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Micronucleus assay in buccal and urothelial epithelial cells of wildland firefighters exposed to wildfire smoke

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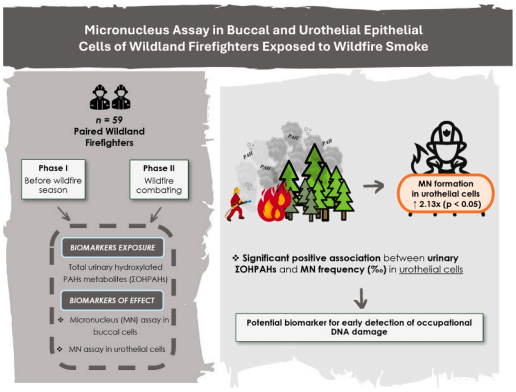
HIGHLIGHTS

- Micronucleus (MN) frequency is considered a potential early cancer risk biomarker.
- MN assay in buccal and urothelial cells is minimally invasive.
- MN‰ in firefighters' urothelial cells were over 2x higher during wildfire season.
- Urinary total OHPAHs significantly increased MN‰ in firefighters' urothelial cells.
- MN assay in urothelial cells may serve as a biomonitoring tool for firefighters.

GRAPHICAL ABSTRACT



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ABSTRACT

Occupational exposure as a firefighter is classified as carcinogenic to humans. However, information on the biological effects of wildland firefighting remains limited. This study aimed to assess genotoxicity in a group of wildland firefighters and evaluate the contribution of total concentration of urinary hydroxylated polycyclic aromatic hydrocarbons (ΣOHPAHs) to selected endpoints. A group of 59 northern Portuguese wildland

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Wildland firefighters
Wildfire smoke

firefighters (mean age: 35.5 ± 9.0 years) was evaluated before and during the wildfire season. Sociodemographic, lifestyle, occupational-related information was collected via questionnaire. The micronucleus (MN) assay in buccal and urothelial cells was applied to paired samples to assess the association with exposure, as indicated by urinary ΣOHPAHs levels. The risk of MN formation in urothelial cells was over twofold higher during the wildfire season [Frequency Ratio (FR): 2.13, 95 % CI: 1.99–2.27, $p = 0.01$]. A 35 % increase in MN frequency in buccal cells was observed during the wildfire season (FR: 1.35, 95 % CI: 0.76–2.40, $p > 0.05$). Urinary ΣOHPAHs exhibited a significant positive association with MN‰ in urothelial cells (FR:1.04, 95 % CI: 1.01–1.08, $p < 0.05$). This study provides the first assessment of MN frequency in urothelial cells of wildland firefighters, offering novel evidence of genotoxic risks and potential long-term health impacts associated with wildland firefighting.

1. Introduction

Wildfires are among the most concerning natural disasters posing significant threats to ecosystems, property, and human safety [1]. Wildland firefighters provide vital services to the community, protecting the environment, heritage and human life [2]. During their activity, wildland firefighters are exposed to multiple occupational hazards such as heat stress, physical and psychological stressors, and the inhalation of smoke pollutants [1].

Through the years, epidemiological studies have reported an increased risk of cancer mortality among firefighters [3], however, studies exploring the underlying biological effects remain scarce, particularly in wildland firefighters [4-6]. Molecular epidemiology enables the evaluation of biological effects associated with environmental and occupational exposures [7,8]. Micronuclei frequency is among the most used biomarkers for genomic instability [9]. The micronucleus (MN) originates from chromosome fragments or whole chromosomes that fail to be incorporated into daughter cells nuclei during mitosis, serving as indicators of cytogenetic alterations and genotoxic exposure [10,11]. They may reflect both structural and numerical chromosomal aberrations and are formed as a consequence of exposure to occupational, environmental and lifestyle genotoxic compounds [12,13]. It is well known that chromosomal damage plays an important role in the aetiology of human cancer [14] and other diseases [15]. The MN assay in lymphocytes is a well-established and validated biomarker for the prediction of cancer in humans [16,17]. However, this traditional method is invasive, requiring blood collection and primary cell culture. Assessing MN in less invasive biological matrices, such as buccal epithelial cells, constitutes a valid alternative method [10]. This approach is particularly relevant because up to 90 % of all cancers are of epithelial origin [18]. Moreover, a significant positive correlation has been observed between the frequency of MN in buccal cells and lymphocytes [17]. This was supported by a recent systematic review conducted by Nersesyan and colleagues (2025), which found a significantly high correlation between the two MN assays [19], supporting the reliability of the buccal MN assay in reflecting genotoxic exposure. In recent decades, the Buccal Micronucleus Cytome (BMCyt) assay has been widely used in human biomonitoring studies to assess environmental and occupational exposure to genotoxic agents [20]. The use of this method is particularly valuable in occupational health surveillance, as buccal cells can be collected through simple, minimally invasive procedures, offering a practical alternative to blood sampling. Furthermore, as the BMCyt assay combines cytological and cytogenetic evaluation, multiple endpoints can be assessed, including genetic instability (MN and nuclear buds), cell death (condensed chromatin, karyorrhexis, pyknotic and karyolytic cells), proliferative activity (basal cell frequency) and cytokinetic defects (binucleated cell frequency) [21,22]. The major route of exposure to smoke air-contaminants among wildland firefighters is inhalation [1]. As humans are oral breathers, the buccal mucosa is directly exposed to airborne pollutants released during wildfire combat activities [22], serving as their primary barrier [23,24]. Thus, the buccal mucosa is a suitable tissue, and the BMCyt is an optimal approach for monitoring early genotoxic effects in wildland firefighters, whose main routes of contaminant exposure are inhalation and ingestion [25-28].

