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Firefighters exposure to fire emissions: Impact on levels of biomarkers of exposure to polycyclic aromatic hydrocarbons and genotoxic/oxidative-effects

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ABSTRACT

Firefighters represent one of the riskiest occupations, yet due to the logistic reasons, the respective exposure assessment is one of the most challenging. Thus, this work assessed the impact of firefighting activities on levels of urinary monohydroxyl-polycyclic aromatic hydrocarbons (OHPAHs; 1-hydroxynaphthalene, 1-hydroxyacenaphthene, 2-hydroxyfluorene, 1-hydroxyphenanthrene, 1-hydroxypyrene, 3-hydroxybenzo(a)pyrene) and genotoxic/oxidative-effect biomarkers (basal DNA and oxidative DNA damage) of firefighters from eight firehouses. Cardiac frequency, blood pressure and arterial oxygen saturation were also monitored. OHPAHs were determined by liquid-chromatography with fluorescence detection, while genotoxic/oxidative-effect biomarkers were assessed by the comet assay. Concentrations of total OHPAHs were up to 340% higher ($p \leq 0.05$) in (non-smoking and smoking) exposed workers than in control subjects (non-smoking and non-exposed to combat activities); the highest increments were observed for 1-hydroxynaphthalene and 1-hydroxyacenaphthene (82–88% of ΣOHPAHs), and for 2-hydroxyfluorene (5–15%). Levels of biomarker for oxidative stress were increased in non-smoking exposed workers than in control group (316%; $p \leq 0.001$); inconclusive results were found for DNA damage. Positive correlations were found between the cardiac frequency, ΣOHPAHs and the oxidative DNA damage of non-smoking (non-exposed and exposed) firefighters. Evidences were raised regarding the simultaneous use of these biomarkers for the surveillance of firefighters' health and to better estimate the potential short-term health risks.

1. Introduction

Climate changes and global warming have substantially contributed to increase forest fire episodes, with longer fire season and more potent fires (de Rigo et al., 2017; San-Miguel-Ayanz et al., 2018). Forest fire emissions release large amounts of several hazardous gaseous and particulate pollutants: particulate matter, carbon monoxide, nitrogen dioxide, and volatile organic compounds (including polycyclic aromatic hydrocarbons (PAHs), acetaldehyde, formaldehyde, benzene, toluene, phenol, xylene, acrolein, and ethylbenzene) (Abrard et al., 2019; Adame et al., 2018; de la Barrera et al., 2018; McClure and Jaffe, 2018; Wentworth et al., 2018; Oliveira et al., 2015, 2017a; Oliveira et al., 2017b; Fent et al., 2013, 2014; Fent et al., 2017, 2015; Park et al., 2015; Keir et al., 2017; Hsu et al., 2011; Pleil et al., 2014). Some of these compounds are classified by the International Agency for Research on Cancer (IARC) as potential/possible carcinogens to humans. PAHs are a large group of ubiquitous compounds formed during combustion processes that are included in the US Environment Protection Agency list of priority pollutants (US Environmental Protection Agency, 2005). Emissions from forest fires are an important source of PAHs as well as the burning of fossil fuels, petroleum, coal tar, gas, and wood (Oliveira et al., 2019). Some PAHs are referred as persistent organic pollutants and endocrine disrupting chemicals (World Health Organization, 2013) with benzo(a)pyrene being classified as carcinogen (group 1) by IARC (2010a); whereas other 9 congeners (naphthalene, benz(a)anthracene, benzo(b)fluoranthene, benzo(j)fluoranthene, benzo(k)fluoranthene,

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chrysene, dibenzo(a,l)pyrene and dibenz(a,h)anthracene and indeno (1,2,3-cd)pyrene) are included in group 2A/2B (*i.e.* probable/possible carcinogens) (IARC (2010a); IARC, 2002). People are environmentally and/or occupationally exposed to PAHs through inhalation of polluted air, dermal contact, and ingestion of contaminated food (Oliveira et al., 2019). In the human body, PAHs are distributed by blood root to several tissues, being primarily metabolized in the liver by cytochrome P450 enzymes *via* different oxidative mechanisms to produce a complex mixture of hydroxylated metabolites that are eliminated through the urine, milk, and feces (Oliveira et al., 2019). Exposure to PAHs can cause formation of active carcinogenic intermediary molecules responsible for the formation of DNA adducts, resulting in mutations, alteration of gene expression profiles, and tumorigenesis (Alhamdow et al., 2017; Barth et al., 2017; Kamal et al., 2015; Moorthy et al., 2015; White et al., 2016; Bocchi et al., 2017; Franken et al., 2017; Jasso-Pineda et al., 2015). PAHs are also known to cause reproductive, developmental, hemato-, neuro-, and immune-toxicities in humans (Agency for Toxic Substances and Disease Registry, 1995). Therefore, fire emissions represent a public health problem and may cause serious risks not only for the general population but also for occupationally exposed workers (Adetona et al., 2016; Alman et al., 2016; Analitis et al., 2012; Black et al., 2017; Reid et al., 2016; Waldman et al., 2016; Le et al., 2014; Johnston et al., 2012; Cascio, 2018; Youssouf et al., 2014; Semmens et al., 2016; Gianniou et al., 2016). Analitis and coworkers (Analitis et al., 2012) reported an increase of 5% in the daily total number of deaths, in a densely populated area, associated with exposure to forest fire emissions, and 6 and 15% increase in the number of cardiovascular and respiratory deaths, respectively.

Firefighters' occupational exposure is classified as possible carcinogen to humans (IARC, 2010b; NIOSH, 2007). During firefighters' work tasks, exposure to hazardous pollutants may induce the generation of reactive species and cause the activation of oxidative pathways that may culminate in pulmonary and cardiovascular inflammatory processes (Alhamdow et al., 2017; Moorthy et al., 2015; Gianniou et al., 2016). The regular and active participation in fire combat has been linked with excess morbidity and mortality among firefighters, being the cardio-respiratory diseases the leading causes of death (Gianniou et al., 2016; Gaughan et al., 2014a, b; Soteriades et al., 2011). Some authors have also associated firefighters' occupational exposure with a possibility of increased risk to develop site-specific cancers, such as leukemia, esophageal, lung, kidney and bladder, skin melanoma, testicular, and urothelial cancer (LeMasters et al., 2006; Daniels et al., 2014; Glass et al., 2016; Golka and Weistenhöfer, 2008; Pukkala et al., 2014; Stec et al., 2018; Youakim, 2006). Furthermore, smoking workers are at a higher risk of suffering from the potential cumulative health risks associated with a regular exposure to fire emissions and tobacco consumption (Fernando et al., 2016; Oliveira et al., 2017c).

