurs rapidly after DNA DSB as part of the cellular mechanism to signal the activation of DNA repair pathways (Sánchez-Flores et al., 2015). Once the damage is

repaired, γH2AX is dephosphorylated or removed, making it undetectable. However, if γH2AX persists after repair, it may indicate ongoing genomic instability (Podhorecka et al., 2010). The use of γH2AX holds great potential for environmental and occupational monitoring, allowing early detection and prevention of potential long-term adverse health outcomes (Laffon et al., 2021). Some studies, for example, have identified other DNA alterations associated with firefighting occupations. For instance, Zhou et al. (2019) reported significant differences in DNA methylation patterns between experienced firefighters and new recruits. These methylation alterations were found to correlate with cancer-related pathways, suggesting that such epigenetic modifications may contribute to increased cancer risk in firefighters. Similarly, Goodrich et al. (2022) verified that firefighting was associated with changes in DNA methylation, further supporting the hypothesis that occupational hazards may induce long-term genetic modifications. Given the role of epigenetic modifications in the development of various diseases, including cancer (Quaid et al., 2024), monitoring these changes, along with biomarkers of DNA damage and repair, could be crucial for identifying occupational risks in wildland firefighters.

In accordance with previous studies (Paiva et al., 2024b), all urinary OHPAH levels in this study were increased after exposure to woodsmoke. The urinary OHPAHs with the highest concentrations after firefighting exposure were 1-OHNaph+1-OHAce, followed by 2-OHFlu, 1-OHPhen and 1-OHPyr. The concentrations of 1OHNaph+1OHAce in this study were higher than those reported in previous studies conducted on firefighters exposed to wildfire emissions (Oliveira et al., 2020). Regarding 2-OHFlu and 1-OHPhen, levels verified in this study were similar to those found in previous studies focused on Portuguese wildland firefighters (Oliveira et al., 2016, 2017, 2020). 1-OHPyr was the PAH metabolite found at the lowest concentration, which is consistent with other studies that assessed urinary OHPAHs in firefighters exposed to woodsmoke and verified that 1-OHPyr is one of the OHPAHs detected at lower concentrations (Adetona et al., 2017; Oliveira et al., 2020; Li et al., 2016).

Concerning the relationship between genotoxicity endpoints and urinary OHPAHs, we found that levels of γH2AX and oxidative DNA damage were positively correlated with urinary 2-OHFlu levels, correspondingly. In addition, we observed a significant contribution of urinary 2-OHFlu for the observed variation (post/pre-exposure) of oxidative DNA damage. Previous studies have also explored correlations between genotoxicity biomarkers and firefighting exposure to PAHs (Andersen et al., 2018a, 2018b; Oliveira et al., 2020). Similarly to our results, Oliveira et al. (2020), observed a positive correlation between oxidative DNA damage and urinary 2-OHFlu levels among two groups of Portuguese wildland firefighters (smokers vs non-smokers) exposed to forest fires. In the Andersen et al. (2018a) study, a positive correlation was found between primary DNA damage levels and urinary levels of 1-OHPyr. Furthermore, both primary and oxidative DNA damage were positively associated with total PAHs levels retrieved from skin wipes (Andersen et al., 2018a). In our study, we found a significant positive association between primary and oxidative DNA damage and the levels of Cs (133Cs) excreted in urine. According to our understanding, this is the first study correlating blood genotoxic/oxidative biomarkers with wildfire smoke-derived metals among wildland firefighters enrolled in real scenarios of fire suppression activities. Up to date, information available on health effects and toxicity of Cs in humans is limited (Yermishev, 2023). Previous studies have shown that stable Cs compounds are able to induce genomic instability, namely, chromosomal aberrations in cultured human primary cells (Ghosh et al., 1993) and micronuclei in mouse bone marrow cells *in vivo* (Santos-Mello et al., 1999). Additionally, a recent study in rats found that stable Cs had pronounced damaging effects on skeletal and cardiac striated muscle tissue, linking it to adverse cardiovascular outcomes (Yermishev, 2023).

In the environment Cs is naturally present only in one stable form - the isotope 133 Cs (non-radioactive). The lithological formations in the Northern region of Portugal - where our study was conducted - is predominantly characterised by granite (pegmatite formation) and schist (Agroconsultores, 1991; Meireles, 2011), where Cs-bearing minerals may occur in different concentrations (Williams, 2004). We hypothesise that wildland burning may promote the release of this element into the environment (Al-Malki, 2009) thereby increasing the risk of exposure. In fact, a study conducted by Wolfe et al. (2004) found higher Cs (133Cs) concentrations in the urine of firefighters enrolled in wildfire combat when compared to NHANES III reference values (Third National Health and Nutrition Examination Survey) (Paschal et al., 1998). Paiva et al. (2024a) characterised the impact of real-life wildland firefighting operations on urinary levels of pollutant metal(loid)s among Northern Portuguese firefighters, and found significant higher post-exposure urinary Cs levels, when compared to baseline levels. Nevertheless, more studies are needed to confirm our results, to better understand the impact of wildland burning on the release and bioavailability of metal(loid)s, and their effects on human health and to facilitate the comparison of urinary metal(loid)s concentrations among wildland firefighters. When examining the link between wildland firefighters' symptomatology during fire combat and genotoxicity biomarkers, we found that those who reported breathing difficulties had significantly higher levels of primary DNA damage compared to those who did not. Although not statistically significant, a similar trend was also observed for oxidative DNA damage and γH2AX. During active fire combat usually, firefighters do not wear adequate respiratory protection, furthermore, they continue to be exposed after combat, exposing them to high concentrations of pollutants in both gaseous and particulate form (Esteves et al., 2024). Consequently, reported breathing difficulty during fire combat may reflect greater inhalation of smoke contaminants, explaining the results obtained.