Excretion represents another significant route of exposure to toxicants. While buccal cells may serve as the first local tissue for the initial contact with airborne pollutants, it is equally important to investigate excretion pathway tissues, such as the urothelium. The application of the MN assay to urothelial cells (e.g., from urine samples) is relatively recent and has gained increasing attention as a non-invasive method for early cancer risk prediction and human biomonitoring [29], particularly relevant given the high prevalence of bladder cancer among firefighters [4].

Wildfire smoke contains several harmful pollutants, including polycyclic aromatic hydrocarbons (PAHs), some of which are known human carcinogens [3], while others are classified as probable (Group 2 A) or possible (Group 2B) human carcinogens [30]. Some of the compounds present in wildfire smoke are excreted in urine, where they can be detected either in their original form (e.g., metals) or as more reactive metabolites, such as hydroxylated PAHs (OHPAHs) [31-33].

In 2022, the International Agency for Research on Cancer (IARC) [4] classified firefighting as carcinogenic to humans, highlighting that such exposure exhibits key characteristics of carcinogens, including genotoxicity, induction of epigenetic alterations, oxidative stress, chronic inflammation, and receptor-mediated effects. Despite this classification, few human molecular epidemiological studies have investigated the biological effects at the molecular and cellular levels among wildland firefighters [4]. Most existing data pertain to structural firefighters (e.g., municipal) or to controlled exposure scenarios such as training exercises, prescribed burns, or fire-simulation chambers [4]. The limited number of studies addressing real-time exposure to wildfires is likely due to the unpredictable nature of such events and the logistical challenges of conducting fieldwork under such demanding conditions, within a predominantly volunteer-based workforce [4].

Biomonitoring constitutes a crucial approach to overcome these difficulties, as it enables the retrospective assessment of internal exposure and early biological effects [1]. The characterisation of early biological effects in wildland firefighters involved in real wildfire-combat activities, and their association with internal exposure biomarkers (e.g., urinary ΣOHPAHs), is essential to generate evidence that can inform the development of preventive and protective measures to mitigate potential health outcomes in this population. This need is particularly important given the projected global increase in wildfire frequency and intensity, which will inevitably impose greater demands on wildland firefighters and increase their exposure burden.

To the best of our knowledge, no previous study has applied the MN assay in urothelial cells within the firefighting context, nor has any study investigated the association between MN endpoints (in both buccal and urothelial cells) and urinary ΣOHPAHs in wildland firefighters.

In this context, the present study aimed to longitudinally evaluate the genetic instability and other cellular endpoints in buccal and urothelial cells of a group of wildland firefighters at two distinct timepoints: before the wildfire season (Phase I) and during the wildfire season, after real wildfire-combat activities (Phase II). Additionally, the study sought to assess the contribution of total urinary PAHs (ΣOHPAHs) to the observed changes (Phase II/Phase I) in the studied endpoints.

2. Material and methods

2.1. Reagents

Sodium chloride (NaCl; Cat. No. 106406), sodium hydroxide (NaOH; Cat. No. 106497), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA; Cat. No. E5134), hydrochloric acid (HCl; Cat. No. 100319), Schiff's reagent (Cat. No. 109034), Fast Green FCF for microscopy (Cat. No. 104022), and Entellan™ (Cat. No. 107961) were purchased from Merck KGaA (Darmstadt, Germany). Tris-hydrochloride (Tris-HCl; Cat. No. A3452) was obtained from AppliChem (Darmstadt, Germany). Ethanol absolute (Cat. No. 20821330), methanol (Cat. No. 20903368), and glacial acetic acid (Cat. No. 20104.334) were supplied by VWR Chemicals (Radnor, PA, USA).

2.2. Study population

The population included in this longitudinal study consisted of a convenience sampling of 59 healthy wildland firefighters (including both volunteer and full-time contracted firefighters) from five fire stations located in the northern region of Portugal. This region is predominantly rural and considered a high-risk area for wildfires, due to its climate (characterised by hot and dry summers), mountainous terrain and vegetation heterogeneity [34-36]. The presence of firefighter units dedicated to wildland firefighting has allowed the study to focus on wildland firefighters. All participants were engaged in wildland firefighting activities and had at least one year of experience as firefighters. All wildland firefighters used the appropriate personal protective equipment (e.g., structural helmet with eye protection, flash hood, gloves, respiratory protection - typically a cotton bandana for wildfire operations - boots, and fire-resistant clothing), in compliance with Portuguese legislation (Order no. 3974/2013; Order no. 4958/2014) [37], and therefore there were no differences in the use of this equipment among participants. Further details on the study population and recruitment procedures have been described previously [6]. A pre-post study design was used, with participants serving as their controls at two time points of their activity: Phase I (baseline) – before the wildfire season, when participants were located at the fire station and had not recently been involved in firefighting activities; Phase II (exposure) – during the wildfire season when firefighters were actively engaged in wildfire combat activities. All participants signed an informed consent form after being fully briefed on the study's objectives, procedures for data collection and biological sample collection, confidentiality of data, and their right to withdraw from the study at any time without consequence. In Phase I, participants completed a comprehensive questionnaire that included questions on sociodemographic characteristics (e.g., sex, age), lifestyle factors (e.g., smoking), occupational details (e.g., years of service) and diet. In Phase II, following active deployment to a wildfire event and upon return to their fire stations, firefighters completed a brief questionnaire to collect data on individual exposure duration (in hours per wildfire event), the total duration of the respective wildfire (in hours), and any acute symptoms experienced during wildfire combat. Biological samples, specifically urine and buccal cells, were collected in both phases of the study.

This study was conducted in accordance with the Declaration of Helsinki and received approval from the Ethics Committee of the University of Porto (reference number 92/CEUP/2020).

2.3. Biological sample collection

In Phase I, before wildfire season, urine and buccal cells were collected at the fire station during the firefighters' shift (morning period). In Phase II, during wildfire season, biological samples were collected at the fire station, immediately after firefighters returned from wildfire-combat activities. Biological samples were collected within a similar post-exposure timeframe for all firefighters.