Monitoring of firefighters' exposure during fire combat is a very complicated task due to unpredictability and challenges of the respective environment (fire locations, atmospheric conditions, dangerous and rapidly changing situations). Thus, biomonitoring represents a crucial tool to overcome some of the logistical difficulties. Biomonitoring data reflect the individual total internal dose regardless the exposure source and the route. The combination of biomarkers of exposure with (bio)markers of effects and/or susceptibility represents a valuable tool for assessing the potential health effects in the exposed subjects (Alhamdow et al., 2017; Barth et al., 2017; Dominguez-Ortega et al., 2016; Zhou et al., 2018; Oliveira et al., 2017d). Some studies have been emerging regarding characterization of firefighters' occupational exposure *via* biomonitoring assays, with emphasis on active firefighters participation in prescribed burns and/or wildland fires combat (Fent et al., 2014; Park et al., 2015; Keir et al., 2017; Gaughan et al., 2014a, b; Fernando et al., 2016; Oliveira et al., 2017c; Edelman et al., 2003; Abreu et al., 2017; Adetona et al., 2017; Oliveira et al., 2016; Caux et al., 2002; Wingfors et al., 2018; Andersen et al., 2018a). Available data come mostly from studies conducted in USA and Canada; however, the obtained findings may not be directly applicable to European subjects due to the different meteorological conditions, types of vegetation, and firefighting practices that affect composition of smoke and consequently human exposure. Only five studies were conducted in European countries (Oliveira et al., 2017c; Abreu et al., 2017; Oliveira et al., 2016; Wingfors et al., 2018; Abreu et al., 2017; Adetona et al., 2017; Oliveira et al., 2016; Caux et al., 2002; Wingfors et al., 2018; Andersen et al., 2018a) with only one considering the simultaneous assessment of biomarkers of exposure and of effect (Andersen et al., 2018a). Thus, this work aimed to contribute to fill this research gap and assessed the occupational exposure of (non-smoking and smoking) firefighters during fire combat activities by biomarkers of exposure and effect. For that purpose, six urinary biomarkers of exposure to PAHs [1-hydroxynaphthalene (1OHNaph), 1-hydroxyacenaphtene (1OHAce), 2-hydroxyfluorene (2OHFlu), 1-hydroxyphenanthrene (1OHPhe), 1-hydroxypyrene (1OHPy), and 3-hydroxybenzo(a)pyrene (3OHB(a)P)] and two genotoxicity biomarkers (basal DNA damage and oxidative DNA damage) were determined in firefighters from eight different stations. Cardiorespiratory parameters were also monitored and correlated with the levels of the selected biomarkers.

2. Materials and methods

2.1. Study location and population characterization

The present study was conducted in the district of Bragança (north of Portugal). During the last five years, this district registered a total of 2 513 fire occurrences, with 43% being forest fires; an area of 60 301 ha was burnt (Instituto da Conservação da Natureza e das Florestas, 2017). Subjects that voluntary agreed to participate in this study were firefighters serving at eight units of professional fire stations from the district of Bragança. All subjects fulfilled a structured questionnaire that was previously adapted from a validated form (World Health Organization, 2002). Relevant personal (age, height, general medical history, existence of diagnosed chronic disease, health status and weight) and professional (employment duration) information was collected. Firefighters perform different tasks at the fire departments but not all subjects were directly involved in firefighting. Therefore, exposure duration in active forest fire combat in the last 48 h and the use of personal protective equipment, as well as information on other relevant PAH exposure sources, namely personal smoking habits, recent environmental exposure to tobacco smoke, and the most frequently consumed meals within the last week, were also retrieved from the questionnaire. Only firefighters with a recent diet without the consumption of grilled, barbecued, and smoked foods and with no history of chronic diseases were selected and considered in this work. A total of 171 firefighters were included in this study and signed an informed consent form that was previously reviewed and approved by the Ethic Committee of University of Porto. Based on the data collected from the questionnaires, firefighters were organized into three different groups according to their active participation in firefighting activities (within the 48 h before sample collection) and their smoking habits: (i) non-smoking and non-exposed subjects (Control group - firefighters that stayed at the fire stations and did not participate in fire combat), (ii) non-smoking and exposed subjects (*i.e.* non-smoking individuals who were directly involved in firefighting activities; Group A), and (iii) smoking and exposed subjects (*i.e.* smoking firefighters exposed to fire emissions; Group B).

2.2. Firefighters' biomonitoring

Subjects allowed monitoring of selected cardio-respiratory parameters, and sampling of spot urine and venous blood samples. Procedures and sampling (urine and blood) were performed following the common health sampling hygiene practices with the help of qualified personnel at the end of the firefighters' 8 h work-shift. Arterial

oxygen saturation was monitored with an Oxy-100 pulse oximeter (Gima, Italy), and the blood (diastolic and systolic) pressure and cardiac frequency were determined with a monitor for upper arm (Geratherm Medical AG Desktop, Geschwenda, Germany). Urine samples were collected by each firefighter in a sterilized polycarbonate container. Blood samples were collected by venipuncture from an antecubital vein in ethylenediamine tetra-acetic acid tubes. Blood samples were suspended in an equal amount of 1:4 (v/v) mixture of dimethyl sulfoxide and RPMI 1640 medium (in 200 µl aliquots) for cryopreservation. After collection, urine and blood samples were immediately coded. All samples were adequately transported (within 1–2 h) and immediately frozen at −20 and −80 °C, respectively.

2.3. Urinary OH-PAHs extraction and chromatographic analysis

Solid-phase extraction of urinary OH-PAHs and the chromatographic analysis were done according to previous works (Oliveira et al., 2017c, 2016). Briefly, 10 mL of urine previously buffered with acetate buffer (pH 5.0) were incubated during 2 h at 37.0 °C with 80 µl of β-glucuronidase/arylsulfatase from *Helix pomatia* (EC 3.2.1.31/EC3.1.6.1; 5.5/2.6 U/ml; Roche Diagnostics, Indianapolis, USA). PAH metabolites were extracted from the hydrolyzed urine samples using C_{18} cartridges (Sep-Pak® Light Plus C18, Waters, Sigma-Aldrich, Steinheim, Germany) and 20.0 mL of methanol/ethyl acetate (10:90; v/v). After evaporation till dryness at room temperature ((Büchi R200 rotavapor and a Büchi Vac V-500 pump), extracts were redissolved in 500 µl of methanol before chromatographic analysis. OHPAHs were analyzed with a Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) equipped with a fluorescence detector in a C_{18} column (CC 150/4 Nucleosil 100–5 C18 PAH, 150 × 4.0 mm; 5 µm particle size; Macherey–Nagel, Duren, Germany). The optimal chromatographic characteristics and further details are presented in Table 1S (Supplementary Material).

