AMERICAN UNIVERSITY OF BEIRUT

EVALUATION OF AROMATIC HYDROCARBON RECEPTOR REPRESSOR (*AHRR*) METHYLATION STATUS AS A SENSITIVE BIOMARKER OF AMBIENT POLYAROMATIC HYDROCARBONS (PAHS) EXPOSURE AND A RISK FACTOR FOR CORONARY ARTERY DISEASE (CAD)

by
IBRAHIM ABDULRAHMAN RIDANY

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
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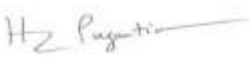

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ABSTRACT
OF THE THESIS OF

Ibrahim Abdulrahman Ridany for Master of Science
Major: Pharmacology and Toxicology

Title: Evaluation of Aromatic Hydrocarbon Receptor Repressor (*AHRR)* Methylation Status as A Sensitive Biomarker of Ambient Polyaromatic Hydrocarbons (PAHs) Exposure and A Risk Factor for Coronary Artery Disease (CAD)

Background Coronary artery disease (CAD) and morbidities from cigarette smoking and pollution are among the leading causes of deaths world-wide. CAD risk factors include pollution exposure and cigarette smoking. Cigarette smoke contains thousands of chemicals like polyaromatic hydrocarbons (PAHs). PAHs are also bound to particulate matter (PM), a major air pollutant, as a product of incomplete combustion. Studies have shown that methylation on the CpG site (cg05575921) in the aryl hydrocarbon receptor repressor (*AHRR*) gene is a marker of cigarette smoke exposure as it is hypomethylated among smokers and partially reversible after smoking cessation. Other studies associated this same *AHRR* CpG site hypomethylation in non-smoking subjects exposed to ambient pollution as measured based on geographic distribution. Furthermore, *AHRR* cg05575921is hypomethylated in CAD patients.

Aim The aim of this study is to evaluate *AHRR* CpG ROI (cg05575921) methylation status as a sensitive biomarker for ambient PAHs exposure and a risk factor for CAD.

Methods Whole peripheral blood and complete data of three urinary PAHs metabolites are available from subjects admitted to American University of Beirut Medical Center (AUBMC) in the Vascular Medicine Program (VMP) for cardiac catheterization, which is considered the gold standard for CAD diagnosis. In addition, data on smoking and CAD status were obtained. DNA was extracted from whole peripheral blood and bisulfite converted. PCR was performed on bisulfite converted samples followed by PCR melting temperature analysis using methylation sensitive high-resolution melting (MS-HRM) and bisulfite sequencing in order to evaluate methylation % and correlate them with urinary PAHs levels and CAD status.

Results Data of the MS-HRM and sequencing methylation analyses were highly correlated. There were however no correlations between each of MS-HRM PCR melting temperature, sequencing average methylation and that of CpG site (cg05575921) and the studied urinary OH-PAHs on Pearson's test. Nevertheless, although the results were not statistically significant, there was a trend of hypomethylation and obstructive CAD among ever and never smokers, with more hypomethylation in smokers. These trends were most apparent with the methylation results based on direct bisulfite sequencing which is the most specific and sensitive method when compared to MS-HRM.

Conclusions Preliminary data showed that there was no correlation between *AHRR* methylation status and three of the analyzed OH-PAHs. This lack of correlation could be due to the fact that the OH-PAHs are based on spot urine collection which may not reflect chronic exposure. In fact, *AHRR* methylation status is currently an established biomarker for chronic smoking exposure, and may hence be a better marker of PAH exposure from ambient pollution as shown in the trend among the never smokers in this study. It is hoped that with further experiments on the rest of the cohort, there would be enough power to show significant results and adjust for potential confounders. To our knowledge, this is the first study where samples were collected from subjects undergoing cardiac catheterization, the gold standard for CAD diagnosis, and where pollution exposure level was measured based on biomarkers rather than atmospheric measurement, which may not reflect actual exposure.