For urine sampling, firefighters were asked to collect a spot urine sample into a 100 mL sterile plastic container. The urine samples were transported to the laboratory under refrigerated conditions (4°C) for processing for the MN assay in urothelial cells and thereafter stored at −20°C for further analysis of urinary OHPAHs.

The protocol for buccal cell collection followed the methods described by Costa et al. [38], Esteves et al. [22] and Thomas et al. [18] with minor modifications. Firefighters were asked to rinse their mouth with tap water to remove unwanted debris. A sample from each cheek was collected using two separate cytobrushes (Deltalab) - one for each cheek - which were gently but firmly rotated against the inside of the cheek wall in a circular motion, not touching in mouth or teeth, in a circular motion for 30 s. Buccal cells were collected into two 15 mL Falcon tubes labelled LC (left cheek) and RC (right cheek), each containing 10 mL Buffer solution (0.01 M Tris-HCl, 0.1 M EDTA, 0.02 M NaCl, pH 7). Samples were transported in refrigerated conditions (4∘C) to the laboratory and processed on the same day for the BMCyt assay.

2.4. Urinary OHPAHs analysis

Quantification of OHPAHs metabolites in urine samples collected both in Phase I and II, followed the methods described by Barros et al. [39] and Paiva et al. [31]. Briefly, analytes were extracted by solid-phase extraction and quantified using high-performance liquid chromatography with fluorescence detection. Calibration curves, limits of detection (LOD) and quantification (LOQ) were established for each metabolite. Quality control included daily analysis of blanks, standards, and spiked pooled urine samples. Total urinary OHPAHs (ΣOHPAHs) represented the sum of the following metabolites: 1-hydroxynaphthalene (1-OHNaph), 1-hydroxyacenaphthene (1-OHAce), 2-hydroxyfluorene (2-OHFlu), 1-hydroxyphenanthrene (1-OHPhen) and 1-hydroxypyrene (1-OHPyr). Concentrations of urinary OHPAHs were normalised to creatinine levels (μmol/mol creatinine).

2.5. Micronucleus cytome assay

2.5.1. Buccal cells

The protocol was adapted from Thomas et al. [18] with minor changes described by Esteves et al. [22] and Costa et al. [40]. Briefly, after being washed and fixed with a cold solution of ethanol: glacial acetic acid (3:1, v/v), the buccal cell pellet was resuspended in 5 mL of the fixative solution and stored at −20°C. Upon slide preparation, fixed buccal cell suspension was centrifuged for 10 min at 500 rcf, supernatant discarded, and the remaining pellet dropped (2–3 non-overlapping drops) into clear coded pre-cleaned slides. Slide staining was performed using the Feulgen-Fast Green method as described in our previous study [22].

2.5.2. Urothelial cells

The method followed protocols previously described [29,41,42], with slight modifications. Urine samples (~30 mL) were separated into two centrifuge tubes and washed at 940 rcf for 20 min. After carefully discarding the supernatant, the cell pellet was washed twice with 10 mL of 0.9 % NaCl at 780 rcf for 10 min. On the last wash, the supernatant was removed leaving around 0.5 mL to ensure no cell loss [29]. The cell pellet was then resuspended with 5 mL of fixative solution (methanol: glacial acetic acid, 3:1) and stored at −20°C. Upon slide preparation, fixed cell suspension was centrifuged for 15 min at 780 rcf, the supernatant discarded, and the remaining pellet dropped into clear coded pre-cleaned slides (2–3 non-overlapping drops). Urine samples from males typically contain fewer cells, and as a result, require to be concentrated in a smaller volume of fixative solution [42]. After air-drying overnight in a horizontal position, slides were sequentially submerged in Coplin jars for 1 min each in a gradient of ethanol solutions, 50 % and 20 % v/v, followed by immersion in deionised water for 2 min. For Feulgen-Fast Green staining, slides were immersed in 5 M

HCL, for 30 min and rinsed in running tap water for 3 min. Slides were then stained with Schiff's reagent (Merck) in the dark, at room temperature, for 90–120 min. After this period, slides were rapidly dipped in deionised water and counterstained for 20 sec in 0.2 % (w/v) with Fast Green (Merck). This staining method minimises false-positive results, often observed with non-specific DNA stains. This specificity enhances the reliability and comparability of the results, making it a valuable tool for accurate assessment of chromosomal and nuclear integrity in cytogenetic analyses [22]. Slides were immersed 3x in absolute ethanol and let to air dry. Slides were covered with coverslips (Manzel-Gläser), mounted with Entellan®, and kept in the dark to dry until microscope scoring.

2.5.3. Slide scoring

Scoring was performed according to the criteria proposed by Thomas et al. [18] and Bolognesi et al. [21]. Stained coded slides were blindly analysed by a single trained operator on a Nikon Eclipse E400 attached to an epi-fluorescence illuminator Nikon C-SHG1 power supply for HG 100 W, under 400x magnification. Cells containing MN and other studied endpoints were further confirmed under fluorescence microscopy using a G-2A filter. For urothelial cells, only cells with typical morphology were analysed, excluding squamous cells from female urine samples. Only cells that were not clumping or overlapping were chosen. The frequency of basal cells, binucleated cells and cells undergoing death (i.e., condensed chromatin, karyorrhectic cells, karyolitic cells, and pycnotic cells) was determined in 1000 cells, whereas the genetic instability (i.e., MN frequency) and gene amplification biomarkers (NBUDs) were scored in 2000 differentiated cells per individual [18].

