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EPIDEMIOLOGY AND BIOMARKERS OF AIR POLLUTION AND BURDEN OF CORONARY ARTERY DISEASE

by
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AN ABSTRACT OF THE THESIS

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In Lebanon, studies indicated significant increase in the risk factors associated with cardiovascular diseases (CVD). This was manifested by an onset of CVD of 12 years earlier than patients in other parts of the world. Several studies have linked PAH exposure to CVD in general population, and were based on self-reported CVD events related to coronary heart disease (CAD), angina, stroke and heart attack. To our knowledge, this is the first time to examine the association between polycyclic aromatic hydrocarbons (PAH) exposure and CAD based on cardiac catheterization results.

Considering the high levels of PAHs in Lebanon due to heavy traffic, abundance of diesel generators in densely populated areas, and poorly maintained power plants in some areas, this study aims at identifying the association between PAH urinary metabolites and CAD based on already available cardiac catheterization results and associated urine samples.

The study consists of assessing the CAD burden of a cohort of 258 patients who have been clinically evaluated at the American University of Beirut Medical Center (AUBMC) since 2014. It also includes quantifying hydroxylated polyaromatic hydrocarbons (OHPAH) concentrations in the urine provided by the cardiac cath patients using High Performance Liquid Chromatography coupled with Fluorescence Detector (HPLC-FLD). SPSS software was used to estimate the association between PAH exposure and CVD. PAH urinary metabolites can be considered biomarkers of CVD burden and may be used as a tool for early detection of CVD development.

It was determined that OHPAHs concentrations that were measured for non-smokers in this study were higher than the levels that were reported for smokers and some occupational workers which implies that nonsmoker patients are highly exposed to sources of PAH mixtures. Such sources are assigned to old car fleet, diesel generators, incinerators and second hand smoking. In particular, 1-OHPYR showed a significant association with CAD

status after adjusting for covariates like age, sex, and diabetes, and it is considered a specific marker for the diesel generator and incineration sources.

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ABBREVIATIONS

1-OHPYR	1-pyrenol
TCDD	2,3,7,8-Tetra-Chloro-Dibenzo-p-Dioxin
2-OHFLU	2-fluorenol
2-OHNAP	2-napthol
3-OHPHEN	3-phenethrol
ACGIH	The American Conference of Governmental Industrial Hygienists
ACN	Acetonitrile
ATSDR	Agency for Toxic Substances and Disease Registry's
AUBMC	American University of Beirut Medical Center
BaP	Benzo(a)pyrene
CO	Carbon Monoxide
CVD	Cardiovascular Disease
CDC	Centers for Disease Control and Prevention
CAD	Coronary Artery Disease
EPA	Environmental Protection Agency
ETS	Environmental Tobacco Smoke
EU	European Union
FDA	Food and Drug Administration

GC-MS	Gas Chromatography coupled with Mass Spectrometry
GSH	Glutathione
HDL	High Density Lipoprotein
HPLC-DAD-MS	High Performance Liquid Chromatography coupled with Diode Array Detector and Mass Spectrometry
HPLC-FLD	High Performance Liquid Chromatography coupled with Fluorescence Detector
IRB	Institutional Review Board
Pb	Lead
LLE	Liquid-Liquid Extraction
LDL	Low Density Lipoprotein
LDVs	Light Duty Vehicles
NAAQS	National Ambient Air Quality Standards
NO_2	Nitrogen Dioxide
O_3	Ozone
OHPAH	Hydroxylated Poly-Aromatic Hydrocarbons
PM	Particulate Matter
PAHs	Poly-Aromatic Hydrocarbons
RSD	Relative Standard Deviation
rpm	rotations per minute
SAA	Serum Amyloid-A
SPE	Solid Phase Extraction

SO_2	Sulfur Dioxide
IARC	International Agency for Research on Cancer
VMP	Vascular Medicine Program
WHO	World Health Organization

CHAPTER I

BIOMARKERS OF AIR POLLUTION AND BURDEN OF CORONARY ARTERY DISEASE

A- Air pollution and related risk factors

A growing body of literature has linked air pollution to fatal heart, and respiratory diseases [1]. The World Health Organization (WHO) related an estimate of 91% of the global population living in regions where the quality of air goes above the WHO limit recommendations [2]. Even more, ambient air pollution resulted in 4.2 million premature mortalities around the world [2] where continuous and extensive exposure to outdoor air pollution is expected to be the main cause of early deaths by 2050, having a greater effect than malaria and water quality [3, 4]. Air pollution was associated with cardiovascular disease (CVD) as it was ranked among the highest four risk factors related to early deaths globally [5]. Major principle air pollutants include Carbon Monoxide (CO), Sulfur Dioxide (SO_2), Nitrogen Dioxide (NO_2), Lead (Pb), Ozone (O_3), and Particulate Matter (PM), defined by the National Ambient Air Quality Standards (NAAQS) by the Environment Protection Agency (EPA) [6].

1. Particulate matter

PM size varies from nanometers to micrometers. PM with diameter ranges between 2.5 and 0.25µm are called fine particles, where PM with diameter less than 0.25µm are called ultrafine particles [7, 8]. Fine and ultra-fine PM composition contain metals , organic compounds and elemental carbons that are mainly originating from combustion practices [8, 9]. A short and long term exposure to PM ($PM_{2.5}$ = 10 µg/m^3) increases the percentage and the relative risk of CVD mortality from 0.4 %to 1.0% and from 1.06 to 1.76 respectively [10].

2. PM levels in Lebanon

In Lebanon, ambient $PM_{2.5}$ levels have exceeded WHO guidelines in greater Beirut area by 100% [11, 12]. Furthermore, several studies on the $PM_{2.5}$ measured by the roadside indicated high levels in Beirut when compared with Los Angeles [12]. Nowadays, air pollution in Beirut is even much worse due to the increased number of construction sites, heavy traffic of diesel trucks, and to the elevated number of diesel generators working as a primary backup power supply during outage due to the lack of a continuous national power generation system.

3. Poly-Aromatic Hydrocarbons (PAHs)

PAHs are a part of the organic compounds in PM [30]. PAHs are lipophilic and a semi-volatile group of more than several hundred different chemical compounds, formed of two or more fused aromatic rings [13]. PAHs having less than four aromatic rings are categorized as low molecular PAH and exist in vapor phase, while those having four or higher aromatic rings are classified as high molecular weight PAH and are bound to

particles [14]. Moreover, the carcinogenicity and mutagenicity effect they possess made them ubiquitous environmental pollutants where they are highly distributed in the environment owing that to their physiochemical properties [15, 16].

a. Sources of PAHs

The pervasive existence of PAHs in the environment is from both natural and anthropogenic sources which are a result of pyrolytic and incomplete combustion of organic matter at high temperature [17, 18]. In addition, natural sources are derived from open burning like forest and prairie fires, petroleum or coal deposits, plant synthesis and volcanic eruption activities [19]. However, major anthropogenic activities consist of incomplete burning of fuels in industrial activities, garbage, tobacco and plant material, road transport like diesel and gasoline automobiles, waste incinerators, residential heating, emission from traffic, and diesel generators [13, 19-22]. Furthermore, PAHs emitted from natural sources are minor compared to anthropogenic sources which are considered the main contributor to high PAH levels in the environment [21].

b. Exposure routes

There are several routes whereby humans are exposed to PAH, mainly inhalation of air, ingestion of contaminated food and water, and dermal contact with dust or soil in occupational and non-occupational settings [19]. Ingestion of food and drinking water is usually an indoor activity, dermal contact to soil is an outdoor activity, while inhalation of air occurs in both indoor and outdoor [23]. PAH concentrations in air can range from five to 200,000 ng/m^3 [24]. Occupational exposure to PAHs generates higher levels compared to environmental exposure, but in both cases it remains a common interest due to adverse health effects on humans that would disseminate over urban and rural population [24].

c. PAHs in air

The Agency for Toxic Substances and Disease Registry's (ATSDR) released information about background levels of PAHs ranging from 0.02-1.2 ng/m^3 in rural regions and 0.15-19.3 ng/m^3 in urban areas [25]. Measured Σ PAH levels, in an occupational exposure related to the inhalation route, fluctuated between 46.4–428 ng/m^3; with two to three aromatic rings were considered as 63.9–95.7% of PAHs [24]. PAHs with four and higher aromatic rings accounted 0.789–2.05% and 2.54–34.7% of Σ PAHs, respectively [26]. However, in non-occupational exposure, PAH levels were ranged from 0.03-921 ng/m^3 [27]. The highest value (921 ng/m^3) is attributed to naphthalene since it is a specific biomarker for indoor activities related to heating, cooking , and the use of repellents [27, 28]. Other important factor that contributes to the inhalation source of PAHs is environmental tobacco smoke (ETS) where measured concentrations of benzo(a)pyrene (BaP), pyrene, and chrysene were 26.7 ng/m^3, 25 ng/m^3, and 70.5 ng/m^3 respectively [29, 30].

d. PAH in food

Humans who are not exposed to occupational or environmental tobacco smoke can absorb 70% of PAH dosage from their diet [31]. Detected PAHs in raw food such as fresh vegetables and fruits is a result of background contaminations from the transportations and deposition of airborne PAH particles originating from industrial and highway areas [13, 32]. Moreover, the quantity of PAHs in food is influenced by the techniques followed in cooking, preservation and storage, and varies from one individual to another depending on the eating habits they follow [33]. Also, PAHs can be identified in fruits, leafy vegetables, meat, fish and grains, where the highest amount is accounted for the charcoaled grilled food

in addition to the fats and oil category [34, 35]. Total amount of mean daily PAHs intake from diet is estimated to be 5-17μg/Kg [36].

e. PAH in water

PAHs have low solubility in water due to their physio-chemical properties [33]. Water contamination occurs from oil spilling and petroleum material, discharging of toxic material from industrial and municipal settings into water sources, along with the atmospheric deposition [32, 33, 37].

f. PAH in soil

Soil medium is full of PAH particles [19]. The concentrations can range from five to 100 μg/kg [27]. Soil contamination sources include deposition of airborne PAH particles originating from vehicle exhaust [13, 38]. Measured levels of PAHs in soil next to road highways with heavy traffic is ≤ 2000 μg/kg [39].

g. Combination of all sources of exposure

It is generally impossible to limit the human exposure to PAHs to a single route because inhalation of air and consumption of food are inevitable to human beings. Inhalation of air is likely to be a smaller route of exposure compared to ingestion of contaminated food in any population exposed to PAH [19]. However, dermal contact and drinking water are considered negligible exposure routes in comparison to inhalation and ingestion in non-occupational setting [19]. The percentages of PAH exposure contributed to diet, air, water, and soil in a non-smoking male were 96.0%,1.6%,0.2% and 0.4% respectively [19]. The daily dose of PAHs intake in a non-smoking male could range between 3-15 μg/kg [19]. On the other hand, the dose would be doubled for a smoker male [19]. These percentages would vary depending on the circumstances related to the PAH

exposure sources. In some cases, air would be the contributing factor in ETS setting where PAH concentrations intake would range between 4–62 ng/day [30].