Calibration curves (calibration points: n ≥6) of 2OHFlu, 1OHPhe, 1OHPy, and 3OHB(a)P were prepared with mixed standards in methanol, whereas a matrix-matched calibration curve was used for 1OHNaph and 1OHAce. The detection (LOD) and quantification (LOQ) limits of PAH metabolites were determined based on 3 and 10 times the standard deviation of the analytical response divided by the slope of the calibration curve for each analyte, respectively (Miller and Miller, 2000). Limits of detection varied between 0.84 ng/l urine (for 2OHFlu) and 0.19 µg/L urine (for 1OHNaph and/or 1OHAce), with limits of quantification ranging between 2.8 ng/l urine and 0.65 µg/L urine, respectively. Blank and standards were daily prepared and analyzed to check instrument performance. The precision of the methodology was evaluated through relative standard deviation (RSD) with intra- and inter-day assays during 6 consecutive days. RSD values varied between 1.3% for 2OHFlu and 6.4% for 1OHPhe (intra-precision assays) and ranged from 1.3% to 8.1% for 1OHNaph + 1OHAce, and 1OHPy (inter-precision assays). Validation of the methodology was achieved with recovery assays performed on a pooled urine sample. Recovery experiments resulted in values between 70.0% and 117%.

Urinary levels of creatinine were determined by the Jaff colorimetric method according to the methodology proposed by Kanagasabapathy and Kumari (2000).

All determinations were performed in triplicate.

2.4. Alkaline comet assay

Before the assay, the frozen blood samples were rapidly thawed at room temperature and washed twice (centrifugation at 223 *g* for 10 min) with Dulbecco's Modified Eagle Medium supplemented with 2% fetal bovine serum. The alkaline comet assay was performed as described by Singh et al. (1988) with minor modifications (Abreu et al., 2017). A medium-throughput version of the comet assay 12-Gel Comet Assay Unit ™ (Severn Biotech Ltd) was used. Briefly, two mini-gels were prepared for each subject in three slides (2 × 3 slides); one slide to assess basal DNA damage and two slides to evaluate oxidized purines. Electrophoresis was carried out for 20 min at approximately 1.2 V/cm. The semi-automated image analysis system Comet Assay IV (Perceptive Instruments, UK) was used for image capture and analysis. A total of 150 cells were scored for each subject. The DNA damage was measured as %TDNA (percentage of DNA in the comet tail).

2.5. Enzyme-modified alkaline comet assay

The comet assay enzyme version was performed as described by Azqueta and Collins (2013). formamidopyrimidine DNA glycosylase (FPG) was the enzyme selected to measure the amount of DNA oxidized purines. Briefly, after lysis, slides for enzyme treatment were washed three times with buffer F (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES, 0.2 mg/ml BSA, pH 8). Gels were incubated for 30 min (37 °C). Electrophoresis was performed as previously described for alkaline comet assay. Net FPG-sensitive sites were calculated by subtracting the %TDNA values of control gels and enzyme-treated gels.

2.6. Statistical analysis

Data were treated with SPSS (IBM Statistics 20) and Statistica (v. 7, StatSoft Inc., USA) software. Concentrations of OHPAHs were presented in µg/L of urine and normalized with urinary creatinine levels. Whenever the concentration of a OHPAH was below its LOD, the value was substituted with LOD/√2 (Hornung and Reed, 1990). Data were compared through the Mann-Whitney U test, since normal distribution was not verified by Shapiro-Wilk´s test. Spearman correlation coefficients (r) were used to evaluate the possible relation between the concentrations of individual and total OHPAHs and the dependency between the levels of biomarkers of exposure with the biomarkers of effect, and cardiovascular parameters. Statistical significance was defined as $p \leq 0.05$.

3. Results and discussion

3.1. Subjects characterization

The biometric characteristics of the three groups of firefighters considered in this study are presented in Table 1. The median age of the study populations varied between 30–36 years. The firefighters reported a long-term exposure to forest fire emissions, with medians ranging between 11 (Group A) to 15 (Control group) years (Table 1). Furthermore, 48 h prior to the sampling campaigns, exposed firefighters (Group A and Group B) were directly involved in firefighting activities for a median period of 3 consecutive hours.

Regarding cardio-respiratory parameters, firefighters showed a similar profile regardless of the group. Overall, arterial oxygen saturation values (97–99%) were within the acceptable range of 95–100% (Booth et al., 2009). The cardiac frequency of firefighters (68–82 heart beats/min) were also within the recommended range of values of 60–100 heart beats/min (Booth et al., 2009); less than 10% of firefighters had cardiac frequency exceeding 100 heart beats/min (Table 1). Diastolic and systolic blood pressure of the study populations varied between 81–83 mmHg and 130–135 mmHg (Table 1), being considered as normal blood pressure levels (≤90 mmHg and ≤140 mmHg, respectively). However, 21% (Group A) to 33% (Control group) and 14% (Group A) to 40% (Group B) of firefighters had elevated diastolic and systolic blood pressure, respectively; 12% of the subjects presented values of both diastolic and systolic blood pressure higher than the accepted normal levels. Elevated blood pressure is a major risk factor for the development and/or aggravation of cardiovascular diseases (Kales et al., 2009). Positive and moderate to strong Spearman correlation coefficients ($0.366 < r < 0.939$; $p \leq 0.001$) were found between the three cardiovascular parameters determined for the majority of firefighters, which indicated dependency between individual cardiac

Table 1
Biometric data and characterization of study populations: non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters.