2.6. Statistical analysis

Wildland firefighters' characteristics are reported as means ± standard deviation (SD) for continuous variables and as absolute numbers and frequencies for categorical variables. Group comparisons by sex were conducted using the independent *t*-test for continuous data and the chi-square test for categorical data. Whenever the study endpoints did not follow a normal distribution (verified through the one-sample Kolmogorov-Smirnov test) non-parametric tests were used. The frequency of MN assay endpoints was reported as the number of a specific anomaly per 1000 cells (‰) [12]. The presence of association between MN frequencies and potential confounding factors, i.e., age, sex, smoking status (current vs former/never smoking), alcohol consumption [12], and dietary habits [22], was first investigated through univariate analysis. Differences between phases were checked with the Man-Whitney *U* test, whereas for variables with more than two groups Kruskal-Wallis Test (with Bonferroni adjustment) was used. Correlations within variables were checked by Spearman's rank correlation Test. Paired comparisons of biomarker levels between Phase I (baseline) and Phase II (wildfire season) were performed using the Wilcoxon signed-rank test. The frequency ratios (FR) with corresponding 95 % asymptotic confidence intervals (95 % CI) comparing post-exposure and pre-exposure mean values (Phase II/Phase I) were estimated using multiple log-linear regression analysis. Biomarkers showing significant changes between phases in univariate analysis were included as covariates. Logarithmic transformation was applied to normalise distribution and to enable the calculation of FR using exponentiated regression coefficients (e^{β}) [43]. Age, sex, and smoking were included as fixed covariates in multivariate models. The level of statistical significance established for α was 5 %. The software Statistical Package for the Social Sciences (SPSS) version 29.0 for Windows was used for the statistical analysis of data collected.

3. Results

The characteristics of the study group are described in Table 1. In sum, the study sample included 59 wildland firefighters, aged 20–55 years (mean 35.5 ± 9.0), comprising 46 (78 %) males and 13 (22 %) females. Regarding BMI, most firefighters (50.8 %) were classified as overweight, with a higher prevalence among males. Thirty-two firefighters (54.2 %) were current smokers. In terms of occupational factors, study subjects reported a mean of 15.9 ± 8.9 years of service as a firefighter (from 3.0 to 34.0 years). Male subjects were more often part of the permanent intervention teams (full-time contracted firefighters) (54.3 % vs 8.3 % of females, $p < 0.05$).

Table 2 summarises firefighters' occupational exposure and reported acute symptoms during real-time wildfire suppression (Phase II, wildfire season). The average wildfire event lasted 43.5 ± 46.4 h (range: 1–144 h). Firefighters reported that wildfires occurred in areas

Table 1
General characteristics of the study population (n = 59), stratified by sex.

Wildland firefighters' characteristics	Male (n = 46)	Female (n = 13)	All (n = 59)
Individual characteristics			
Age (years)[a]	35.6 ± 9.3 (20.0–55.0)	35.1 ± 8.0 (21.0–45.0)	35.5 ± 9.00 (20.0–55.0)
Body Mass Index (BMI) (kg m^{-2})[a]	26.7 ± 3.3 (19.4–35.9)	26.0 ± 8.0 (21.2–33.0)	26.5 ± 3.3 (19.4–35.9)
Normal weight, *n* (%)	17 (37.0 %)	5 (38.5 %)	22 (37.3 %)
Overweight, *n* (%)	23 (50.0 %)	7 (53.8 %)	30 (50.8 %)
Obesity, *n* (%)	6 (13.0 %)	1 (7.7 %)	7 (11.9 %)
Lifestyle-related			
Smoking habits			
Non-current smoker	19 (41.3 %)	8 (61.5 %)	27 (45.8 %)
Current smoker	27 (58.7 %)	5 (38.5 %)	32 (54.2 %)
Occupational-related			
Years of service[a]	16.1 ± 9.4 (3.0–34.0)	14.8 ± 6.7 (7.0–28.0)	15.9 ± 8.9 (3.0–34.0)
Permanent Intervention Teams			
No, n (%)	21 (45.7 %)	11 (91.7 %)	32 (55.2 %)
Yes, n (%)	25 (54.3 %)	1 (8.3 %) *	26 (44.8 %)

[a] Mean ± SD (min-max). * Statistical significance was determined using the Chi-square test ($p < 0.05$).

Table 2 summarises firefighters' occupational exposure and reported acute symptoms during real-time wildfire suppression (Phase II, wildfire season). The average wildfire event lasted 43.5 ± 46.4 h (range: 1–144 h). Firefighters reported that wildfires occurred in areas

Table 2
Characteristics of firefighters' occupational exposure to a real-time fire event and acute symptomatology. Data retrieved on Phase II (during wildfire season).

Active wildfire exposure	Male (n = 46)	Female (n = 13)	All (n = 59)
Duration of wildfire event (hours) [a]	44.3 ± 47.6 (1.5–144.0)	40.5 ± 43.9 (1.0–120.0)	43.5 ± 46.4 (1.0–144.0)
1–4 h, n (%)	17 (37.0 %)	6 (46.2 %)	23 (39.0 %)
> 4–72 h, n (%)	19 (41.3 %)	5 (38.5 %)	24 (40.7 %)
> 72 h, n (%)	10 (21.7 %)	2 (15.4 %)	12 (30.3 %)
Exposure duration (hours per event)	11.7 ± 12.3 (1.0–55.0)	8.7 ± 9.2 (1.0–24.0)	11.0 ± 11.7 (1.0–55.0)
1–3 h, n (%)	17 (37.0 %)	7 (53.8 %)	24 (40.7 %)
> 3–12 h, n (%)	15 (32.6 %)	3 (23.1 %)	18 (30.5 %)
> 12 h, n (%)	14 (30.4 %)	3 (23.1 %)	17 (28.8 %)
Acute symptomatology *			
Eyes & respiratory irritation, n (%)	11 (23.9 %)	2 (15.4 %)	13 (22.0 %)
Breathing difficulty, n (%)	9 (19.6 %)	3 (23.1 %)	12 (20.3 %)
Headaches, n (%)	6 (13.0 %)	1 (7.7 %)	7 (11.9 %)