B- Toxic kinetics and metabolism

PAHs are xenobiotic, and their biological fate is governed by absorption, distribution, metabolism, and excretion [40]. Although PAHs are chemically inert hydrophobic compounds, but their metabolism leads to active metabolites [41, 42].

1. Absorption

After intra-gastric administration of PAH, in animal models, an observation of a peak in the lymph was reported four hours later [43].

However, after administration of aerosol particles in rats, PAHs absorption through pulmonary track showed clearance of PAHs from lungs to other organs in a biphasic manner; two-hour half-life for the fast phase followed by two days' half-life for the slower phase [44]. PAHs bound to particles would take 20 times more to be cleared from the lungs compared to free PAHs [45]. The process of clearance from the lungs depends on the size and chemical composition of the bounded particles, and varies with the PAH skeleton [45-47].

Several animal model studies showed that PAHs can easily penetrate the skin when administered to the back of mice, and later showed a biphasic clearance; 40 hours for the fast phase and 104 hours for the slow phase [48]. The amount of PAH administered to mice was recovered later in feces which proves the percutaneous absorption of PAHs [48].

2. Distribution

Irrespective of PAHs exposure routes, in vitro studies and animal models showed a similar fast and extensive distribution of PAHs after inhalation, ingestion, and dermal contact [49]. PAHs and their metabolite levels can be detected in several internal organs from minutes to hours after administration [48, 50]. PAHs might accumulate and get stored in fat deposits, and then released into the blood circulation [50]. Most of PAHs and their metabolites are centered in the gut area, since liver is the main responsible organ for metabolism [50]. Moreover, high level of PAHs are found in the gut due to the mucocillary clearance that occurs after inhalation exposure [51].

Following inhalation exposure, the distribution of PAH into different body organs started with PAHs clearance from pulmonary tissues, where the half-life ranged between two to three hours [51]. High levels of PAHs were reported in liver half an hour post exposure [51]. However, PAH concentrations in kidney were double the amount found in liver six hours post exposure [51]. The highest PAH levels were found in small intestine and stomach one hour after the exposure, but decreased to later increase in the large intestine [51].

3. Metabolism

Lipophilic PAHs can be converted into polar compounds by microsomal mixed function oxidase system enzymes [52]. The enzymes that are responsible for metabolizing PAHs into polar derivatives are widely spread in all body organs, tissues, and cells [53]. Liver is the principle site for metabolizing PAHs followed by lung, intestines, skin and kidneys [54]. PAHs trigger the induction of cytochrome P450 enzyme, this was evident in a

study conducted on groups of mice that were dermally administered an increasing dose of PAH [55]. PAHs saturation regarding the absorption and metabolism was observed [55]. However, the rate of absorption and metabolism increases when these mice where induced by 2,3,7,8-Tetra-Chloro-Dibenzo-p-Dioxin (TCDD) which is a specific compound that triggers the induction of cytochrome P450 [55, 56].

The enzymes that are responsible for PAH metabolism can be divided into two groups, where each group is responsible to catalyze a certain type of reaction in the biotransformation process as phase one and two reactions [41, 42].

The fate of phase one metabolism is the addition of a polar functional group to the xenobiotic compounds through oxidation, reduction or hydrolytic reactions [56, 57]. Cytochrome P450 is the most common enzyme in phase one since it has a wide substrate selectivity and specificity [58, 59].

The new polar compounds that are formed from phase one reactions undergo further synthetic reactions in phase two with internal compounds that originates from carbohydrates or amino acids such as glucuronides, sulphate esters and mercapturic acids [56, 60].

Biotransformation will be further discussed using BaP as an example on PAH metabolism. Most of in-vitro and in-vivo studies focused on BaP since it is specified as type one carcinogen by EPA [61]. In principle, PAH biotransformation has attracted scientific attention since they are unreactive chemical compounds that can cause major diseases like cancer when they form later the electrophilic derivatives which bind to macromolecules like DNA and initiate mutations [62]. The international agency for

research on cancer (IARC) [41] has summarized the metabolism of BaP in the following schematic diagram (Figure 1)[63-65] .

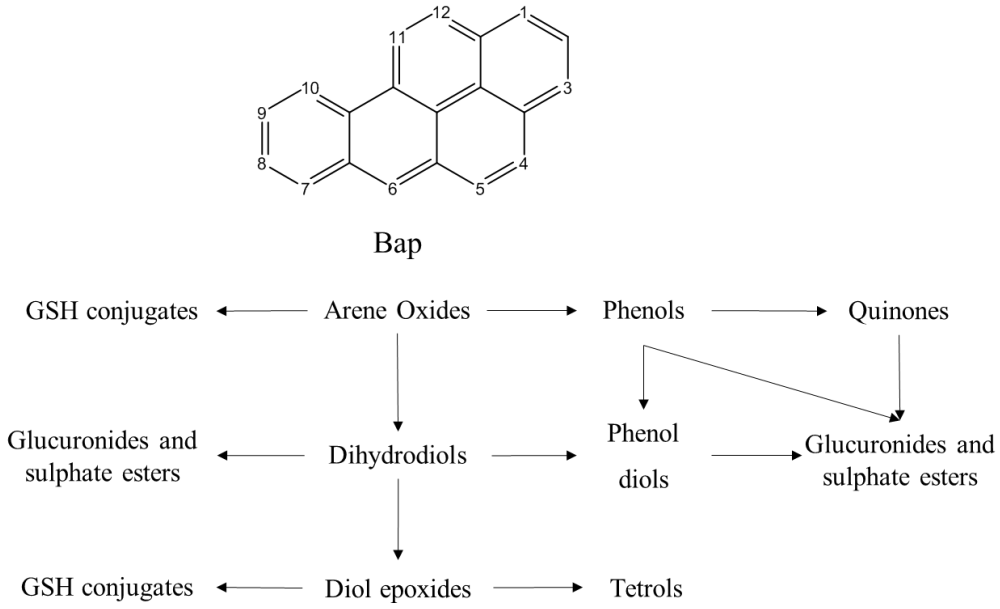

Figure 1: Benzoapyrene metabolism.

As shown in Figure 1 cytochrome P450 metabolize benzo(a)pyrene to many arene oxides [66], where arene oxides can transform to phenols by spontaneous rearrangement or they can transform to dihydrodiols by undergoing hydration with epoxide hydrolase enzyme [67, 68]. Another mechanism for the phenol production is the direct oxygen insertion of parent BaP [41, 69]. The phenol metabolites of BaP can be transformed to quinones by oxidation reaction [70, 71]. Also, the dihydrodiol metabolites can be transformed to phenol dihydrodiol after oxidation that is followed by hydration [41, 72].

Some dihydrodiol metabolites can be metabolized further to diol epoxide by oxidation reaction [73], which can be transformed to tetrol metabolites by epoxide hydrolase enzyme [41, 74].

Regarding phase two biotransformation, once polar electrophilic derivatives are formed, they can covalently bind with Glutathione (GSH), sulfate esters or glucuronic acid at any stage of the different metabolites formation [41, 67], either spontaneously or through catalyzed reactions [75, 76] (Figure 2).

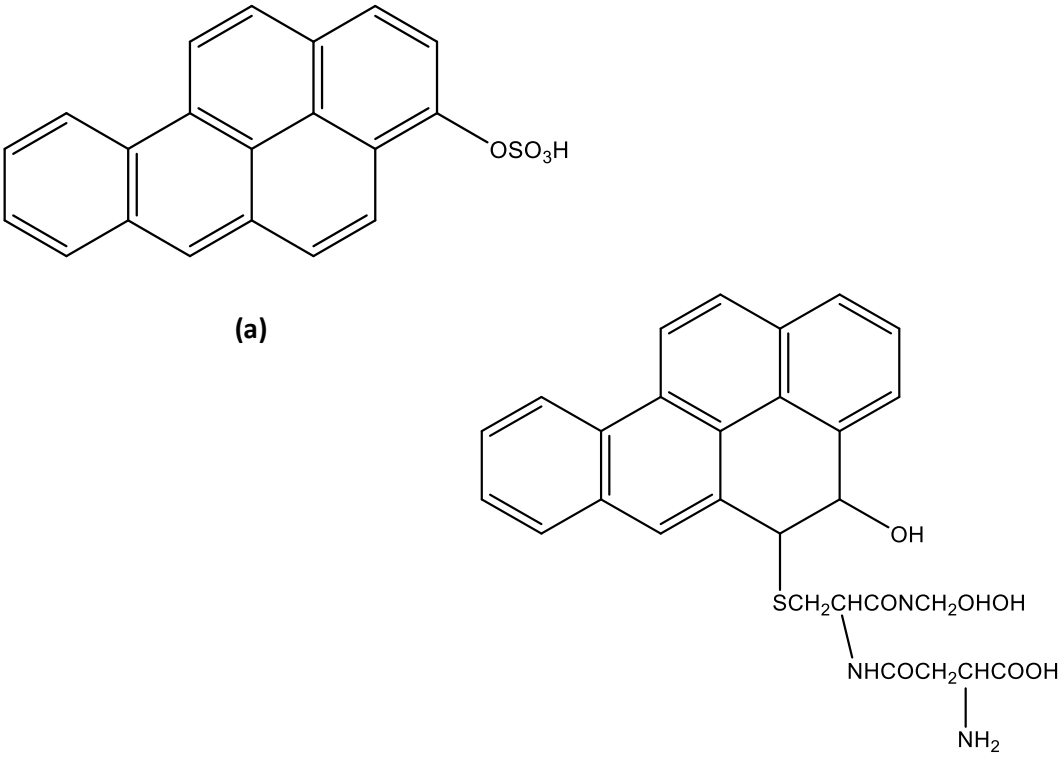

Figure 2: Sulphate ester conjugate **(a)** and Gluthathione conjugate **(b)**.

4. Excretion

Irrespective of the administration route, PAH metabolites will be excreted via urine or feces, since they are polar and more water soluble in comparison to their parent compounds [77, 78].

PAH absorbed from the gastrointestinal tract showed higher rates of excretion and lower storage in different organs and tissues in comparison to other route of exposure [41, 79]. This can be attributed to the fact that orally absorbed PAHs reached the systemic circulation after entering the liver where they undergo first pass effect [79, 80]. The excretion rates from different routes of administration were studied in animals [41], and showed that intraperitoneal injection resulted in the excretion of 30% of the original dose in 72h, whereas orally absorbed dose caused in 82% excretion within 24h [41, 80].