	Control group	Group A	Group B
Study population (n)	93	48	30
Age (median; range; years)	36 (22–55)	30 (21–52)	32 (26–50)
Occupational exposure to fires			
Long-term exposure: years worked as firefighter (median; range; years)	15 (2–30)	11 (2–30)	12 (7–25)
< 10 years (%)	29	40	20
10–20 years (%)	52	47	60
> 20 years (%)	19	13	20
Recent exposure (hours[a])	0	3 (2–12)	3 (2–8)
Recent exposure to tobacco smoke (median; range[b])	n.a	n.a	20 (10–25)
Cardio-respiratory parameters			
Arterial oxygen saturation (median; range; %)	97 (95–99)	98 (88–99)	99 (97–99)
Cardiac frequency (median; range; heart beats/min)	73 (56–105)	68 (54–94)	82 (54–112)
Diastolic blood pressure (median; range; mmHg)	83 (67–122)	81 (49–93)	81 (67–96)
Systolic blood pressure (median; range; mmHg)	134 (108–178)	130 (119–148)	135 (116–156)
Respiratory pathologies including allergies			
Yes (n; %)	10	20	10
No (n; %)	90	80	90

n.a. – not applicable.
[a] number of hours directly involved in firefighting activities within the 48 h before sample collection.
[b] number of cigarettes smoked per day during the sampling period.

frequency and blood pressure (diastolic and systolic).

3.2. Biomarkers of exposure

Urinary biomarkers of exposure, namely 1OHNaph and 1OHAce, were detected in all firefighters while 1OHPhe, 2OHFlu and 1OHPy were detected in more than 90% of the subjects. 3OHB(a)P was not detected, thus it was not included in the further results analysis. These findings are in agreement with previous works as absence of this biomarker in the urine of firefighters has been reported (Oliveira et al., 2017c, d; Oliveira et al., 2016; Wingfors et al., 2018). Regarding other occupationally exposed workers, 3OHB(a)P has been detected with very low rates which can be attributed to the complex metabolism of organic compounds with high molecular weights (Alhamdow et al., 2017; Fernando et al., 2016; Yamano et al., 2014; Díaz-Merchán et al., 2013; Barbeau et al., 2014, 2015; Lutier et al., 2016a). Furthermore, according to the existent literature, 3OHB(a)P is predominantly eliminated through the feces rather than urine (Li et al., 2012; Marie et al., 2010).

Individual and total levels of urinary PAH metabolites (ΣOHPAHs) are summarized in Table 2. Since creatinine is eliminated from the human body at a constant rate, levels of PAH metabolites were normalized with creatinine concentrations in order to compensate for fluctuations caused by differences in diuresis and to minimize the influence of individual parameters, such as daily water intake, internal body temperature, and physical exercise. Moreover, creatinine concentrations below 0.3 g/l indicate much diluted urine while values higher than 3 g/l may suggest the existence of some kidney disease (World Health Organization, 1996). Overall, creatinine levels in the selected firefighters ranged between 0.70–2.90 g/L, being within the range of values proposed by the World Health Organization (1996). The inter-comparison of ΣOHPAH concentrations among the three different groups was (by decreasing order): Group B (6.96 μmol/mol creatinine) > Group A (1.68 μmol/mol creatinine) > Control group (1.59 μmol/mol creatinine); significant differences were observed among the three groups ($p \leq 0.004$) showing the impact of smoking and fire emissions exposure on ΣOHPAH levels. Similar profiles were obtained for the urinary levels of 1OHNaph + 1OHAce (5.61 *versus* 1.54 *versus* 1.40 μmol/mol creatinine, respectively for Group B, Group A and the Control group; $p \leq 0.010$) and 2OHFlu (0.62 *versus* 0.09 *versus* 0.06 μmol/mol creatinine; $p \leq 0.025$). Levels of urinary 1OHPhe in non-smoking exposed firefighters (Group A) were significantly higher than in control subjects (0.06 *versus* 0.04 μmol/mol creatinine; $p = 0.005$; Table 2); no significant differences were found between the urinary levels of 1OHPhe in firefighters from Group B with the other individuals, suggesting that this biomarker may not be appropriate or sufficiently sensitive for assessment of cumulative exposure to tobacco smoke and fire emissions. Oliveira et al. (2016) also suggested that urinary 1OHPhe excretion was the less affected PAH metabolite in firefighters involved in combat activities. Median concentrations of 1OHPy in the urine of all firefighters were similar (0.03-0.04 μmol/mol creatinine; $p > 0.05$). Urinary excretion rates of 1OHPhe and 1OHPy

Table 2
Descriptive statistics of PAH biomarkers of exposure (median, percentile 25–75, and range; μmol/mol creatinine) in non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters.

PAH biomarker*	Control Group		Group A		Group B	
	Median (P25–P75) (μmol/mol creatinine)	Range	Median (P25–P75)	Range	Median (P25–P75)	Range
1OHNaph + 1OHAce	1.40 (0.60–1.82)[a]	0.03–4.14	1.54 (0.85–3.20)[b]	0.60–121	5.61 (3.61–8.28)[c]	1.18–47.8
2OHFlu	0.06 (0.04–0.12)[a]	5.67×10^{-4}–0.48	0.09 (0.05–0.21)[b]	5.67×10^{-4}–0.47	0.62 (0.41–1.08) [c]	0.29–1.61
1OHPhe	0.04 (0.02–0.10)[a]	6.71×10^{-3}–0.21	0.06 (0.04–0.08)[b]	0.02–0.29	0.04 (0.03–0.09)[ab]	0.02–0.19
1OHPy	0.03 (0.02–0.04) [a]	1.84×10^{-3}–0.23	0.04 (0.02–0.07)[a]	1.84×10^{-3}–0.19	0.04 (0.02–0.10)[a]	3.69×10^{-3}–0.85
ΣOH-PAHs	1.59 (0.75–2.19)[a]	0.10–4.27	1.68 (1.09–3.39)[b]	0.82–121	6.96 (4.32–8.82)[c]	1.52–48.6

Different superscripts (a, b, c) correspond to statistically different distributions between each group of firefighters ($p \leq 0.05$).
* 1OHNaph + 1OHAce: 1-hydroxynaphthalene and 1-hydroxyacenaphthene; 2OHFlu: 2-hydroxyfluorene; 1OHPhe: 1-hydroxyphenanthrene; 1OHPy: 1-hydroxypyrene; ΣOHPAHs – represents the sum of all individual PAH metabolites.