[a] Mean ± SD (min-max); * Self-reported symptoms experienced during firefighting activities.

containing gorse, scrub, chestnut, oak, pine, olive trees, and various types of underbrush. Most participants experienced exposure between 1 and 72 h: 39.0 % for 1–4 h, 40.7 % for 5–72 h, and 30.3 % for over 72 h. Individual exposure duration averaged 11.0 ± 11.7 h (males: 11.7 ± 12.3 h; females: 8.7 ± 9.2 h), ranging from 1 to 55 h. Exposure times were distributed as follows: 40.7 % for 1–3 h, 30.5 % for 4–12 h, and 28.8 % for more than 12 h. The most common acute symptoms reported were eye/respiratory irritation (22.0 %), breath difficulty (20.3 %), and headaches (11.9 %), with slight variations between sexes.

The relationship between exposure duration (hours per wildfire event) and self-reported acute symptoms during firefighting activities was investigated (Fig. 1, 5). A statistically significant association was observed between exposure duration and the incidence of eye and respiratory irritation among wildland firefighters involved in a real wildfire event (χ^2=8.43, p = 0.01). Specifically, 4.2 % of wildland firefighters reporting 1–3 h of exposure experienced these symptoms, which increased to 27.8 % among those with 3–12 h of exposure, and 41.2 % in individuals exposed for more than 12 h. Breathing difficulty was reported by 4.2 %, 16.7 %, and 17.6 % of participants across the same exposure categories, respectively. Headaches were reported by 16.7 % of firefighters exposed for 1–3 h, 33.3 % for 3–12 h, and 11.8 % for more than 12 h. No associations were found between exposure duration and the other reported symptoms (i.e. headaches and difficulty breathing).

Regarding the pre-post exposure variation of biological endpoints, the mean MN frequency in buccal cells was 0.40 ‰ ± 0.07 in Phase I vs 0.54 ‰ ± 0.13 in Phase II (Table 3), showing a 35 % post-exposure increase (95 % CI: 0.76–2.40, p > 0.05) (Fig. 1). Although this increase was not significant, it suggests a trend toward higher genetic instability during the wildfire season compared to baseline.

For the frequency of MN in urothelial cells, a significant increase was found (0.24 ‰ ± 0.09 vs 0.51 ‰ ± 0.12, Table 2) with an FR of 2.13 (95 % CI: 1.99–2.27, p = 0.01), reflecting a 113 % higher risk of genetic instability during the wildfire exposure (Fig. 1).

As regards other endpoints, there was a statistically significant increase in the frequency of binucleated buccal cells (0.90 ‰ ± 0.20 vs 1.97 ‰ ± 0.23, Table 2), with a FR of 2.20 (95 % CI: 1.60–2.99, p < 0.01) (Fig. 1), indicating a doubling of cytokinetic defects following wildland firefighting exposure. No significant changes were observed for the other cellular endpoints investigated (Table 3).

Demographic variables (sex, BMI, age), lifestyle (smoking habits, alcohol consumption), or diet (consumption of vegetables, fruit, tea or coffee) had no significant effect on the studied endpoints during Phase I (5, respectively – 5). However, with respect to occupational-related factors, wildland firefighters with more than 20 years of service exhibited significantly higher levels of BN urothelial cells than those with less than 10 years of service (mean ± S.E., 4.56 ± 1.33 vs. 1.31 ± 0.79; p < 0.01) (5 – 5). In Phase II, no statistically significant associations were observed between the evaluated biological endpoints (including both effect and exposure biomarkers) and wildfire duration, duration of firefighters' exposure during suppression activities, or self-reported symptomatology (data not shown).

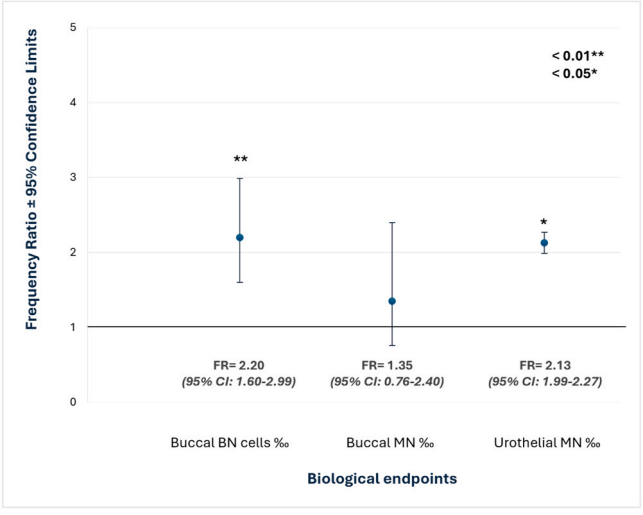

Fig. 1. Frequency ratios (FR) of biological endpoints showing a significant positive variation within study phases (i.e. buccal BN cells and MN in urothelial cells), along with FR of MN in buccal cells. * and ** indicate the statistical significance of post/pre-exposure ratios, with p-values < 0.05 and < 0.01, respectively.

Table 3 -
Descriptive statistics of MN assay (buccal and urothelial cells) in Phase I (before wildfire season - baseline) and Phase II (during wildfire season).