C- PAH in Lebanon

In 2015, an ambient PAH measurement was conducted during summer and winter at three coastal locations in Lebanon; AUB, Dora, and Zouk Mikael, and the PAH levels were 36.30 ± 2.8 ng/m^3 in winter and 13.15 ± 1.18 ng/m^3 in summer at AUB, 25.08 ± 3.31 ng/m^3 and 27.65 ± 2.05 ng/m^3 in winter and summer, respectively at Zouk Mikael, and 91.88 ± 8.18 ng/m^3 in winter and 69.43 ± 5.86 ng/m^3 in summer in Dora which was the highest [81]. In addition, numbers showed that BaP concentration have exceeded the European Union (EU) air quality standard of 1 ng/m^3 which were 2.07 ng /m^3 and 2.9 ng/m^3 at Zouk Mikael and Dora respectively [81]. Diagnostic ratios were measured and attributes PAH sources to traffic, diesel combustion, paved roads dust, fuel combustion for power generation, and waste incinerators [81].

PAH emissions from private diesel generators were measured in Beirut which resulted in an elevated background levels by 40% in PAHs [82]. A growing body of literature is studying the health effects of PM of which are PAHs [83].

D- Significance of CVD

In Lebanon, several studies showed an increasing development in the occurrence of CVD and associated age-related risk factors [84, 85]. It is common as Lebanon is one of the low and middle-income countries where the onset of CVD is earlier in comparison with reports in other countries [86]. To be more specific, CVD patients in the Middle East are 12 years younger than patients residing in Western Europe, and China [86]. In addition, it is important to study CVD since it is number one in mortality worldwide by WHO were 17.9 million people die every year from CVD which constitute 31% of the total global death [87]. Moreover, another WHO report related to Lebanon showed that non-communicable diseases are expected to account for 91% of total deaths, where CVD alone accounts for 47% of mortality, followed by cancer, respiratory diseases, and diabetes [88].

E- PAH and CVD

Many findings showed a positive association between exposure to PAH and CVD [83] where air pollution can cause CVD via elevating blood pressure and increasing heart rates [89], as well as through atherosclerosis development [90]. Asweto in his review about cardiovascular health risk posed by PAHs and ultrafine particles explains the mechanism of action [91]. The proposed mechanism by which PAH cause CVD starts when PAH bounded to particles enter the blood stream and deposit on the endothelial cells of the

vascular vessels [91]. It is important to mention that endothelial cells work as a protective barrier against any exogenous compounds [92]. After the deposition, atherogenesis is induced where damage is identified in the endothelial wall causing endothelial dysfunction via inflammation [91, 93]. Endothelial dysfunction can lead to injury to the vascular wall, and the creation of the atherosclerotic plaque [94, 95]. However, there is a gap in the literature regarding the full mechanism, but studies have proven that reactive oxygen species (ROS) that are formed from oxidative stress and its relation to the immune system changes in addition to the systemic inflammation and its DNA bindings could be the mediator [91, 96, 97].

F- Hydroxylated PAHs metabolites and CVD

There is a huge gap in the literature evaluating hydroxylated metabolites with CVD rather than others. As shown in Figure 1, the biotransformation process produces different metabolites like phenols, diols, quinones, and epoxides [41]. However, the focus of this study will be on hydroxylated (phenolic) metabolites for several reasons. An animal study showed the different percentages of BaP metabolites formation where phenol isomers had the highest percentage ranging from 39-49%, followed by dihydrophenol isomers having the percentage between 27 to 39%, and finally quinones had the lowest percentage ranging from 14-17% [98]. Another animal study on the formation rates of different metabolites revealed that the highest formation rate at 0.76 nmol/min/nmol P450 was for phenols, where a rate of 0.46 nmol/min/nmol P450 was given to quinones metabolites, and diol isomers had a rate ranged from 0.04 and 0.10 nmol/min/nmol P450 [99]. As observed, the high percentage of hydroxylated metabolites makes it easier for the detection purposes.

Also, diol epoxides are extensively studied in cancer research as it is proven that diols are correlated with lung cancer [100]. However, phenol metabolites don't have a mutagenicity effect as the diol metabolites [101].

G- Analytes of interest

1. Sources of 2-naphthol (2-OHNAP)

2-OHNAP is a direct biomarker for naphthalene exposure, which can be found in both indoor and outdoor air exposure [102]. Main sources of naphthalene include the use of moth repellent which is considered the second specific biomarker after incomplete burning [103] ,gaseous pesticides in agriculture field, household deodorizers, in addition to the biomass burning [104]. The incomplete combustion of gasoline and diesel in vehicular emission is a major source of naphthalene that can reach a concentration of 50 and 505 µg/km [105, 106]. Side stream of cigarettes smoke is considered to be an important source of naphthalene where second hand smokers can absorb up to 15 µg/ cigarette of naphthalene [107]. Another interesting source are the mosquito coils that has a maximum level of 19.5 µg/h [108]. Processed food at higher temperature like frying of fish can increase the amount of naphthalene to 4.4 $\mu g/m^3$ [109].

2. Sources of 2-fluorenol (2-OHFLU)

2-OHFLU is a direct biomarker for fluorene exposure [110]. Tobacco smoke is a major source of fluorene metabolites [111]. A study done in US and Poland population [111], showed that hydroxyfluorene metabolites are specific biomarkers for cigarette smoke

[111]. Fluorene is a significant component of coal tar [112], where 3.7 µg/m^3 had been quantified upon bitumen and coal-tar exposure [113]. In addition, gasoline engine exhaust had detected fluorene [114]. For diesel powered engines, low molecular weight PAHs were mainly measured including fluorene [115].

3. Sources of 3-phenanthrol (3-OHPHEN)

3-OHPHEN is a direct biomarker for phenanthrene exposure [110]. Phenanthrene metabolites were highly found in exposed workers to diesel exhaust where post shift metabolites concentrations were three fold higher than the pre shift levels [116]. Crowded vehicular traffic generates high amount of phenenthrene as it was recorded that 81.1 ng/m^3 was measured in road tunnel environments [117]. Waste incinerators are a major source of phenethrene [118]. Also, the incomplete combustion of fossil fuel in industrial activities generates high levels of phenenthrene [21].

4. Sources of 1-pyrenol (1-OHPYR)

1-OHPYR is a direct biomarker for pyrene exposure [119]. Cigarettes smoke is a significant and specific source of pyrene metabolites where smokers recorded higher 1-OHPYR metabolites than non-smokers by four folds [111]. In addition, pyrene is present in gasoline and diesel fuel emission as the emission factor can reach a level of 1000µg/kg [120]. Oil combustion and incinerations generates high levels of pyrene [118, 121]. Furthermore, extensive biomass burning in rural area generates 1-OHPYR levels that were six folds higher than what was generated from traffic and vehicular emission [122].

H- Half-lives of OHPAH

Several studies have been done to determine the half-lives and the percentages of different OHPAH that reached the urine after the ingestion of known dose of PAH in barbecued chicken [94]. The median maximum concentrations of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR after the ingestion were 33, 28, 27, and 29 folds higher than the pre-exposure levels respectively [94]. In addition, the median half-lives of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR were 2.5h, 2.9h, 4.1h, and 3.9h respectively [94]. Further, measured excreted mean percentages of the previously mentioned OHPAHs metabolites were 182%, 60%, 11%, and 6.8% respectively [94]. As a conclusion, the main excretion route for the low molecular weight OHPAHs is urine, while the main excretion route of high molecular weight OHPAHs is in feces [95].

Half-lives from the inhalation and dermal routes were higher than the ingested routes. Example is the half-lives of 1-OHPYR from the inhalation and dermal routes which ranged from 6-29h and 11.5 -15h respectively [96-98].

I- Objective of the study

Several studies have linked PAH exposure to CVD in general population, and were based on self-reported CVD events related to coronary heart disease, angina, stroke and heart attack [83, 123], serum biomarkers such as C-reactive protein, homocysteine, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and triglycerides [124], and blood levels of acute phase proteins Serum amyloid-A (SAA) [125]. To our knowledge, this is the first time the association between PAH exposure and CAD based on cardiac catheterization results is examined.

Our goal is to determine whether air pollution urinary biomarkers are a risk factor for CAD by first assessing the CAD burden of 258 patients who have been clinically evaluated at the AUBMC Cardiac Catheterization lab as a first step, and second by quantifying OHPAH concentrations in the urine provided by the Cardiac Catheterization patients using HPLC-FLD, and finally using statistical analysis to test whether the levels of PAH metabolites in the urine of patients admitted for Cardiac Catheterization associate with CAD burden.

CHAPTER II

ANALYTICAL METHOD DEVELOPMNT, OPTIMIZATION, AND VALIDATION

A- Study participants

The current study involved subjects residing in Lebanon (n = 315) who are more than 40 years of age and have been clinically evaluated at the AUBMC Cardiac Catheterization Lab for angiography since 2014. Baseline characteristics of each patient were collected through the Vascular Medicine Program's (VMP) data collection forms and stored in the VMP databases. Variables on demographics were extracted from patients' medical charts after getting their consent, and details on their angiograms are added to their data collection forms. Participants with un-recommended levels on urine creatinine were not included in the final model (the recommended levels of urine creatinine by WHO ranges between 30 and 300 mg/dl)[126, 127]. This resulted in 258 participants included in the final analyses. Of those, 170 (65.90 %) had obstructive disease (obstruction of at least 50% in at least one of the arteries) despite the fact that more than half of them are nonsmokers (n = 153 (61.00 %)). Information related to smoking habits were collected from medical charts. The study protocol was approved by the institutional Review Board (IRB) for the human subjects' aspect (number of IRB application; FAS.NS.01).

B- Materials

1. Chemicals and reagents

2-naphthol (2-OHNAP), 2-fluorenol (2-OHFLU),3-phenanthrol (3-OHPHEN) ,1-pyrenol (1-OHPYR), and 1- pyrenol-d9 (D9-1-OHPYN) were purchased from Toronto Research Chemicals (Toronto, ON Canada). β- Glucuronidase/arylsulfatase from Helix pomatia was provided by Sigma –Aldrich (Indianapolis, IN, USA). Acetonitrile (HPLC gradient grade, ≥ 99.9%), water HPLC gradient grade, and sodium acetate anhydrous laboratory reagent grade were obtained by fisher scientific (UK). Formic acid (LC/MS grade) was purchased from Fisher Scientific (Poland). n- Hexane (CHROMASOLV for HPLC, ≥ 97.0 %) was obtained from Honeywell Riedel-de Haën (Germany). Acetic acid of glacial 100% extra pure was obtained from Chemical Management Consulting (CMC) (Germany). Zorbax 300extend -C18 column (4.6 X 150 mm, 3.5 µm) was from Agilent (USA). Creatinine Assay Kit (ab204537).