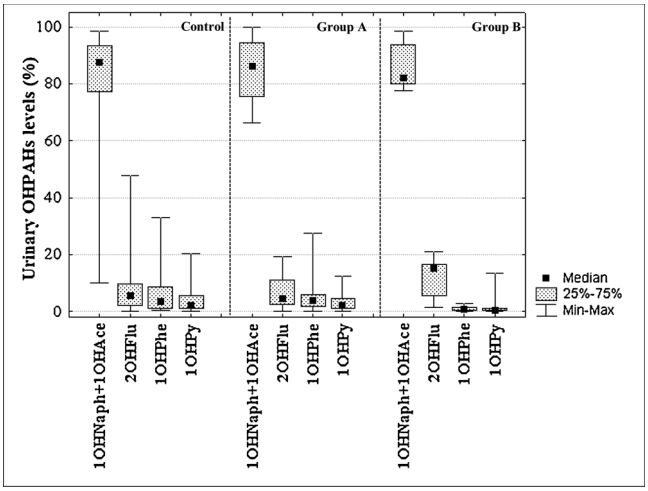

Fig. 1. Urinary levels (%) of PAH metabolites (1OHNaph + 1OHAce: 1-hydroxynaphthalene and 1-hydroxyacenaphthene; 2OHFlu: 2-hydroxyfluorene; 1OHPhe: 1-hydroxyphenanthrene; 1OHPy: 1-hydroxypyrene) in the characterized groups: non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters.

may help to understand these findings since their median half-life time (13.8 and 23.5 h, respectively) are much higher than the ones determined for 1OHNaph (6.6 h) and 2OHFlu (8.4 h) (Li et al., 2016). The American Conference of Governmental Industrial Hygienists proposed a benchmark level of 0.5 μmol/mol creatinine of 1OHPy as evidence to occupational exposure to PAHs (American Conference of Governmental Industrial Hygienists, 2010); this limit was exceeded only in some smoking and exposed subjects (Group B; Table 2). Some authors assessed firefighters' occupational exposure to ambient $PM_{2.5}$-bound PAHs at different fire stations and reported that compounds with 2–3 rings (including naphthalene, acenaphthene, fluorene, and phenanthrene) represented more than 64% of total PAHs; compounds with higher molecular weight (including pyrene) were less abundant in firefighters' breading air zone (Oliveira et al., 2017b, d; Wingfors et al., 2018; Fent and Evans, 2011; Kirk and Logan, 2015). 1OHNaph + 1OHAce were by far the most abundant PAH biomarkers in the characterized subjects (82–88% of ΣOHPAHs), being followed by 2OHFlu (5–15%); 1OHPhe (0.7–3.8%) and 1OHPy (0.5–2.1%) had very low contributions to ΣOHPAHs (Fig. 1). Levels of OHPAH are strongly related with the molecular weight of the un-metabolized congener compounds, being the highest urinary concentrations associated with the lower molecular weight compounds (Adetona et al., 2017; Li et al., 2016).

Urinary levels without creatinine normalization of individual and ΣOHPAHs (in μg/L of urine) in firefighters are presented in Table 2S. Concentrations of ΣOHPAHs were 6 and 316% higher in exposed non-smoking (Group A) and smoking (Group B) firefighters, respectively, in comparison with the control subjects (Table 2; $p \leq 0.004$). Other authors also observed significantly increased concentrations of OHPAH in the urine of post-shift firefighters when compared to non-exposed subjects (Keir et al., 2017; Fernando et al., 2016; Oliveira et al., 2017c; Adetona et al., 2017; Oliveira et al., 2016; Wingfors et al., 2018). 1OHNaph + 1OHAce and 2OHFlu were the compounds with the highest increments in non-smoking (Group A; 10 and 50%, respectively) and smoking (Group B; 300 and 930%) exposed subjects comparatively with Control group (Table 2). Exposure to fire emissions promoted a significant increase ($p \leq 0.05$) in levels of 1OHNaph + 1OHAce (1.54 *versus* 1.40 μmol/mol creatinine; $p \leq 0.010$), 2OHFlu (0.09 *versus* 0.06 μmol/mol creatinine; $p \leq 0.025$), and 1OHPhe (0.06 *versus* 0.04 μmol/mol creatinine; $p \leq 0.05$), and as a consequence in ΣOHPAHs, in subjects from Group A comparatively with control subjects (Table 2). These findings are in line with the results reported by other authors (Oliveira et al., 2017c; Edelman et al., 2003; Adetona et al., 2017; Oliveira et al., 2016; Robinson et al., 2008; Laitinen et al., 2010). Regarding exposed firefighters, levels of ΣOHPAHs were 314% higher in smoking (Group B) than in non-smoking (Group A) subjects ($p \leq 0.001$). Since both groups of exposed firefighters reported a similar recent median exposure to fire emissions (3 consecutive hours; Table 1), the differences found between the urinary levels of individual and ΣOHPAHs may be attributed to the individual smoking habits (Group B). Smoking contributed to increments of 260 and 590% in the urinary concentrations of 1OHNaph + 1OHAce and 2OHFlu, respectively (Table 2). Variability in the urinary levels of PAH biomarkers of exposure among the groups of firefighters is also affected by other factors. It is known that elimination kinetics of PAH metabolites from the human body vary from compound to compound and are strongly dependent on the route of exposure and on the tasks performed by workers (Li et al., 2012; Brzeznicki et al., 1997; Gendre et al., 2002, 2004; Lutier et al., 2016b).

Despite the similar distribution profile of PAH metabolites among the three groups of firefighters (Fig. 1), the ratios between various biomarkers of exposure differed (Table 3). For non-smoking exposed firefighters (Group A), the ratio of (1OHNaph + 1OHAce)/2OHFlu (23%) was slightly increased while the other ratios were lower (6% for 1OHPhe/1OHPy to 50% for 2OHFlu/1OHPy) in comparison with the Control group. These findings suggest higher impact and contribution of fire emissions exposure on urinary levels of 1OHNaph + 1OHAce, 1OHPhe and 1OHPy. Cumulative impact of recent exposure to fire emissions and regular tobacco consumption resulted in a significant reduction in 1OHNaph + 1OHAce/2OHFlu (64–71%; $p = 0.005$) and significant increases in the other ratios (280 and 380% for 1OHNaph + 1OHAce/1OHPy to 470 and 1000% for 2OHFlu/1OHPy; $p \leq 0.001$), except for the ratio 1OHPhe/1OHPy (Table 3). These results may indicate the strong contribution of tobacco smoke to the urinary levels of PAH biomarkers with augmentation of 1OHNaph + 1OHAce, 2OHFlu and 1OHPy levels. Previously, St. (Helen et al. (2012)) reported 1-, 2-, and 3OHFlu as the PAH metabolites that exhibited the greatest difference between non-smoking and smoking individuals, being followed by

Table 3
Ratios between PAH urinary biomarkers of exposure in non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters.