Micronucleus test endpoints	*n*	Phase I – Pre-wildfire season Mean ± SE (min-max)	Phase II – Wildfire season Mean ± SE (min-max)
Buccal cells			
Micronuclei ‰	58	0.40 ± 0.07 (0.00–2.00)	0.54 ± 0.13 (0.00–4.00)
Nuclear buds ‰	58	0.03 ± 0.02 (0.00–1.00)	0.09 ± 0.03 (0.00–1.00)
Binucleated cells ‰	58	**0.90 ± 0.20 (0.00–9.00)**	**1.97 ± 0.23 (0.00–6.00)** **
Condensed chromatin ‰	58	0.03 ± 0.03 (0.00–2.00)	0.03 ± 0.02 (0.00–1.00)
Pyknosis ‰	58	0.09 ± 0.04 (0.00–2.00)	0.07 ± 0.04 (0.00–2.00)
Karyolysis ‰	58	1.22 ± 0.48 (0.00–18.00)	0.24 ± 0.12 (0.00–4.00)
Karyorrhexis ‰	58	0.21 ± 0.08 (0.00–4.00)	0.14 ± 0.06 (0.00–3.00)
Urothelial cells			
Micronuclei ‰	43	**0.24 ± 0.09 (0.00–4.00)**	**0.51 ± 0.12 (0.00–1.00)** *
Nuclear buds ‰	43	0.03 ± 0.03 (0.00–1.00)	0.01 ± 0.01 (0.00–1.00)
Binucleated cells ‰	43	2.30 ± 0.60 (0.00–18.00)	2.28 ± 0.98 (0.00–40.00)
Condensed chromatin ‰	43	0.00 ± 0.00 (0.00–0.00)	0.00 ± 0.00 (0.00–0.00)
Pyknosis ‰	43	0.02 ± 0.02 (0.00–1.00)	0.05 ± 0.05 (0.00–2.00)
Karyolysis ‰	43	0.16 ± 0.07 (0.00–2.00)	0.00 ± 0.00 (0.00–0.00)
Karyorrhexis ‰	43	0.12 ± 0.08 (0.00–3.00)	0.09 ± 0.04 (0.00–1.00)

The Wilcoxon signed-rank test was used for two-group comparisons. Bold values represent statistically significant differences within groups (p < 0.05); * p < 0.05; ** p < 0.01; n refers to the number of individuals per group. SE indicates the standard error of the mean.

The possible contribution of post-exposure levels of urinary ΣOHPAHs to the observed variation (△) in MN‰ in urothelial cells was investigated with multiple log-linear regression analysis (Table 4). Urinary ΣOHPAHs showed a significant positive association with △ MN ‰ in urothelial cells (FR = 1.04, 95 % CI: 1.01–1.08, p < 0.05), suggesting a 4 % increase in MN frequency for each unit increase (μmol/mol creatinine) in urinary ΣOHPAH. Age, sex, and smoking status did not significantly influence △ MN‰.

4. Discussion

The frequency of micronuclei is considered a valuable biomarker for assessing chromosomal damage and genetic instability [17]. The use of the MN assay in epithelial cells, such as buccal mucosa and urothelial cells, is particularly advantageous for human biomonitoring studies. This suitability stems from the minimal invasiveness of cell collection, low cost, ease of storage, simple slide preparation, and the assay's ability to detect genotoxic effects [13]. The buccal mucosa is the first site of contact with hazardous compounds [29], while urothelial cells reflect

Table 4 –
Regression analysis of the association between selected variables [i.e., urinary ΣOHPAHs, sex, age and smoking status] and the variation (△) in MN frequency (‰) in urothelial cells.

	△ MN‰ Urothelial Cells		
	β ± SD	**FR [95 % CI]**	***p*-value**
Exposure			
Phase I - Before wildfire season	-	1.00	-
Phase II - During wildfire season	0.75 ± 0.13	2.13 [1.99–2.27]	**< 0.05** *
Urinary ΣOHPAHs (μmol/mol creatinine) *(n = 43)*	0.04 ± 0.01	1.04 [1.01–1.08]	**< 0.05** *
Age (n = 43)	−0.01 ± 0.03	0.99 [0.91–1.08]	n.s.
Sex			
Female (*n = 9*)	-	1.00	-
Male (*n = 34*)	−0.29 ± 0.35	0.75 [0.29–1.95]	n.s.
Smoking status			
on-smoker (*n = 20*)	-	1.00	-
Current Smoker (*n = 23*)	0.09 ± 0.53	1.09 [0.25–4.80]	n.s.

β: Regression coefficient; * Statistically significant ($p < 0.05$); n.s.: Not statistically significant.

the final site of exposure to hazardous substances excreted by the body [41].

In the present study, MN frequency increased in both urothelial and buccal cells during the wildfire season among wildland firefighters, although statistical significance was observed only in urothelial cells. To date, only two human biomonitoring studies - one of ours - have assessed MN frequency among firefighters, both using buccal cells [22,44]. Ray et al. (2005) reported a higher frequency of this cytogenetic biomarker in the buccal cells of municipal firefighters in India (n = 47) compared to a matched control group of office workers (n = 40) [44]. The second study, previously published by our research group, conducted in fire stations during the 2021 pre-wildfire season, investigated the association between MN frequency in buccal cells and inhaled doses of particulate matter (PM10 and PM2.5) in both indoor and outdoor environments of fire stations, in a group of 80 firefighters [22]; no statistically significant association was found between estimated PM exposure and MN frequency [22]. The lack of association was attributed to the relatively low concentrations of both outdoor and indoor particulate matter measured in this study [22], possibly influenced by the post-COVID-19 deconfinement period (2021), with overall lower levels of air particulate pollution.

As mentioned above, a significant increase in MN frequency in urothelial cells was observed during the wildfire season, following wildland firefighters' involvement in real-wildfire events, when compared to pre-season levels. The use of the MN assay in urothelial cells has a relatively recent application in human biomonitoring studies. So far, and to our knowledge, no studies have examined MN frequency in urothelial cells among wildland firefighters. However, this biomarker has been used in other occupational studies, some of which have reported significant positive associations between MN frequency and exposure to occupational hazardous compounds [45]. Sukuroglu et al. (2024), for example, assessed MN frequency in urothelial cells of Turkish hairdressers exposed to chemicals and found higher MN frequency in the exposed workers compared to the control group [46]. In another study, an almost two-fold increase in MN frequency was observed in plastic factory workers exposed to 4,4'-methylene-bis-2-chloroaniline compared to control groups [47]. Murray et al. (2005) explored the biological effects of occupational exposure to bitumen fumes, which are known to contain PAHs, and found a higher mean MN frequency in the urothelial cells of exposed workers than in controls [48].