Our results exploring the association of work-related variables, gathered at Phase II, after the fire-event combat, have shown a significant positive influence of fire exposure and event duration on DNA damage endpoints.

Longer-lasting wildfires may release higher amounts of pollutants due to prolonged biomass combustion, leading to increased pollutant accumulation in the atmosphere and greater exposure. Kganyago et al. (2021) for example, observed that larger burned areas were associated with higher concentrations of smoke pollutants (i.e., PM and black carbon). Furthermore, prolonged exposure to smoke pollutants during long-lasting wildfires may occur through several factors, including the permanence of wildland firefighters near the affected area, their geographical proximity to the wildfire event (residential area or fire station), or ongoing exposure to contaminated personal protective equipment (PPE)/firefighting tools/vehicles, in the fire station. These factors may simultaneously contribute to increased risk of exposure to smoke pollutants and, consequently, enhance biological damage.

Despite our findings, our study has some limitations. Since data on social-demographic and work-related factors were collected by self-administered questionnaires, information bias may have occurred, inclusively regarding the precise number of hours spent on wildland firefighting activities and regarding the smoking status (e.g., recall bias or social desirability bias). Further studies should assess cotinine levels to accurately determine tobacco exposure. Additionally, it is important to consider the metabolism and excretion rates of certain combustion-derived products (Bader et al., 2021), which may lead to an underestimation of exposure levels due to the time interval between the exposure event and biological sample collection. Moreover, further studies are needed to confirm our results.

Nonetheless, our study uses a pre/post-study design, where participants serve as their own control, reducing the risk of potential confounders. This longitudinal analysis includes repeated measurements at different time points of the occupational exposure, enrolling the same individuals engaged in real-wildfire scenarios, *in situ*, providing more accurate, close-to-reality, insights into potential causal associations between exposure and observed outcomes.

In occupational hygiene, once a health risk is identified, the primary

mitigation measure is to eliminate, reduce, or substitute the source of exposure, thereby minimising its presence and lowering the associated risk (Schoket, 1999). However, for wildland firefighters, this may constitute a challenge. Therefore, the proper use of PPE, shift-rotation, and decontamination practices, and comprehensive training on these procedures, are of extreme importance to reduce contact with wildland smoke contaminants (Esteves et al., 2024). In this regard, our data seems to suggest the importance of rotative shifts during fire combat, rest areas protected from fire contaminants and effective decontamination procedures. Moreover, adopting a healthy lifestyle is essential for improving overall health and mitigating potential health effects of firefighting occupational exposure. Healthy behaviours include maintaining a balanced diet, rich in antioxidants to support cellular redox balance, engaging in regular physical exercise, practicing good sleep hygiene, monitoring mental/emotional health and avoiding risky behaviours such as tobacco and alcohol consumption (IARC, 2023).

5. Conclusion

Despite the obvious challenges that firefighters face, little is known about the biological mechanisms underlying the health outcomes associated with wildland firefighting. Thus, understanding exposure-induced biological mechanisms is crucial for the application of clinical tools in the preliminary identification of potential diseases, including cancer, and for designing measures to prevent disease onset.

Our findings showed that occupational exposure to real wildfire events induced systemic genomic instability, measured by comet assay and γH2AX, among firefighters. Furthermore, a positive association between effect biomarkers and smoke combustion by-products (PAHs and metals), duration of exposure, wildfire dimension, and firefighters' acute symptomatology, was found. To our knowledge, this is the first longitudinal study investigating the impact of firefighters' occupational exposure to real wildfire events on systemic DNA damage biomarkers. Moreover, this is the first time that γH2AX was evaluated among wildland firefighters exposed to forest fires.

Our data is of paramount importance to support recommendation measures and policy development vis-à-vis the protection of firefighters' health. The identification of occupational risk factors is crucial for the development of mitigation strategies to minimise exposure risks and adverse health impacts. Future longitudinal studies should focus on the assessment of the impact of firefighting exposures through cancer-related biomarkers, particularly on local-targeted tissues.

CRediT authorship contribution statement

Filipa Esteves: Writing – review & editing, Writing – original draft, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joana Madureira:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Carla Costa:** Writing – review & editing, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Joana Pires:** Software, Methodology, Investigation, Data curation. **Bela Barros:** Methodology, Investigation, Formal analysis, Data curation. **Sara Alves:** Methodology, Investigation, Formal analysis, Data curation. **Josiana Vaz:** Writing – review & editing, Resources, Project administration, Methodology. **Marta Oliveira:** Resources, Methodology, Investigation, Formal analysis, Data curation. **Klara Slezakova:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adília Fernandes:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **Maria do Carmo Pereira:** Writing – review & editing, Project administration, Methodology, Funding acquisition. **Simone Morais:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Vanessa Valdiglesias:** Writing – review & editing, Resources, Methodology, Data curation. **Stefano Bonassi:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **João Paulo Teixeira:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition. **Solange Costa:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Ethics approval

This work received approval for research ethics by the Accredited Ethics Committee of the University of Porto, Portugal, Report Nr. 92/CEUP/2020, under the project BioFirEx project (PCIF/SSO/0017/2018): "A panel of (bio)markers for the surveillance of firefighter's health and safety".

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2025.114576.

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