Ratio	Control Group Median (range)	Group A Median (range)	Group B Median (range)
(1OHNaph + 1OHAce)/2OHFlu	15.5 (0.24–2566)[a]	19.1 (3.85–1089)[ab]	5.5 (3.71–71.4)[c]
(1OHNaph + 1OHAce)/1OHPhe	25.7 (0.31–211)[a]	23.1 (2.44–2698)[ab]	116 (29.6–4621)[c]
(1OHNaph + 1OHAce)/1OHPy	43.7 (0.49–1222)[a]	35.0 (5.37–6481)[ab]	168 (5.95–1172)[c]
2OHFlu/1OHPhe	2.1 (3.0×10^{-3}–7.33)[a]	1.8 (3.0×10^{-3}–5.84)[ab]	10.8 (5.64–1032)[c]
2OHFlu/1OHPy	3.2 (0.01–11.4)[a]	1.6 (0.02–11.5)[ab]	18.2 (0.48–86.7)[c]
1OHPhe/1OHPy	1.6 (0.42–6.89)[a]	1.5 (0.45–22.7)[a]	1.8 (0.02–5.99)[a]

1OHNaph + 1OHAce: 1-hydroxynaphthalene and 1-hydroxyacenaphthene; 2OHFlu: 2-hydroxyfluorene; 1OHPhe: 1-hydroxyphenanthrene; 1OHPy: 1-hydroxypyrene. Superscripts (a, b, c) correspond to statistically different distributions between each group of firefighters ($p \leq 0.05$).

Table 4
Spearman correlations between the concentrations of urinary PAH metabolites in non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters.

	2OHFlu	1OHPhe	1OHPy	ΣOH-PAHs
Control group				
1OHNaph + 1OHAce	0.220*	0.196	0.233*	0.978**
2OHFlu	–	0.526**	0.448**	0.310**
1OHPhe	–	–	0.784**	0.324**
1OHPy	–	–	–	0.371**
Group A				
1OHNaph + 1OHAce	0.218	−0.222	−0.115	0.982**
2OHFlu	–	0.327*	0.330*	0.276
1OHPhe	–	–	0.680**	−0.127
1OHPy	–	–	–	−0.056
Group B				
1OHNaph + 1OHAce	0.539**	0.620**	0.331	0.994**
2OHFlu	–	0.442*	0.608**	0.570**
1OHPhe	–	–	0.432*	0.622**
1OHPy	–	–	–	0.356

1OHNaph + 1OHAce: 1-hydroxynaphthalene and 1-hydroxyacenaphthene; 2OHFlu: 2-hydroxyfluorene; 1OHPhe: 1-hydroxyphenanthrene; 1OHPy: 1-hydroxypyrene; ΣOH-PAHs: Total PAH metabolites.

* Statistically significant ($p < 0.05$).

** Statistically significant ($p < 0.001$).

2-naphthol and 1OHPy. However, (Oliveira et al. (2017c)) observed that 1OHNap and 1OHAce exhibited more pronounced increments after tobacco consumption while 2OHFlu was the most affected PAH metabolite by fire combat activities. Determination of these ratios can be an useful tool, but they may vary greatly according to the performed activity and the existent emission sources (Barbeau et al., 2014).

Spearman correlation coefficients were determined among the urinary levels of individual and ΣOHPAHs to estimate the relation among compounds for each group of firefighters (Table 4). The obtained correlations were all positive, and mostly moderate to strong ($0.220 < r < 0.978$; $p \leq 0.05$) in Control individuals; only correlation between 1OHNaph + 1OHAce with 1OHPhe resulted in low associations ($r = 0.196$). These findings point towards a common source of exposure to PAHs. Correlations between urinary levels of 1OHNaph + 1OHAce with ΣOHPAHs were strong for firefighters from Group A (non-smoking exposed; $r = 0.982$; $p \leq 0.001$); a similar conclusion was found for 1OHPy with 1OHPhe ($r = 0.680$; $p \leq 0.001$). Moderate correlations were also obtained between the concentrations of 2OHFlu with 1OHPhe ($r = 0.327$; $p \leq 0.05$) and with 1OHPy ($r = 0.330$; $p \leq 0.05$). However, some biomarkers of exposure were weakly correlated in subjects from Group A, thus evidencing the exposure to other PAH sources (Table 4). For smoking and exposed firefighters (Group B), the obtained associations were moderate to strong ($0.432 < r < 0.994$; $p \leq 0.05$), with exception of 1OHPy with ΣOHPAHs ($r = 0.356$; $p > 0.05$) and 1OHPy with 1OHNaph + 1OHAce ($r = 0.331$; $p > 0.05$). Once again, these findings suggest the existence of a major PAH exposure source in individuals from Group B. Since these subjects were simultaneously exposed to fire emissions and tobacco smoke, and based on the urinary PAH ratios, it is assumed that a tobacco median consumption of 20 cigarettes per day exerted a more pronounced effect in the firefighters from Group B than the respective exposure to fire emissions [3 (2–8) h].