In the present study, a significant association was found between urinary concentrations of ΣOHPAHs and increased MN frequency in urothelial cells among wildland firefighters. To the best of our knowledge, to date, this is the first study assessing the relationship between urinary ΣOHPAHs and MN frequency induction in urothelial cells of firefighters. The fact that a statistically significant difference was found only for MN frequency in urothelial cells may reflect tissue-specific sensitivity related to the systemic metabolism and excretion of toxicants. Urothelial cells are directly exposed to urinary metabolites of absorbed substances [41], including PAHs, which are metabolised in the liver and excreted via urine [31,32]. Moreover, contrary to buccal cells, which primarily reflect respiratory exposure and may benefit from partial protection by PPE (e.g., cotton bandanas), urothelial cells integrate cumulative exposure from multiple pathways, including inhalation, ingestion, and dermal absorption.

Although we found no occupational studies linking PAHs exposure with MN in urothelial or buccal cells, some studies have reported increased MN frequencies in other cell types associated with occupational exposure to PAHs and combustion by-products. For instance, MN formation was noted in rat lung cells exposed to dibenz[*a*,*h*]anthracene [49], and increased MN frequency was reported in human lung epithelial A549 cells after PAH exposure [50]. Increased MN frequencies have also been documented in lymphocytes of chimney sweeps, barbecue workers, and cook-oven workers compared to controls [51-53]. Notably, a recent study demonstrated a strong positive correlation between MN frequencies in peripheral blood lymphocytes and epithelial cells [19]. Given that lymphocyte MN is a validated early biomarker of cancer risk, this correlation might support the relevance of our findings of increased MN frequency in firefighters' urothelial cells.

Our findings suggest that the use of MN frequency may be a suitable biomarker of chromosomal damage induced by occupational PAHs exposure, particularly in high-risk populations such as firefighters. The established association between PAHs exposure and elevated incidence of bladder cancer among firefighters [4] underscores the relevance of assessing MN frequency in urothelial cells. Previous clinical studies have documented significantly higher MN frequencies in urothelial cells from bladder cancer patients than controls (18.29 ± 10.04 vs 14.40 ± 8.49, $p = 0.01$), with MN frequency positively correlating with tumour grade and stage ($p = 0.03$) [54]. These findings support the potential of MN frequency as a biomarker for early detection and prognosis of bladder cancer [54]. Thus, our study provides insights into the potential genotoxic effects of PAHs exposure in wildland firefighters and reinforces the value of MN frequency in urothelial cells as a sensitive biomonitoring endpoint, offering a non-invasive method to detect genotoxic effects in tissues relevant to urinary excretion pathways.

In our study, we did not find any association between wildland firefighters' exposure duration (hours per wildfire event) and the studied biological endpoints. It is important to note that wildfire exposure is highly variable; smoke intensity and composition can differ depending on the wildfire's behaviour and each wildland firefighter's location and task. Despite the absence of association with biological markers, exposure duration was significantly related to acute eye and respiratory symptoms, likely due to short-term exposure to wildfire smoke gases and particles known to trigger acute inflammatory responses [6].

The application of the MN assay in epithelial cells, including both urothelial and buccal cells, is of particular interest because it allows the assessment of additional biomarkers beyond genetic instability [18]. Our results also revealed a significant increase in binucleated buccal cells among wildland firefighters during the wildfire season compared to baseline levels before the season. Literature suggests that an elevated ratio of binucleated cells may indicate a high rate of aneuploidy (abnormal chromosome number), which has been associated with an increased risk of cancer and neurodegenerative diseases [55,56]. We further identified a significant association between the frequency of urothelial binucleated cells and years of service as a firefighter. Specifically, wildland firefighters with more than 20 years of service exhibited significantly higher frequencies of binucleated urothelial cells compared to those with less than 10 years. These findings suggest that a prolonged

career as a wildland firefighter may contribute to defects in cytokinesis, reflected in increased binucleated urothelial cells, potentially indicating underlying cytotoxic or genotoxic stress affecting the basal epithelial layer. This may reflect increased tissue renewal or abnormal turnover due to exposure to smoke contaminants or to the consequent inflammation, leading to cumulative effects for long-term exposures. In a study by Ray et al. [44], the authors found a significant association between MN frequency in buccal cells and years of service as a firefighter. Specifically, they observed that firefighters with longer careers ($\geq$ 20 years) had higher MN frequency in buccal cells (4.43 ± 0.32 vs 3.21 ± 0.24, $p < 0.05$) compared to those with shorter careers (< 20 years) [44]. The lack of association between other evaluated cellular endpoints (e.g., condensed chromatin, karyorrhexis, pyknotic, and karyolytic cells) in both urothelial and buccal cells may be attributed to the transient nature of these cytotoxic effects, which are often rapidly repaired. The low observed prevalence of these cellular endpoints contributed to the low frequencies observed in both phases, limiting the ability to detect significant differences and associations.

Overall, this study provides evidence of genotoxic effects associated with wildland firefighting, reflected by the significant increase in MN frequency in urothelial cells and its association with urinary ΣOHPAHs, a recognised biomarker of PAHs exposure. Together with our previous findings of systemic DNA damage and inflammation in the same population [5,6], these results reinforce the potential health impact of wildland firefighting occupational exposure. Recent work in firefighters has also demonstrated DNA methylation alterations implicating cancer-related pathways [57,58], underscoring the value of integrating genotoxic, inflammatory and epigenetic endpoints for a comprehensive assessment of exposure-related biological effects.