C- Methodology

1. Preparation of standard solutions in Acetonitrile

The stock standard solutions (1000 µg/ml) of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR were prepared by dissolving 1mg of the solid standard in 1ml of acetonitrile (ACN) (for each standard). The stock standard solution of D9-1-OHPYR was prepared in the same way and diluted to the final concentration of 1 µg/ml. Mixtures of calibration standard were then prepared by diluting the standard solution with ACN to reach lower concentration ranges. The internal standard solution was added in such a way that the final concentration is 2 µg/l in each calibration mixture.

2. *Optimization of sample preparation*

a. Urine pretreatment

The optimization experiments were established using pooled human urine samples that were collected from six healthy volunteers. After collection, urine specimens were frozen and stored at −80 °C. Aliquots of urine samples were transferred into 5ml centrifuge tubes and stored at - 20 °C until analysis. Ahead of use, urine samples were thawed at room temperature and centrifuged for five minutes at 4000 rpm to remove solid impurities.

Urine as a complex matrix is composed of electrolytes, nitrogenous compounds, vitamins, hormones, organic acids, and miscellaneous organic compounds [128]. In addition, urine contains metabolites of any xenobiotic molecule coming from different sources including drugs and pollutants [111]. In order to ensure the removal of interferences, the following steps which include sample pretreatment and sample clean up were implemented. The general scheme of the method was adopted by Centers for Disease Control and Prevention (CDC) with slight modifications [129]. The systematic profiling of the urine sample pretreatment is shown in Table 1.

Table 1 : Urine pretreatment..

Dilution factor (volume of urine: volume of sodium acetate buffer)	Buffer concentration (M)	Buffer pH	Efficiency
(2:2)	1	5	No
(2:2)	0.5	5	No
(2:2)	0.05	5	No
(1:0.5)	1	5	No
(1:0.5)	0.5	5	No
(1:0.5)*	0.05	5	Yes

**The highlights in red row indicates the best outcomes*

b. Urine clean up

Urine is a biological matrix known to have interfering components like metabolites of different xenobiotic, decomposition products, and other contaminants that might suppress or enhance the peak analytes [130] .Several extraction protocols are recommended for urinary OHPAH mainly solid phase cleanup and liquid-liquid extraction (LLE) [129, 131]. In this study, both cleanup methods were optimized to compare the efficiency as well as the recovery of each protocol.

i. Solid Phase Extraction (SPE):

The percent of the organic modifier and the pH of the solution were varied in order to determine the optimal extraction conditions of the analytes. Considering the relatively non-polar nature of OH-PAHs compared to other substances present in urine, a C18 silica based SPE (ENVI-C18) cartridge was selected for isolation followed by pre-concentration of these compounds. The optimized SPE clean-up protocol is reported in Table 2.

Table 2: SPE Cleanup workflow.

Trials	Condition	Equilibrate	Load	Wash 1	Wash 2	Elute	Wash/Elute Profile
1	3ml Methanol	3ml sodium acetate buffer	0.5ml urine sample	3ml sodium acetate buffer	ACN: water (1:1) 5ml	6ml ACN	2-OHNAP, 2-OHFLU, and 3-OHPHEN eluted in wash 2 1-OHPYR eluted in ACN All analytes were eluted in ACN fraction
2	3ml Methanol	3ml sodium acetate buffer	0.5ml urine sample	3ml sodium acetate buffer	ACN: water (1:2) 5ml	6ml ACN	
3	3ml Methanol	3ml sodium acetate buffer	0.5ml urine sample	3ml sodium acetate buffer	ACN: water (1:4) 5ml	6ml ACN	
4*	3ml Methanol	3ml sodium acetate	0.5ml urine	3ml sodium	ACN: water	6ml ACN	

	buffer	sample	acetate buffer	(1:5) 6ml

**The highlights in red row indicates the best outcomes of the SPE procedure of the urine samples*

ii. Liquid-Liquid Extraction (LLE):

In the optimization of LLE, hexane, ethyl acetate, toluene and different ratios of toluene hexane were tested. Samples were extracted two times with 8ml aliquots of solvent, the organic extracts were combined, and then evaporated under gentle stream of nitrogen at room temperature. The optimized LLE protocol is reported in Table 3.

After each addition of organic solvent, the samples were placed on a shaker at 250 rotations per minute (rpm) to ensure mixing, which was followed by centrifugation at 3000 rpm for 30 min to get a clear separation of the immiscible solvents. The organic layer was collected from each extraction, and then combined. In both SPE and LLE, the final solvent containing the analytes of interest was dried completely in a nitrogen sample concentrator, and then reconstituted by adding 150µl of acetonitrile.

Table 3: LLE protocol workflow.

Solvent	Buffer Ph	Buffer strength	Number of extraction	Outcomes
Toluene	-	-	2x	Low analytes recovery
Ethyl acetate	-	-	2x	
Toluene: Hexane (1:1) (2:8)	-	-	2x	
Hexane*	-	-	2x	High analytes recovery
	8	-	2x	Low analytes recovery
	7	-	2x	

	6	-	2x	
	5	-	2x	High analytes recovery
		1M	2x	High recovery of napthol and fluorenol only
		0.5M	2x	
		0.05M	2x	High analytes recovery
			3x 4x	Recovery increased by less than 5%

**The highlights in red row indicates the best outcomes of the LLE procedure of the urine sample*

3. Optimization of extraction protocol

Two different analytical approaches were tested for the isolation of OH-PAHs from urine. With regard to unsatisfactory results obtained by SPE with C18 sorbent, mainly low method recovery and poor purification of extract since SPE cartridges are made up of polypropylene material, interfering peaks, probably from polypropylene plastic housing and polyethylene frits [127] appeared in place of the chromatographic peaks of all OHPAHs under study, therefore, we decided to isolate the target compounds by LLE using n-Hexane as a medium to which they are to be transferred.

In LLE, highest recoveries were obtained using n-hexane for, 2-OHNAP, 2-OHFLU, 3-OHPHEN and 1-OHPYR, as it gave much cleaner chromatographic graphs, has acceptable recovery for all the OH-PAHs of interest, and was quickly evaporated by nitrogen evaporation due to its high volatility.

4. The influence of pH

The pH of the medium is one of the important variables for solvent extraction since the analytes should not be ionized to anion species that can be generated at higher pH values which in return reduces extraction efficiency [132]. The influence of the pH on the extraction recoveries for analytes was assessed in the range of pH 5-8. As shown in Table 3, the relatively high extraction recoveries were obtained at pH 5. As a result, sodium acetate buffer of pH 5 solution was selected.

5. Analytical method development

Several analytical methods were used for the determination and quantification of OHPAHs in urine samples using chromatographic techniques such as gas chromatography coupled with mass spectrometry (GC-MS) [129], high performance liquid chromatography coupled with diode array detector and mass spectrometry (HPLC-DAD-MS)[133], and high performance liquid chromatography coupled with fluorescence detector (HPLC-FLD) [134].

a. <u>HPLC-FLD</u>

Assays were performed using HPLC system equipped with a fluorescence detector. Detection programs (excitation wavelengths [nm]/emission wavelength [nm]) for each compounds were as follows: 2-OHNAP, 2-OHFLU, and 3-OHPHEN 227 nm/355 nm; 1-OHPYR, 242 nm/388 nm [134]. The following HPLC conditions were used: (A) water (0.1% formic and acetonitrile (B). The elution was performed as follows: 30-min equilibration, followed by an isocratic elution (A) 50% and (B) 50% for 15 min. Flow rate

was set at 0.6 ml/min; column was a C18 column (4.6 X 150 mm, 3.5 µm); and the column temperature was kept at 35°C [134].

In principle, fluorescence is known to be 100-1000 times more sensitive than other absorption spectrometric detectors [135]. Further, using fluorescence, each analyte has specific excitation and emission wavelength which enhance the quantification of OHPAHs along with decreasing the probability of chromatographic interferences.

6. Extraction method development

The problem we had to overcome was the practical unavailability of a truly blank matrix. Given that the human body is exposed to PAHs practically throughout life (inhalation of polluted air or cigarette smoke, ingestion of contaminated food, dermal absorption) [41], traces of OH-PAHs and their glucuronides/ sulphates are unavoidably present in any urine sample [41]. The only way to assess the performance characteristics of both methods during optimization was to spike urine with a deuterated analogue (D9-OHPYR) prior to the hydrolysis. For the most effective enzymatic de-conjugation step, 10, 20 or 50µl of β- glucuronidase was added to the urine sample (pH 5, 37 °C, 16h). Comparable amounts of OH-PAHs were released (10, 20 and 50µl) for both sample preparations. For this reason, for the final validation experiments and examination of real samples, 10µl of β-glucuronidase was adopted [129].

Urine samples (1ml) were thawed at room temperature and diluted with 0.5ml of 0.05M sodium acetate buffer and then adjusted to pH 5.0 with acetic acid or ammonium acetate, fortified with the internal standard (d9-OHPYR) at 2 µg/l, and 10µl of glucuronidase/arylsulfatase enzyme was added to the samples, and the mixture was

incubated at 37ºC for 16h in order to de-conjugate OHPAH from attached macromolecules [129].

The analytes of interest were purified by extraction with 8ml of hexane. After the addition of hexane, the samples were mixed for 10 min (20 rpm) on the shaker, and then centrifuged for 30 min at 3000 rpm. The extraction procedure was repeated again. The combined hexane extracts (16ml) were concentrated to dryness under a gentle stream of nitrogen (lower than 5 psi) and reconstituted with 150µl acetonitrile for instrumental analysis.

D- Determination of creatinine

The urine creatinine values were used to normalize the urine concentration in individual samples, thus ensuring improved data comparability and adjusting for variable dilutions among participants [126]. Samples with excessive physiological dilution or concentration outside the range of 30 mg/dl ≤ creatinine ≤ 300 mg/dl were excluded according to WHO recommendations [126, 127]. A calorimetric method for the estimation of urinary creatinine was based on Jaffé's reaction [136], where a colored complex of creatinine with alkaline picrate is formed. The measurements were done based on a calorimetric creatinine assay kit. The reaction mixture consisted of 100 mg/dl creatinine standard diluted with double distilled water to reach the following calibration curve concentrations: 0.3126, 0.625, 1.25, 2.5, 5, 10, and 20 mg/dl together with 50µl of creatinine detection reagent that were transferred directly into a 96 well of a microliter plate. Jaffé s reaction was carried out for 30 min at room temperature, and then the

absorbance was measured using microplate reader at 490 nm. The same reaction was applied to urine samples that were diluted 20 times with double distilled water.