3.3. Biomarkers of effect

Two early genotoxic/oxidative-effect biomarkers (basal DNA damage and oxidative DNA damage) were used to estimate firefighters' body response to occupational exposure at a cellular and molecular level. The achieved results for both of these biomarkers in the three characterized groups are presented in Fig. 2. Median values of the oxidative stress biomarker (measured as % NET-FPG) was 316% and 112% higher in non-smoking exposed (Group A) and smoking exposed (Group B) subjects than in Control group [2.7% (Group A) *versus* 0.64% (Control group); $p \leq 0.001$ and 1.4% (Group B) *versus* 0.64% (Control group); $p > 0.05$], respectively (Fig. 2a). Levels of oxidative stress biomarker were significantly lower in smoking exposed firefighters (Group B) than in non-smoking exposed subjects (Group A). It is known that tobacco smoke contains a high number of mutagenic and carcinogenic substances, such as benzene, arsenic and PAHs. The influence of smoking habits on comet assay parameters are yet to be established, since there are conflicting data (Hoffmann et al., 2005; Collins et al., 2014). On the other hand, some authors have reported lower DNA damage (measured as chromosomal breaks) of healthy smokers compared to never-smokers (Lao et al., 2008). Smokers have also showed an increase on baseline repair capacity (Wei et al., 2000) probably as an adaptation resulting from the increased demand for repair stimulated by the continuous damage caused by tobacco carcinogens (Wang et al., 2013). Therefore, the stimulated repair mechanism in smokers may in part explain the results obtained. Nevertheless, it is important to note that the number of smokers and non-smokers in our study limits the value of the data obtained and restricts possible conclusions, further studies are necessary to confirm these results. Results for the basal DNA damage (expressed as %TDNA) were inconclusive since no significant differences were observed between the three groups (Fig. 2b). Both of the exposed groups (A–B) showed positive and moderate correlations between the levels of oxidative stress biomarker with 2OHFlu ($r = 0.456$ for Group A and $r = 0.383$ for Group B; $p \leq 0.05$), 1OHPhe ($r = 0.365$ for Group A, $p = 0.05$ and $r = 0.359$ for Group B, $p > 0.05$), and 1OHPy ($r = 0.313$ for Group A and $r = 0.451$ for Group B; $p \leq 0.05$). Moreover, positive and moderate correlations were also found between the oxidative stress biomarker and the urinary levels of 1OHNaph + 1OHAce ($r = 0.306$; $p > 0.05$), as well as with ΣOHPAHs ($r = 0.305$; $p > 0.05$) in smoking and exposed firefighters (Group B). The DNA oxidative damage measured by comet assay is an effective biomarker of effect, not exposure, and therefore is less specific in identifying a single causative agent. During firefighting, subjects are exposed to a complex mixture of hazardous pollutants, which implies different effects that interact in the organism in different forms that may be additive, synergistic, antagonistic, or potentiating. Therefore, these results indicate subclinical changes in subjects recently involved in fire combat even if for a short period of time. Thus, early genotoxic effects in firefighters might be much higher once they are regularly involved in fire combat for long period of many consecutive hours, sometimes even for days or repetitively during several weeks when the largest wildland fires occur. There are no occupational exposure limits for firefighters, and comparisons can be only made with the scarce related studies. Abreu et al. (2017) reported that firefighting activities and wood smoke exposure were associated with higher values of oxidative and basal DNA damage. More recently, Andersen et al. (2018a,b) demonstrated that exposure to PAHs during firefighting activities was positively linked with genotoxicity in peripheral blood mononuclear cells. Other authors also found moderate correlations between urinary levels of 1OHPy and genotoxic effects (Kuang et al., 2013; Siwińska et al., 2004; Marczynski et al., 2009; Talaska et al., 2014), although some inconsistency was attributed to the high ratios variability of the airborne PAH congeners among the workers from different industrial sectors (Barbeau et al., 2015; Fan et al., 2014; Marczynski et al., 2011). Urinary biomarkers of exposure to PAHs (mainly 2OHFlu, 1OHPhe and 1OHPy) seem to be appropriate to be used as early markers of genotoxic effects in exposed firefighters. Still, much more research studies and data are necessary to confirm these findings.

Principal Component Analysis (PCA) was performed based on the exposure and genotoxic/oxidative-effect biomarkers of the three groups. Three models (A, B and C) are presented in Fig. 1S (the Supplementary Material). The model A allowed the extraction of three principal components (PC) with eigenvalues ≥1.01 and Kaiser-Meyer-Olkin sampling adequacy (KMO) of 0.54. Altogether the three PCs represented 76.07% of the original data (Fig. 1S (a) presents PC1 *versus*

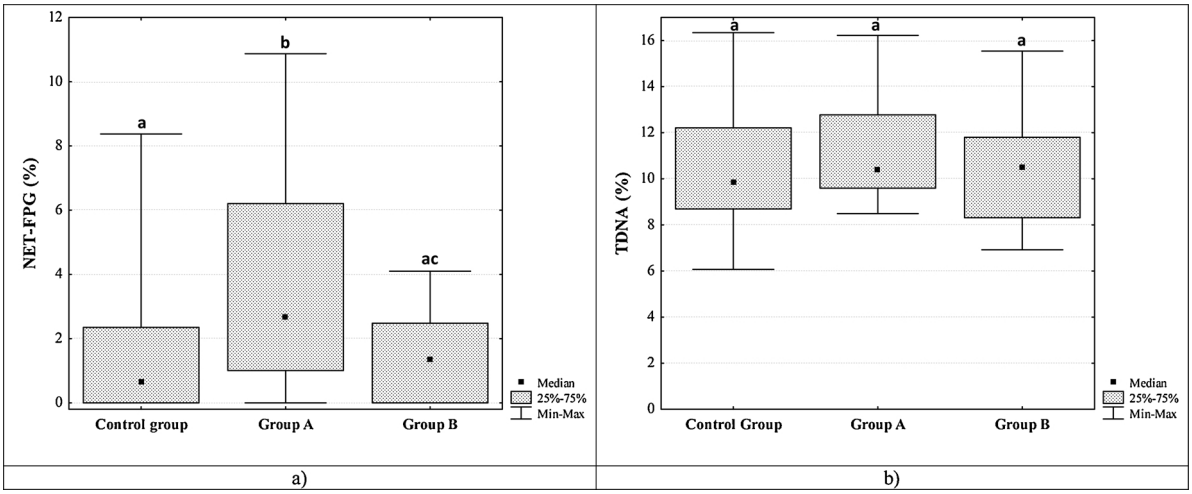

Fig. 2. Levels of DNA damage measured by comet assay: a) oxidative DNA damage (NET-FPG, %) and b) primary DNA damage (TDNA, %) among non-smoking and non-exposed (Control group), non-smoking exposed (Group A), and smoking exposed (Group B) firefighters. Superscripts[a, b, c] represent statistically significant differences between the groups.

PC2; PC3, data not shown, explained less than 15%). PC1 allowed a partial separation between non-smoking exposed firefighters (Group A) from the Control group based on the urinary levels of ΣOHPAHs, 1OHNaph + 1OHAce, 2OHFlu, 1OHPhe, and 1OHPy (square cosine values > 0.461); the biomarker of oxidative stress, NET-FPG, was the highest loaded variable in PC3 (square cosine = 0.518). The model B explained the variability of 71.4% of the original data (2 PCs with eigenvalues ≥1.83 and KMO = 0.54) and shows a moderate separation between smoking exposed firefighters (Group B) and non-smoking exposed subjects (Group A) (Fig. 1S (b)). PC1 presented the highest loadings for urinary ΣOHPAHs, 1OHNaph + 1OHAce and 2OHFlu (square cosine values > 0.589) while PC2 was strongly influenced by 1OHPhe, 1OHPy and NET-FPG (square cosines > 0.496). The model C represented 84.03% of the original data (2 PCs with eigenvalues ≥1.19 and KMO = 0.615) and allowed a good separation between smoking exposed firefighters (Group B) from non-smoking and non-exposed subjects (Control Group) (Fig. 1S (c)). Urinary ΣOHPAHs, 1OHNaph + 1OHAce, 2OHFlu, 1OHPy (PC1: square cosines ≥0.470), and 1OHPhe (PC2: square cosine = 0.473) were the variables that contributed the most for subjects' differentiation. Altogether, the urinary PAH biomarkers of exposure principally ΣOHPAHs, 1OHNaph + 1OHAce and 2OHFlu [the Bartlett sphericity test proved the strong correlation between these biomarkers ($0.535 < r < 0.992$; $p \leq 0.05$)] and, in a less extent, the biomarker of oxidative stress allowed to evaluate the impact of: i) firefighting activities in non-smoking subjects (Model A – Control Group *versus* Group A); ii) tobacco consumption in exposed firefighters (Model B – Group A *versus* Group B); iii) cumulative effect of fire combat activities and tobacco consumption in exposed firefighters (Model C – Control Group *versus* Group B.