Expanding biomonitoring to additional biological matrices may further enhance the specificity and sensitivity of assessing occupational exposure effects. Nasal epithelial MN assay, for example, may provide a minimally invasive method to assess inhalation-specific effects due to the anatomical proximity to the exposure site [59]. In addition, systemic effect biomarkers, such as MN assessment in peripheral blood lymphocytes, or characterisation of blood cell populations, may further improve understanding of the mechanisms underlying firefighters' exposure-related health risks by integrating both local and systemic responses.

The results of the present study should be interpreted considering some limitations. The voluntary nature of participant recruitment may introduce selection bias, and the use of self-reported data is susceptible to information bias (e.g., recall bias or social desirability bias), particularly concerning the accuracy of reported smoking status. Future research should consider incorporating biomarkers of tobacco exposure, such as cotinine levels, to improve the accuracy of exposure assessment. Additionally, the timing of sample collection is crucial when assessing dynamic biomarkers such as urinary OHPAHs and MN assay endpoints. For instance, MN in epithelial cells, such as buccal and urothelial cells, may take up to three weeks to peak due to cell turnover from the basal to surface layers [12,18]. Although in this study, samples were collected within a similar post-exposure window, variability in timing may have contributed to the underestimation of biomarker levels.

Despite these limitations, this study provides valuable findings. Novel information regarding biological effects related to wildland firefighters' occupational exposure is proposed, particularly concerning wildland firefighters' exposure to real fire events. The use of a pre-post design, in which individuals serve as their own controls, improves the reliability of the results. This is the first study investigating the frequency of MN and other cellular endpoints in the urothelial cells of firefighters, regardless of the type of firefighter. Moreover, for the first time, MN frequencies in buccal cells of wildland firefighters were assessed at two different time points of their activity (before and during fire season), as well as investigating the effect of urinary ΣOHPAHs on MN frequency in urothelial cells, specifically on wildland firefighters.

Longitudinal studies with larger sample sizes, ideally including other regions or countries, are needed to confirm the present findings, enhance their generalisability, and clarify the long-term health effects related to wildland firefighters' occupational exposure. Incorporating multiple sampling time points would better capture the temporal dynamics of urinary OHPAHs and buccal MN assay responses, while controlling for additional potential confounders (e.g., oral hygiene) is essential. This approach would be particularly important for assessing the persistence or potential reversibility of genotoxic effects, thereby enhancing our understanding of the biological impact and recovery trajectories following wildfire-related exposures. In addition, environmental air monitoring, particularly within fire stations during wildfire seasons, is warranted to complement biomonitoring data and provide a more accurate characterisation of PAH exposure sources, thereby facilitating the development of targeted and effective mitigation strategies.

Taken together, our findings highlight the genotoxic effects associated with wildfire exposure in wildland firefighters, underscoring the need for preventive measures in this workforce. These measures should include the consistent and proper use of adequate PPE, targeted occupational health education and training, and effective decontamination procedures to minimise exposure to harmful contaminants such as PAHs. In parallel, the promotion of a healthy lifestyle, including a balanced diet, regular physical activity, adequate sleep, stress management, and avoidance of risk factors such as tobacco and alcohol use, may further reduce overall disease risk. Regular occupational health surveillance, including human biomonitoring programs, is essential for early detection of exposure-related biological effects and to guide timely preventive interventions.

5. Conclusion

Warmer and drier weather, mostly driven by climate change, has increased the frequency and intensity of wildfires across the globe. As a result, wildfires are expected to engage more firefighters in the coming years, thereby heightening the occupational health burden associated with firefighting activities.

Our study confirms the potential of the MN assay in urothelial cells as a valuable non-invasive tool for monitoring occupational groups at increased risk of developing bladder cancer, particularly wildland firefighters. This is a useful effect biomarker to evaluate genotoxicity and early cancer risk associated with harmful human exposures.

To mitigate these risks, the implementation of decontamination procedures to reduce firefighters' exposure to woodsmoke-derived air pollutants is imperative. In parallel, regular medical surveillance should be prioritised to facilitate early detection of adverse health outcomes and to inform effective occupational health strategies in this high-risk population.

Environmental Implication

This longitudinal study provides novel mechanistic evidence linking wildfire smoke exposure to genotoxic effects in wildland firefighters - a workforce facing increasing occupational hazards due to climate-driven wildfire activity. By integrating micronucleus assays in firefighters' non-invasive surrogate tissues (buccal and urothelial epithelial cells) with urinary OHPAHs measurements, we demonstrate a dose-response relationship between occupational PAHs exposure and chromosomal damage. These findings underscore the genotoxic potential of wildland firefighting and highlight the need for biomonitoring, effective decontamination protocols, and evidence-based risk reduction strategies among firefighters.

CRediT authorship contribution statement

Filipa Esteves: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Joana Madureira:** Writing – review & editing,

Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bela Barros:** Methodology, Data curation. **Sara Alves:** Methodology, Data curation. **Josiana Vaz:** Writing – review & editing, Resources, Project administration, Methodology, Investigation, Data curation. **Marta Oliveira:** Methodology, Data curation. **Klara Slezakova:** Writing – review & editing, Project administration, Methodology, Investigation, Data curation. **Adília Fernandes:** Resources, Project administration, Methodology, Funding acquisition, Data curation. **Maria do Carmo Pereira:** Project administration, Funding acquisition. **Simone Morais:** Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Stefano Bonassi:** Writing – review & editing, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **João Paulo Teixeira:** Writing – review & editing, Resources, Project administration, Methodology, Formal analysis, Data curation. **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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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.140427.

Data availability

The authors do not have permission to share data.

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