E- Validation of the HPLC-FLD method for 2-OHNAP, 2-OHFLU, 3-OHPHEN and 1-OHPYR

The optimized analytical method was assessed for its process efficiency by determining the following parameters that are set by the food and drug administration (FDA) for bioanalytical method development that includes extracted calibration curve, limit of detection, limit of quantification, selectivity, accuracy, precision, stability , and recovery [130].

1. Extracted calibration curve

A calibration curve is a relationship between the response of an instrument and known prepared analyte concentrations [132]. It undergoes the same sample pretreatment and sample clean up protocols using pooled urine matrix from healthy volunteers to mimic real samples situation [130]. Each analyte of interest has its own extracted calibration curve by spiking the pooled urine matrix with known standard and internal standard concentrations in the expected range of the intended study [130]. A calibration curve is made up of a blank sample (matrix sample prepared without internal standard), a zero sample (matrix sample prepared with internal standard), and five to seven non-zero samples in the expected range of the study of interest [130]. The range of the extracted calibration curve is 0.1-40 µg/l for 2-OHNAP (0.1,1,5,10,20,30,40 µg/l) and 0.5-20 µg/l (0.5,1,2,5,10,16,20 µg/l) for 2-OHFLU, 3-OHPHEN, and 1-OHPYR.

Limit of detection (LOD) is the lowest concentration of the analyte that can be detected but not necessary quantified [132]. It is calculated based on the standard deviation and the analytes slope [132]. It is expressed as the following: LOD=3σ/s, where σ is the standard deviation of ten replicates of blank urine sample injections and s is the slope of the extracted calibration curve [132]. Limit of quantification (LOQ) is the smallest amount that can be measured with reasonable accuracy [132]. It is expressed as the following: LOQ=10σ/s, where σ is the standard deviation of a low-concentration sample and s is the slope of the calibration curve [132]. Both LOD and LOQ are shown in the Table 4.

2. Selectivity

Selectivity is completed to ensure that there are no interferences at the retention time of the analytes of interest [130]. It is determined by checking the urine of six different individuals for interfences [130]. After checking the blank urine for the six different individuals, the optimized method was selective, no interference was shown at the retention time for the analyte of interest except for 2-OHNAP and it was minimal compared to the LOQ.

3. Accuracy

According to FDA, the accuracy of any analytical method illustrates the proximity of the mean values measured by the optimized sample preparation method to a nominal true value [130]. It is calculated by dividing measured concentrations of the target analytes

by the spiked concentration of five sample replicates of each low, medium, and high concentrations [137].

A good accuracy indication is between 80%-120% [130]

The low concentration for 2-OHNAP, 2-OHFLU,3-OHPHEN, and 1-OHPYR is 5 µg/l. The medium concentration for 2-OHNAP is 20 µg/l, and it is 10 µg/l for 2-OHFLU, 3-OHPHEN, and 1-OHPYR. The high concentration for 2-OHNAP is 40 µg/l, and it is 16 µg/l for 2-OHFLU, 3-OHPHEN, and 1-OHPYR.

4. Precision

According to FDA, the precision of any analytical method illustrates the closeness of the mean values measured when the same optimized sample preparation procedure is tested and repeated multiple times(intra- and inter-days) [130]. It is calculated by determining the relative standard deviation (RSD) for pre-spiked and extracted five sample replicates of each low, medium, and high concentrations that are done during the same day (intra-day) and for three consecutive days (inter-day) [130]. The recommended RSD should be less than 20% [130] (Table 4).

5. Stability

The stability of the optimized analytical method can be evaluated by storing three sample replicates of low and high concentrations at - 20°C for 24h for three freeze and thaw cycles [130]. Stability was validated by comparing the concentrations in each sample stored at −20 °C with the concentrations in the corresponding sample that had undergone three

cycles of freezing and thawing [138]. Recommended RSD should be less than 20%, and our results were within the recommended levels [130].

6. Matrix effect experiment

It is important to assess the recovery and the matrix effect in the following experimental procedure, which consists of three sets [139].

Set 1. Pre-spiking: five replicates of each low, medium, and high concentrations were prepared in 1ml of urine buffered with 0.5ml of sodium acetate buffer that are placed inside 12ml glass centrifuge tube followed by spiking of standard and internal standard. After the addition of the 8ml of hexane, mixing is ensured at 250 rpm for 10 min, and extraction of the analytes is performed during centrifugation at 3000 rpm for 30 min. The extraction process is repeated twice followed by complete nitrogen evaporation, and reconstitution using 150µl of acetonitrile.

Set 2. Post-spiking: five replicates of each low, medium, and high concentrations were prepared in 1ml of urine buffered with 0.5ml of sodium acetate buffer that are placed inside 12ml glass centrifuge tube followed by the addition of the 8ml of hexane, then mixing is ensured at 250 rpm for 10 min, and extraction of the analytes is performed during centrifugation at 3000 rpm for 30 min. After which the extraction process is repeated twice, the organic layer is combined and spiked with standards, and internal standards. The final step includes complete nitrogen evaporation, and reconstitution using 150µl of acetonitrile

Set 3. Neat standard: five replicates of each low, medium, and high concentrations were prepared in 150µl of ACN by spiking of standard and internal standard solutions. Evaluation of the matrix effect:

Set 1/Set 2= LLE recovery

Set2/Set3= matrix effect on HPLC-FLD

According to FDA guidelines, a good clean up recovery is between 80%-120%. For the matrix effect, a value of >100% indicates intensity enhancement, and a value of <100% indicates intensity suppression. Validation results are shown in Table 4.

Table 4: Validation results of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR.

Metabolite	2-OHNAP	2-OHFLU	3-OHPHEN	1-OHPYR
Limit of detection (LOD) µg/l	0.09	0.20	0.09	0.06
Limit of quantification (LOQ) µg/l	0.29	0.67	0.31	0.20
QC sample conc. µg/l			Intraday	
			% Accuracy	
Low	90.75	61.07	94.41	82.94
Medium	83.65	55.89	89.55	87.42
High	94.98	71.85	93.57	86.54
			% Precision	
Low	3.66	6.32	5.31	8.27
Medium	4.14	3.01	5.88	4.74
High	2.78	4.46	4.49	3.80
QC sample conc. µg/l			Inter-day	
			% Precision	
Low	8.50	14.40	8.92	10.84
Medium	11.12	9.48	7.33	10.52
High	10.80	9.09	7.90	8.26
% Matrix effect				
Low	90.60	63.89	92.33	63.70
Medium	83.34	61.92	89.91	64.90
High	80.30	62.64	85.03	94.04
% LLE recovery				
Low	100.16	89.06	91.85	119.30
Medium	84.41	82.13	88.52	96.40
High	93.49	95.91	95.33	92.02

F- Statistical Analysis

Differences in participant characteristics by CAD status were examined using chi-square tests for categorical variables and t-tests for continuous variables. The prevalence (N, %) was presented for categorical variables, while the mean ± standard deviation (SD) was presented for continuous variables. Logistic regressions were performed to study the association between exposure to PAH and CAD; which was considered as a predictor, adjusting for age, sex, and classical risk factors for CVD such as diabetes, and dyslipidemia. Correlation analysis between pairwise PAH were conducted and Spearman's correlation coefficients were calculated. Univariate and multivariate analysis were evaluated and corrected odds ratios and their 95% confidence intervals were reported. A p value less than 0.05 was used to indicate significance. Data were analyzed using the SPSS version 23.0.

CHAPTER III

LEVELS OF OHPAHS IN LEBANESE COHORT

A- Concentrations of OHPAHs

The arithmetic mean, median, standard deviation and minimum and maximum value of urinary concentrations of 2-OHNAP, 2-OHFLU,3-OHPHEN and 1-OHPYR are presented in Tables 5 and 6 as µg/g creatinine and as µg/l in Table 7 for all subjects (n=258). Data is further divided according to their smoking status, age, gender, and CAD status. Among the four urinary metabolites analyzed, 2-OHNAP, and 2-OHFLU, were detected in 92.2% of the total urine samples. However, 1-OHPYR and 3-OHPHEN were detected in 80.23 % and 22.09%, respectively. In this study, the mean concentrations of the four urinary OHPAHs among patients who were admitted to do cardiac catheterization were in decreasing order, 2-OHNAP> 2-OHFLU>1-OHPYR> and 3-OHPHEN (Table 5).

Table 5: Concentrations of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR in this study (n=258) categorized based on the smoking habits.

µg/g	2-OHNAP	2-OHFLU	3-OHPHEN	1-OHPYR
All subjects n 258	238	238	57	207
Mean (SD)	23.69 ± 67.84	20.89 ± 38.29	1.35 ± 1.49	2.04 ± 3.09
Median	7.54	10.85	1.00	1.36
Min-Max	0.255-665.53	1.53-368.32	0.20-10.67	0.16-35.20
Not current smoker n.167	149	150	25	128
Mean (SD)	21.19 ± 63.46	15.76 ± 32.48	1.47 ± 2.17	2.01 ± 3.44
Median	5.13	8.97	0.77	1.33
Current smoker n. 84	82	81	30	74
Mean (SD)	29.49 ± 77.68	30.57 ± 47.01	1.28 ± 0.58	2.14 ± 2.51
Median	12.36	17.44	1.27	1.40
p value	0.382	**0.01**	0.64	0.77
Never smoker n.153	137	138	22	119
Mean (SD)	22.55 ± 66.01	16.46 ± 33.73	1.34 ± 2.17	2.05 ± 3.56
Median	5.40	9.22	0.72	1.33
Ever smoker n. 98	94	93	33	83
Mean (SD)	26.45 ± 72.96	27.64 ± 44.57	1.38 ± 0.86	2.06 ± 2.39
Median	10.84	15.29	1.30	1.43
p value	0.67	0.04	0.91	0.98

Although the difference in the OHPAH concentrations relative to the gender was not significant, it is worth mentioning that females exhibited higher mean concentrations of 2-OHFLU, 3-OHPHEN, and 1-OHPYR while, males showed higher concentrations of 2-OHNAP (Table 6). When divided based on age groups, it was noted that the younger age group (40-60) had higher concentrations of 2-OHNAP, 2-OHFLU, and 3-OHPHEN than other age groups (61-70 and 71-90) with the difference between these age groups being not significant (Table 6).

Table 6: Concentrations of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR in this study categorized based on the demographics (age, and gender).