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ABBREVIATIONS

5-mC	Methyl Cytosine
AhR	Aryl Hydrocarbon Receptor
AhRR	Aryl Hydrocarbon Receptor Repressor
ARNT	AhR nuclear translocator
AUBMC	American University of Beirut Medical Center
B[a]P	Benzo[a]pyrene
BRAC1	Breast Cancer Type 1
CAD	Coronary Artery Disease
CO	Carbon monoxide
COHB	Carboxyhemoglobin
COPD	Chronic Obstructive Pulmonary Disease
CPK	Creatine Phosphokinase
CTn I	Cardiac Troponin I
CVD	Cardiovascular Disease
CYP	Cytochrome P450
DBF	Disturbed Blood Flow
DNMT	DNA Methyl Transferase
ECG	Electrocardiogram
GC	Gas Chromatography
HDL	High Density Lipoproteins
HPLC	High-Performance Liquid Chromatography
HScT	High Sensitivity Cardiac Troponin
INOCAD	Ischemic Non-Obstructive Coronary Artery Disease
LDL	Low Density Lipoproteins
MBD	Methyl CpG Binding Domains
MeCP	Methyl Cytosine Binding Protein
MI	Myocardial Infarction
MS-HRM	Methylation Sensitive High Resolution Melting
mTOR	Mammalian Target of Rapamycin
NO_2	Nitrogen Dioxides
NSTEMI	Non-ST-Elevation Myocardial Infarction
O_3	Ground-Level Ozone
OH-Flu	Hydroxylated Fluorene
OH-Naph	Hydroxylated Naphthalene
OH-Phen	Hydroxylated Phenanthrene
OH-Pyr	Hydroxylated Pyrene
oxLDL	Oxidized Low Density Lipoproteins
PAH	Polyaromatic Hydrocarbons
PCR	Polymerase Chain Reaction
PM	Particulate Matter
PPAR-ɤ	Peroxisome Proliferator Activated Receptor Gamma
ROS	Reactive Oxygen Species
SA	Stable Angina
SMC	Smooth Muscle Cells
SO_2	Sulphur Dioxides
STEMI	ST-Elevation Myocardial Infarction

TCDD	Tetrachlorodibenzodioxin
TET	Ten-Eleven Translocator
UA	Unstable Angina
VMP	Vascular Medicine Program
WHO	World Health Organization

CHAPTER I
INTRODUCTION

A. Coronary artery disease (CAD)

1. Epidemiology

CAD is the leading cause of death world-wide. In fact, approximately 18 million patients die yearly from CAD. In 2005, six million deaths were reported in the United States due to CAD, this number tripled in 2009. In 2007, 3.33% of CAD patients died. In 2020, however, this percentage increased to reach 25% of CAD-induced deaths in the US. In Lebanon, the story is not so different. According to the World Health Organization (WHO), 47% of patients died from CAD in 2018 [1-3].

2. Risk factors

Risk factors of CAD include abnormal lipid panel i.e. increased low density lipoproteins (LDL), high triglycerides, high total cholesterol, decreased high density lipoproteins (HDL), pollution, obesity, smoking, aging, heritability of CAD, hypertension, diabetes, hyperuricemia and psychosocial stress [1-4].

3. Pathophysiology

CAD is one of the cardiovascular diseases that starts with coronary endothelial dysfunction (that could be a result of hypertension, hyperlipidemia, smoking, toxins, etc). This dysfunction in the endothelium allows LDL to pass through and accumulate on the coronary endothelium. Once LDL passes through, it is oxidized (oxLDL) by reactive species. When macrophages approach the oxLDL and phagocytize them, they become foam cells which are retained on the endothelial layer. Foam cells start a

signaling pathway that ends by recruiting smooth muscle cells (SMC) that emigrate from the media to adventitia, which also engulf oxLDL, fatty SMC and foamy macrophages. These accumulate to form the fatty streak which is the first sign of atherosclerosis that is visible without magnification. Fatty SMC start producing extracellular matrix and proteoglycans forming a fibrous plaque lesion or fibro-fatty atheroma **(Figure 1)**. The resulting advanced atherosclerotic plaque limits blood flow to the heart causing an imbalance between oxygen demand and oxygen supply causing the classical CAD symptoms [3-5].