3.4. Relation between biomarkers and cardio-respiratory parameters

While on-duty during a fire combat, firefighters are frequently exposed to hazardous pollutants, may have an inadequate nutrition, suffer from posttraumatic stress disorder, sleep disruption/deprivation, and imbalance between job demands and decisional latitude, all of which constitute occupational risk factors for elevated blood pressure, metabolic syndrome, and consequent cardiovascular diseases (Kales et al., 2009). More than 21 and 14% of firefighters in this study presented, respectively, diastolic and systolic blood pressures higher than 90 and 140 mmHg (Table 1). No association was found between the levels of blood pressure and the urinary concentrations of PAH metabolites or the levels of early genotoxic biomarkers. However, significant and positive correlations were found between the cardiac frequency of firefighters with the urinary concentrations of ΣOHPAHs ($r = 0.431$ for Control group, and $r = 0.568$ for Group A; $p \leq 0.001$) and with the biomarker of oxidative stress ($r = 0.382$ for Control group, $r = 0.393$ for Group A; $p \leq 0.001$); inconclusive data were obtained for subjects from Group B. Several factors may affect the impact of fire emissions on the health of exposed firefighters, including levels of gaseous and particulate pollutants within the air breathing zone, exposure duration, exertion levels, and individual susceptibility to the associated health risks (*i.e.* preexisting and/or predisposition to develop cardio-respiratory diseases). The results achieved in this work suggest that the urinary PAH biomarkers, the blood biomarker of oxidative stress and cardiac frequency of non-smoking (non-exposed and exposed) firefighters were correlated, however this cross sectional study could not conduct causal relationship. A long period of work as wildland firefighter has been significantly associated with high blood pressure and heart arrhythmia, two well-established risk factors for cardiovascular diseases (Semmens et al., 2016). Findings from some studies support the evidence that occupational exposure to fire emissions may induce local inflammatory response in firefighters with the subsequently initiation of a systemic response that will culminate in adverse health consequences (Gianniou et al., 2016; Gaughan et al., 2014b; Ferguson et al., 2016). Regarding other occupationally exposed groups, Singh et al. (2018) found significant and positive correlations between urinary 9-hydroxyfluorene and 1OHPy with some acute kidney injury biomarkers (kidney injury molecule 1 and tissue inhibitor of metalloproteinases) in Indian male kitchen workers with microalbuminuria, thus suggesting that occupational exposure to PAHs may cause kidney injury. Alhamdow et al. (2017) reported that urinary PAH metabolites of chimney workers were positively associated with diastolic blood pressure. These exposed workers presented increased levels of homocysteine, cholesterol, and high-density lipoprotein due to their occupational exposure to PAHs in soot (Alhamdow et al., 2017). Brucker et al. (2013) reported strong associations between the urinary concentrations of 1OHPy with pro-inflammatory cytokines and elevated levels of biomarkers of oxidative damage in occupationally exposed taxi drivers. More recently, Barth et al. (2017)) also found increased levels of urinary 1OHPy concentrations and some biological inflammation markers of DNA damage (% of neutrophilis expressing intercellular adhesion molecule-1 and NTPDase activity in platelets) and genotoxicity biomarkers (% tail in DNA and micronucleous frequency) in taxi drivers. Evaluation of the potential health risks associated with occupational exposure is a difficult and complex task. Data describing the

association between urinary PAH metabolites and cardiovascular risks on non-occupationally exposed populations have been slowly emerging. Shiue et al. (Shiue, 2015) reported higher urinary levels of OHPAH in people with diagnosed cardiovascular disease and cancer; urinary concentrations of 2-naphthol, 1OHPy and 4OHPhe were associated with higher rates of cancer, heart attack, and hypertension occurrences. Ranjbar and colleagues (Ranjbar et al., 2015) concluded that exposure to PAHs was directly related with obesity and with the expression of obesity-related cardiometabolic health risk factors such as metabolic syndrome, type 2 diabetes, hypertension, and dyslipidemia. More recently, Poursafa et al. (2018) found that high concentrations of urinary PAH metabolites were directly associated with the incidence of some cardiometabolic risk factors in young children. Based on the available information, there is a need to minimize exposure to PAHs in occupationally exposed groups and to promote environmental mitigation policies to protect human health.

4. Conclusions

Considering the lack of current knowledge on the topic, this study characterized the impact of firefighting activities on firefighters' occupational exposure based on biological monitoring. Urinary concentrations of ΣOHPAHs, 1OHNaph + 1OHAce and 2OHFlu were significantly higher in exposed (non-smoking and smoking) than in non-exposed subjects. Moreover, significant increments of the oxidative DNA damage were found in non-smoking exposed subjects. The main findings of this study suggest that the cardiac frequency, urinary PAH biomarkers of exposure and the blood biomarker of oxidative stress of non-smoking (non-exposed and exposed) firefighters correlate well; however, this cross sectional study could not conduct causal relationship. More comprehensive studies are needed in a larger group of subjects directly involved in firefighting activities to validate these findings. Future studies should include more biomarkers of exposure, cardiovascular markers, and biomarkers of early genotoxic/oxidative-effects to better characterize their interrelation and association with the development and/or aggravation of cardiovascular diseases in firefighters. Surveillance (bio)monitoring programs need to be implemented, principally in the countries that have been severely affected by forest fires, in order to go deeper on the characterization of the health risks and their direct (short- and long-term) impact along the firefighter´s life.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

This work has received approval for research ethics from approved by the Ethic Committee of University of Porto and a proof/certificate of approval is available upon request.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2019.121179.

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