µg/g	2-OHNAP	2-OHFLU	3-OHPHEN	1-OHPYR
Demographics of the samples				
Age				
Mean (SD)				
40-60 n. 110	30.91 ± 76.17	25.84 ± 45.83	1.51 ± 1.85	2.18 ± 4.15
61-70 n. 83	22.58 ± 77.44	19.23 ± 37.71	1.06 ± 0.47	2.24 ± 2.58
71-90 n. 65	12.16 ± 20.76	14.74 ± 20.24	1.44 ± 1.56	1.52 ± 0.86
Median				
40-60	8.81	12.45	1.03	1.33
61-70	6.66	10.37	1.16	1.43
71-90	5.12	9.20	0.66	1.20
P value	0.24	0.18	0.59	0.38
Gender				
Mean (SD)				
Female n. 55	22.66 ± 46.83	22.08 ± 47.70	1.62 ± 2.57	2.40 ± 2.76
Male n. 203	23.98 ± 72.85	20.56 ± 35.36	1.26 ± 0.86	1.94 ± 3.18
Median				
Female	5.96	9.73	0.77	1.57
Male	7.75	11.18	1.08	1.28
P value	0.90	0.80	0.60	0.38

B- Comparison with other cities

The median levels determined in this study were compared to concentrations in various cities around the world as shown in Figure 3. It was found that the median concentrations of 2-OHNAP (5.64 µg/l), 2-OHFLU (8.35 µg/l), 3-OHPHEN (0.70 µg/l) and 1-OHPYR (1.04 µg/l) were higher than what was reported in the National Health and Nutrition Examination Survey (NHANES) in USA [140] (2.28 µg/l, 0.215 µg/l, 0.105 µg/l, 0.092 µg/l) and Kuala Lumpur, Malaysia (1.55 µg/l, 0.112 µg/l, 0.043 µg/l, 0.065 µg/l) [141], but comparable to that in Guangzhou city, China 2 (3.3 µg/l, 9.5 µg/l, 1.5 µg/l)

[134]. The measured median concentrations of 2-OHNAP were found to be lower than that reported in Al-Asma/Al-Jahra governorates, Kuwait (7.33 µg/l) [141].

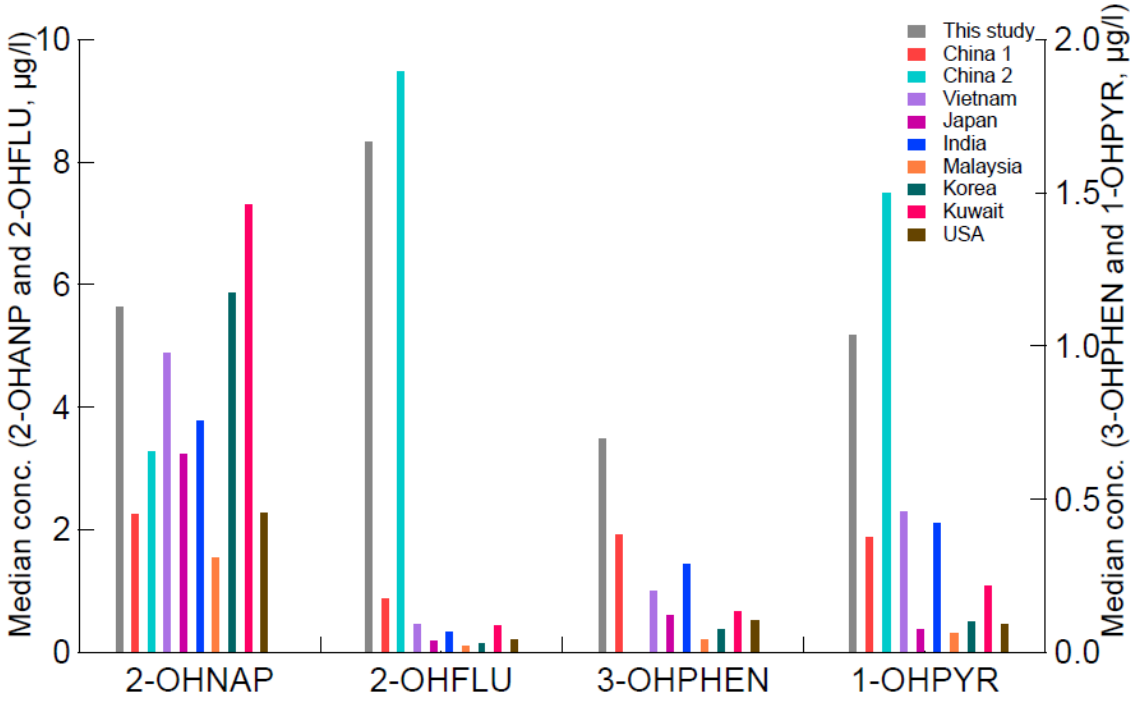

Figure 3: Concentrations of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR in urine from this study and general populations from around the world (median µg/l). China (1): n=84 Guangzhou/Shanghai/Harbin cities; Vietnam: n=23 Hanoi city; Japan: n=34 Ehime/Kumamoto cities; India: n=38 Mettupalayam/Chennai cities; Malaysia: n=29 Kuala Lumpur city; Korea: n=60 Seoul/Busan/Yeosu; Kuwait: n=38 Al-Asma/Al-Jahra governorates [141]; US: n=2500 NHANES [140]; China (2): n=51 school volunteers from Guangzhou city [134].

C- Comparison between smokers and non-smokers (current and not current smokers)

The mean levels of 2-OHNAP, 2-OHFLU, and 1-OHPYR in smokers were higher than non-smokers except for 3-OHPHEN (Table 5). These findings are in agreement with

other published studies [111, 142]. The difference between the smokers and non-smokers was statistically significant for only 2-OHFLU (p= 0.01). Compared to smokers and non-smokers in other cities, the levels of 3-OHPHEN were slightly higher by 1.2 and 1.4 folds, respectively [143]. Also, the concentrations of 2-OHFLU (smokers ;14.17 µg/l and non-smokers;5.57 µg/l) and 1-OHPYR (smokers; 1.29 µg/l and non-smokers;0.97 µg/l) in smokers and non-smokers determined in this study, exhibited higher levels when compared to individuals with similar smoking status in the US (smokers; 0.42 µg/l and non-smokers; 0.04 µg/l) and asphalt workers in Milan and Lodi provinces in Italy (smokers; 0.64 µg/l and non-smokers; 0.44 µg/l) as shown in Figures 4 and 5 [143, 144]. However, the measured concentrations of 2-OHNAP (smokers; 11.09 µg/l, non-smokers; 3.64 µg/l) were lower than what was reported for asphalt workers in Milan and Lodi provinces in Italy (smokers; 16.50 µg/l and non-smokers; 8.00 µg/l)[143].

Most importantly, the measured levels of non-smoker participants showed levels of OHPAHs in urine higher than the levels of smokers in some other cities [143]. Example is the median levels of 2-OHFLU (5.57 µg/l), 3-OHPHEN (0.64 µg/l), and 1-OHPYR (0.97 µg/l) of non-smokers that were measured in this study and were found to be up to 2.8, 1.8, and 2.3 times higher than what was reported for the urine samples of the smokers in the US sample population (2.03 ,0.348, and 0.425 µg/l), respectively [144]. Moreover, the measured levels of OH-PAHs in non-smokers were slightly higher than the levels measured for occupational workers in asphalt mixing and paving [143], but lower by several folds than the levels that were measured in another occupational exposure setting like the workers in Polish coke oven plants (Figure 6) [145].

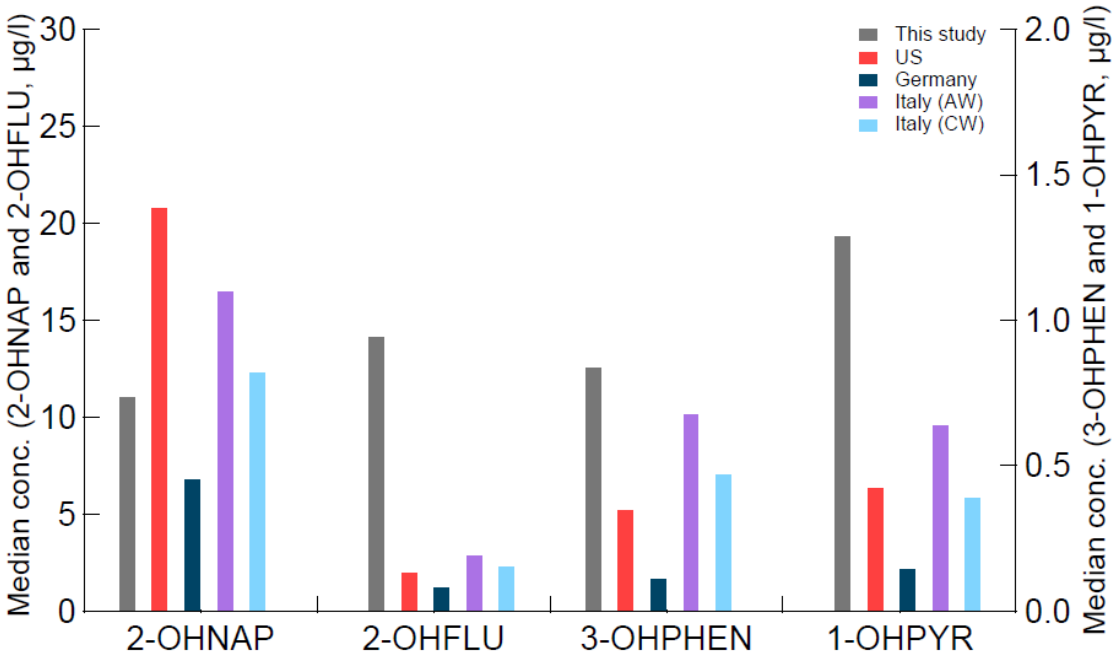

Figure 4: Concentrations of the four different OHPAHs in smokers from different countries. This study: n=84 ,US: n=30 smokers purchased from Bioreclamation[144] , Germany: n=100 smoker adults [146] ,Italy asphalt workers (AW): n=43 and Italy construction workers (CW): n=12 [143].

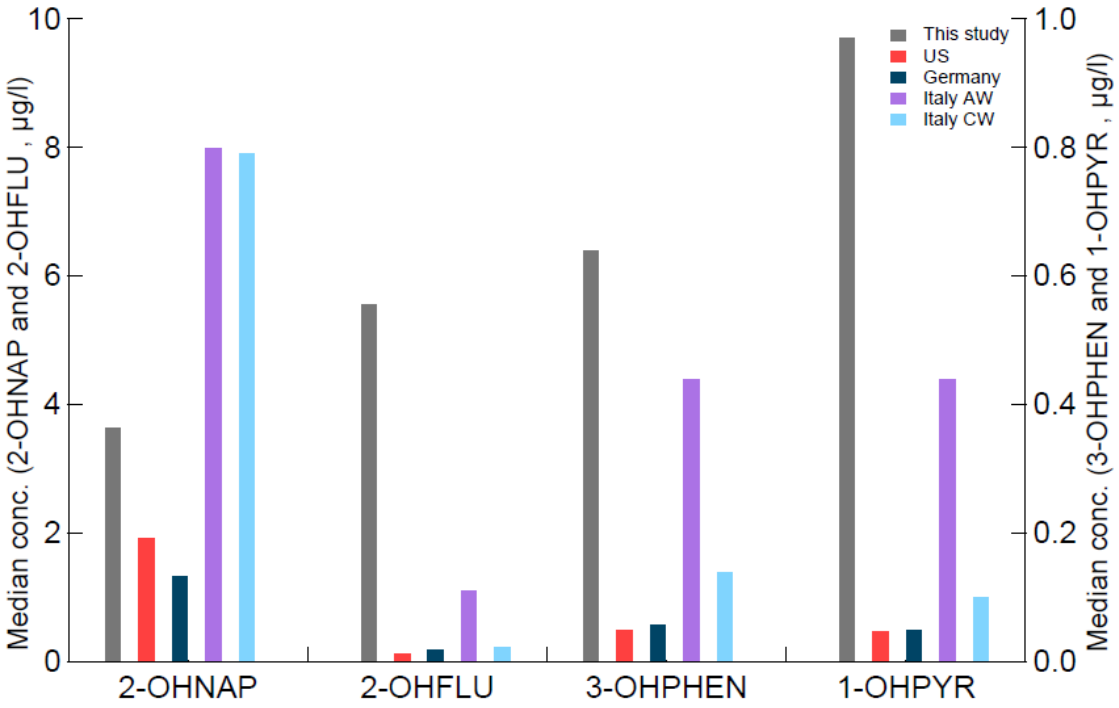

Figure 5 : Concentrations of the four different OHPAHs in nonsmokers from different countries. This study: n=167, US: n=30 adult volunteers at CDC [144], Germany: n=50 adults [146], Italy asphalt workers (AW): n=32, and Italy construction workers (CW): n=25 [143].

Table 7 : Concentrations of 2-OHNAP, 2-OHFLU, 3-OHPHEN, and 1-OHPYR in this study (n=258) categorized based on the smoking habits.

µg/l	2-OHNAP	2-OHFLU	3-OHPHEN	1-OHPYR
All subjects n 258				
Mean (SD)	17.41 ± 36.46	18.21 ± 31.22	1.07 ± 0.92	1.60 ± 2.16
Median	5.64	8.35	0.70	1.04
Min-Max	0.37-256.77	1.01-298.19	0.45-3.96	0.40-25.18
Not current smoker n.167				
Mean	16.03	13.27	0.91	1.46
Median	3.64	5.57	0.64	0.97
Current smoker n. 84				
Mean	20.71	26.62	1.22	1.87
Median	11.09	14.17	0.84	1.29
p value	0.35	**0.00**	0.22	0.19

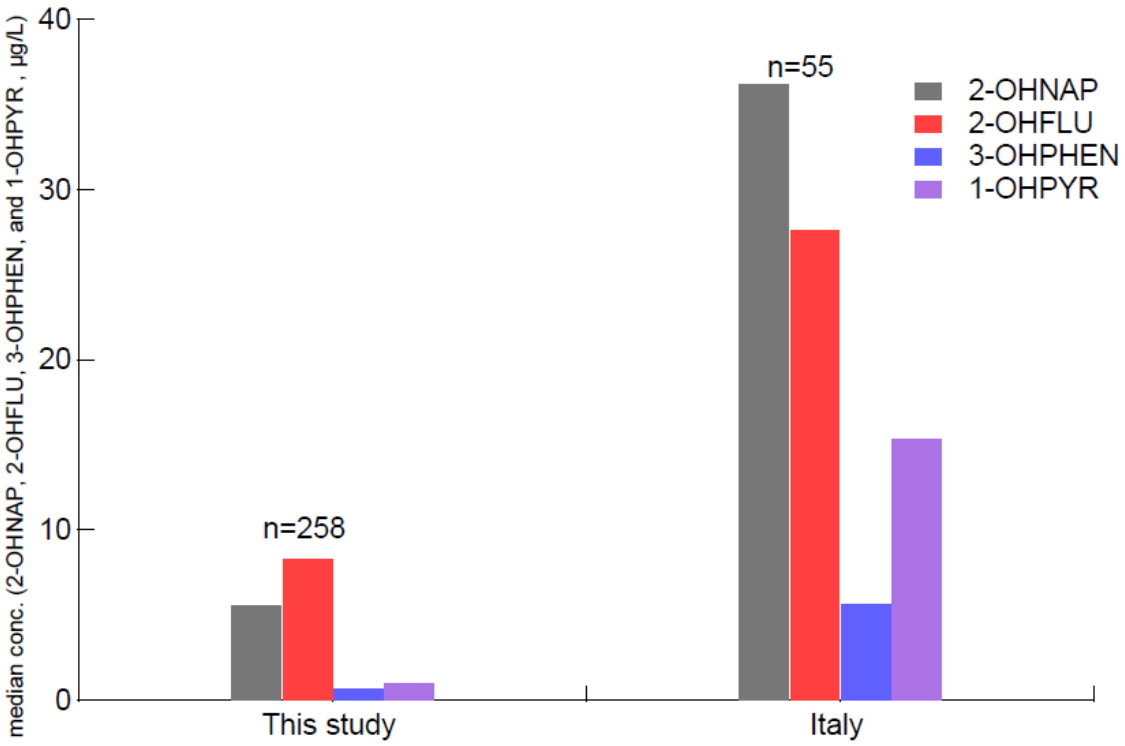

Figure 6 : Comparison of 2-OHNAP, 2-OHFLU,3-OHPHEN and 1-OHPYR in this study to occupational exposure in Italy (Polish coke oven workers)[145].

D- Spearman correlations

Spearman correlations between pair of analytes were calculated. Log-2-OHNAP correlated with Log-2-OHFLU(R=0.18) and Log-1-OHPYR (R=0.18), and Log-1-OHPYR correlated with Log-2-OHFLU(R=0.27) and Log-3-OHPHEN (R=0.31) as shown in Table 8.

Table 8 : Spearman correlations between pairwise OH-PAHs.

	Log-2 OHNAP	Log-2-OHFLU	Log-3-OHPHEN	Log-1-OHPYR
Log-2-OHNAP	1	**0.18**	0.14	**0.18**
Log-2-OHFLU	-	1	0.22	**0.27**
Log-3-OHPHEN	-	-	1	**0.31**
Log-1-OHPYR	-	-	-	1

Bold if $p < 0.05$

E- Discussion

The concentrations of 2-OHFLU,3-OHPHEN, and 1-OHPYR that were reported for individuals in cities like Guangzhou, Hanoi, Kuala Lumpur, and Seoul were lower than the levels that were determined for the admitted CAD sample population represented in this study [141]. Moreover, OHPAH concentrations measured for the non-smoker samples were found to be higher than the levels that are reported for smokers and some occupational workers [144, 145]. This can be attributed to several factors including environmental PAH exposure [147], lifestyle like diet [111, 148], and the individual characteristics that are linked to metabolism [148].

It is well established that the non-volatile PAHs are major components of ambient PM [30]. PMs have been shown to exceed WHO guidelines in the greater Beirut area by at least 100% for $PM_{2.5}$ and 273% for PM_{10} [11]. Accordingly, high PAH exposure would be directly proportional to high levels of OHPAH in the body [147, 149]. The particle PM_{10} bound PAH levels that were determined in three cities along the coast of Lebanon were found to be similar to what was reported in Ningbo, China (46.0 ng/m^3) and Korea (89.3 ng/m^3) [81, 150] and higher than most cities in Europe and the US [151, 152]. The sources causing high PAH concentrations in the air were attributed to heavy and old traffic [153, 154], diesel generators [155], open burning, and second hand smoking [111].

The Lebanese car fleet between 2005 and 2015 registered a high number of light duty vehicles (LDVs) contributing to 88 % of the total car fleet [156, 157]. Reported data showed not only a two-time increase in the vehicle number between 2005 and 2015 but an increase in the average age reaching 19 years in 2015 as well [157]. The linear relationship that is established between mileage and car emission indicates that an increase in the car age will cause the deterioration of the car engine and as such the increase in the release of toxic emissions including hydrocarbons such as PAHs [158, 159].

Alarmingly, since the end of the civil war in 1990, Lebanon has witnessed a surge in the use of diesel generators as a primary backup power supply during power outage. These measures have contributed to an elevated PAH background levels reaching about 40% and an estimated $PM_{2.5}$ emissions per capita from diesel generators that is 5.3 times higher than what is calculated for Delhi [82, 157, 160]. As such, the Lebanese people are exposed to continuous emissions of diesel particles which are expected to contribute majorly to urinary OHPAHs. In fact, the analysis of urinary metabolites post exposure to diesel engine aerosols revealed an increase of 72 and 44 folds in naphthalene and phenanthrene, respectively when compared to the pre-exposure concentration [155, 161]. High levels of phenanthrene (67.95 ng/m^3) and pyrene (22.07 ng/m^3) followed by fluorene (7.04 ng/m^3) and naphthalene (6.25 ng/m^3) [116, 162] that were measured in the smoke of the diesel generator in Henan Province (China) indicate that 3-OHPHEN and 1-OHPYR can be considered a specific biomarker for this diesel emissions especially for exposed non-smokers [162-164].

Additional exposure originates from the open waste burning that has become a common practice in rural and urban areas in the wake of the solid waste management crisis

in 2015 [165]. Measurements of airborne PAHs in residential sites undergoing repeated waste burning incidents showed a 218% increase when compared with PAH levels measured after a rainy period with no waste burning activities [165]. Also, in this case both phenanthrene and pyrene are considered good indicators of incineration as well for diesel generators [118, 166].

PAHs are also important components of cigarette and water pipe tobacco smoke [42, 167]. So, in the absence of the law enforcement of banning smoking indoors, and due to the prevalence of cigarette and water pipe smoking in Lebanon, second hand smoking is expected to add to the levels of PAHs in ambient indoor environment and as such increase the probability of PAH exposure [168, 169].

In this study, females had higher concentrations of 2-OHFLU, 3-OHPHEN and 1-OHPYR than males while, males had higher concentrations of 2-OHNAP. Several studies attribute this difference in metabolism to gender [148].

Correlations using the spearmen's method were calculated between pairwise OHPAHs. It was determined that log-1-OHPYR showed a positive and significant correlations with log-2-OHNAP, log-2-OHFLU and log-3-OHPHEN and log-2-OHNAP correlated with log-2-OHFLU (Table 8). Moderate correlation coefficients that ranged between 0.18-0.31 were determined. Other studies showed a moderate and strong correlations between pairwise OHPAH that ranged between 0.37-0.88 [83]. The variations in the results of this study compared to NHANES (n=3283), which is representative of the US general population [40], could be attributed to the small sample size (n=258)

In brief, OHPAH in urine represent recent exposure to any PAH mixture due to their short half-life that can range from few hours to one day depending on the metabolic

variations, and the lifestyle among different people [96, 170]. Due to the high prevalence of PAHs in ambient air originating from continuous emissions from cars, diesel generators and open waste burning, it is most likely that these emissions have contributed to the high levels of urinary PAH metabolites in the patient samples that were evaluated in this study.

CHAPTER IV

ASSOCIATION BETWEEN OHPAH AND CAD

Using SPSS, four urinary hydroxylated PAH metabolites were analyzed in a sample size of 258 participants who are more than 40 years of age. Regarding the dependent variable which is the CAD status, 88 participants were identified as non-obstructive CAD and 170 as obstructive CAD.

A- Distribution of selected demographic characteristics by CAD status.

Demographic factors distribution divided by non-obstructive and obstructive CAD status are presented in Table 9. Participants who were in the obstructive CAD group are more likely than those in the non-obstructive CAD group to be older in age ($p = 0.02$), male (86.5% vs. 63.6%, $p < 0.0001$), diabetic (52.4% vs. 29.5%, $p < 0.001$), and having dyslipidemia (74.7% vs. 54.5%, $p = 0.001$). However, no significant difference was observed regarding the smoking and hypertension factors between the non-obstructive and obstructive CAD groups.

Table 9 : Distribution of selected demographic characteristics by CAD status.

Demographics	All	Non-obstructive CAD	Obstructive CAD	*p* Value
Sample	258	88 (34.10)	170 (65.90)	
Age	63.37 ± 9.63	61.45 ±10.30	64.36 ± 9.15	0.02
Gender				
Female	55 (21.30)	32 (36.40)	23 (13.50)	0.00
Male	203 (78.70)	56 (63.60)	147 (86.50)	
Smoking habits				0.48
Never	153 (61.00)	55 (64.00)	98 (59.40)	
Ever	98 (39.00)	31 (36.00)	67 (40.60)	
Hypertension				0.48
Yes	183 (70.90)	60(68.20)	123 (72.40)	
No	75 (29.10)	28 (31.80)	47 (27.60)	
Diabetes				0.00
Yes	115 (44.60)	26 (29.50)	89 (52.40)	
No	143 (55.50)	62 (70.50)	81 (47.60)	
Dyslipidemia				0.00
Yes	175 (67.80)	48 (54.50)	127 (74.70)	
No	83 (32.20)	40 (45.50)	43 (25.30)	

Values are n (%) or mean ± SD.

Mean concentrations of the four measured urinary OHPAH analytes were compared between the non-obstructive and obstructive CAD groups as shown in Table 10. Non-obstructive CAD cases had higher 2-OHNAP and 3-OHPHEN concentrations. However, obstructive CAD cases had higher 2-OHFLU and 1-OHPYR concentrations. Only, 1-OHPYR was significantly different between the two groups (2.04 µg/g vs. 2.25 µg/g, p=0.05).

Table 10 : Difference of log-transformed and non-log transformed OHPAHs by CAD status.

	Non-Obstructive CAD N=88	Obstructive CAD N=170	P-value
2-OHNAP	24.80 ± 46.72	23.07 ± 77.42	0.85
Log-2-OHNAP	0.96 ± 0.61	0.83 ± 0.61	0.13
2-OHFLU	18.19 ± 22.77	22.22 ± 43.92	0.45
Log-2-OHFLU	1.04 ± 0.41	1.07 ± 0.44	0.59
3-OHPHEN	1.58 ± 2.09	1.21 ± 0.90	0.37
Log-3-OHPHEN	0.04 ± 0.33	-0.01 ± 0.28	0.58
1-OHPYR	1.60 ± 1.03	2.25 ± 3.67	**0.050**
Log-1-OHPYR	0.12 ± 0.27	0.18 ± 0.32	0.21

Values are mean ± SD.

The log-transformed urinary OHPAH will be used in spearman correlation and binary logistic regression since the data is not normally distributed and it's skewed.

B- Binary logistic regression models

The results of the correlation between OHPAH concentrations and CAD status from adjusted binary logistic regression models are tabulated in Table 11. In the unadjusted model, none of the analytes were significantly associated with obstructive CAD status. After adjusting for age and sex, it was found that 1-OHPYR was significantly associated with obstructive CAD cases (OR= 3.818, 95%CI: 1.203-12.119; p =0.02). Similarly, and by adjusting for additional covariates like diabetes, 1-OHPYR remained significantly associated with Obstructive CAD cases (OR= 4.150, 95%CI: 1.25-13.82; p =0.02). A marginally significant correlation between 1-OHPYR and CAD was noted after adjusting for age, sex, and dyslipidemia, and, after adjusting for age, sex, diabetes and dyslipidemia, where the p values were 0.07 and 0.08 respectively.

Table 11 : Logistic regression analysis to determine the predictors of CAD in the cohort.

OHPAH			p Value
Log-2-OHNAP	Unadjusted OR (95%CI)	0.713 (0.462-1.100)	0.12
	Adjusted OR [a] (95%CI)	0.732 (0.463-1.158)	0.18
	Adjusted OR [b] (95%CI)	0.850 (0.53-1.37)	0.50
	Adjusted OR [c] (95%CI)	0.740 (0.46-1.20)	0.22
	Adjusted OR [d] (95%CI)	0.835 (0.513-1.360)	0.46
Log-2-OHFLU	Unadjusted OR (95%CI)	1.193 (0.632-2.251)	0.58
	Adjusted OR [a] (95%CI)	1.367 (0.695-2.690)	0.36
	Adjusted OR [b] (95%CI)	1.320 (0.66-2.62)	0.43
	Adjusted OR [c] (95%CI)	1.080 (0.53-2.21)	0.83
	Adjusted OR [d] (95%CI)	1.080 (0.527-2.213)	0.83
Log-3-OHPHEN	Unadjusted OR (95%CI)	0.599 (0.101-3.562)	0.57
	Adjusted OR [a] (95%CI)	0.615 (0.101-3.763)	0.59
	Adjusted OR [b] (95%CI)	0.410 (0.06-2.66)	0.35
	Adjusted OR [c] (95%CI)	0.411 (0.063-2.662)	0.35
	Adjusted OR [d] (95%CI)	0.411 (0.063-2.662)	0.35
Log-1-OHPYR	Unadjusted OR (95%CI)	1.910 (0.701-5.204)	0.20
	Adjusted OR [a] (95%CI)	**3.818 (1.203-12.119)**	**0.02**
	Adjusted OR [b] (95%CI)	**4.150 (1.25-13.82)**	**0.02**
	Adjusted OR [c] (95%CI)	**2.938 (0.893-9.672)**	**0.07**
	Adjusted OR [d] (95%CI)	**2.992 (0.868-10.315)**	**0.08**

[a] adjusted for age and sex
[b] adjusted for age, sex, and diabetes
[c] adjusted for age, sex, dyslipidemia
[d] adjusted for Stepwise: age; sex (reference: female); hypertension; diabetes; dyslipidemia; smoker (reference: never)

C- Discussion

Binary logistic regression models showed that 1-OHPYR was significantly associated with obstructive CAD status after adjusting for covariates like age, sex, and diabetes that is considered a classical risk factor for CAD. This implies that a high risk of CAD is associated with high concentrations of 1-OHPYR. A similar study has shown that 1-OHPYR was predictive of SAA in coke oven workers having C-reactive protein as a marker for CVD [125]. 1-OHPYR is considered a significant tracer for total PAH exposure since it represents 90% of pyrene urinary excretion in humans [131]. It is set as a biomarker

for the occupational exposure to any mixtures containing PAH [171] by the American Conference of Governmental Industrial Hygienists (ACGIH) and is treated as a marker for diesel generator and incinerator emissions [162, 165]. The association between the CAD status and the second PAH marker; PHEN could not be established due to the limitation in the adopted analytical method to detect low 3-OHPHEN concentrations. Variations in the associations between CVD and specific PAH metabolites among countries is mainly attributed to the differences of the prevalent emission sources and consequently the individual PAH exposures [83].

Limitations to this study include: (1) the lack of self-reported data on the personal PAH exposure via the different routes (inhalation, dermal and diet) during the time of the sample collection. This information is crucial because OHPAHs in urine represent recent exposure to PAH mixtures with a short half-life ranging from hours to one day [41]. (2) This is a cross-sectional study that is limited in time, and (3) the selection criteria of the participants does not represent the general population but rather a subset of people who were admitted for cardiac catheterization.

Still important findings indicate that emissions from diesel generators and incinerations are shown to have contributed to high level of 1-OHPYR in urine samples even for the non-smokers patients and that the higher levels of 1-OHPYR are associated with a higher the risk of CAD after adjusting for confounders such as age, sex, and diabetes. Those important results call for immediate actions on reducing exposure to PAHs mainly originating from diesel generators and incineration by implementing proper policies.

CHAPTER V

CONCLUSION

Several OHPAHs that are commonly considered biological markers of air pollution, were measured in the urine of 258 patients who were admitted to do cardiac catheterization at AUBMC. Results showed that the levels of OHPAHs for smokers and non-smokers were higher than what was reported in urine samples of similar smoking status in other cities [141]. Most notably, OHPAHs concentrations that were measured for non-smokers in this study were higher than the levels that were reported for smokers and some occupational workers [144, 146] which implies that nonsmoker patients are highly exposed to sources of PAH mixtures. Such sources are assigned to old car fleet, diesel generators, incinerators and second hand smoking [81, 165]. In particular 1-OHPYR which is considered to be a specific marker for the diesel generator and incineration sources [116, 163] was best associated with the CAD burden with high levels of 1-OHPYR being linked with a higher the risk of CAD. Hence, it can be concluded that the exposure to PAHs from diesel generators and incinerators constitute a high risk for CVD [86].

The reported high levels of OHPAHs are concrete evidence of high exposure to PAHs and call for the need to implement strict regulations on the emissions of cars, diesel generators and uncontrolled open burning.

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