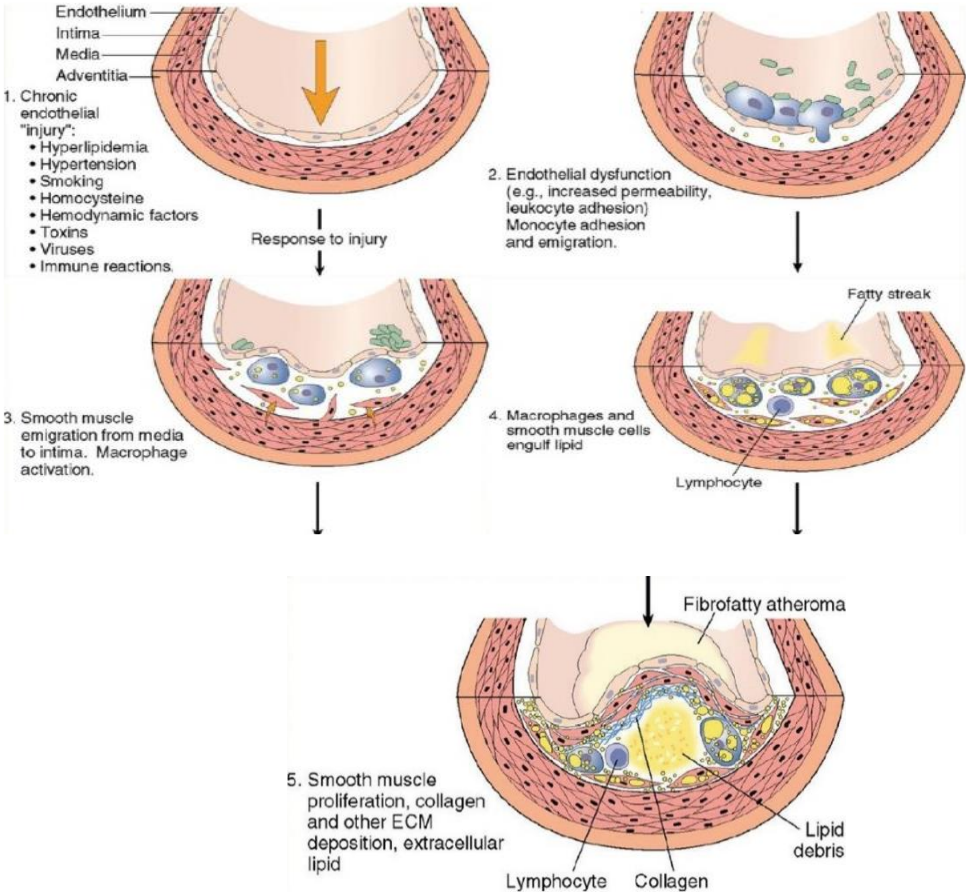


Figure 1 Atherosclerotic plaque formation [6].

4. Symptoms and presentation

The atherosclerotic disease can lead to either stable or unstable angina. Stable angina (SA) is defined as at least 70% atherosclerotic plaque narrowing of one or more of the coronary arteries and its clinical symptoms are triggered by exercise or emotional stress that will eventually worsen the imbalance between oxygen demand and oxygen supply. However, unstable angina (UA) is characterized by an advanced plaque remodeling and a thrombus formation that is white (platelet-rich) in nature. Higher grade UA plaque morphology resembles that of MI, whereas lower grade UA plaque morphology resembles that of SA. Unlike SA, UA is unexpectedly symptomatic even at rest [7-9]. In both cases, patients may present with chest pain radiating to the arms, neck, and jaw. In addition, they may present with non-specific symptoms such as dyspnea, fatigue, sweating, nausea, and vomiting [10].

5. Tests

There are several tests performed to diagnose or assess coronary artery disease. First is the cardiac catheterization **(Figure 2)**, which is the gold standard to diagnose coronary artery disease. It is used to check for blockages or narrowing of the coronary arteries. A needle is inserted into the patient's radial or femoral artery. After that, a guiding wire is inserted through the needle to the coronary arteries. This is followed by a thin hollow tube, called catheter, which is inserted through the guiding wire to the coronary arteries, the guiding wire is then removed. A dye that is visible by X-Ray, is injected into the catheter to allow visualization of any partial or complete obstruction of the coronary arteries (also called angiogram) [11, 12].

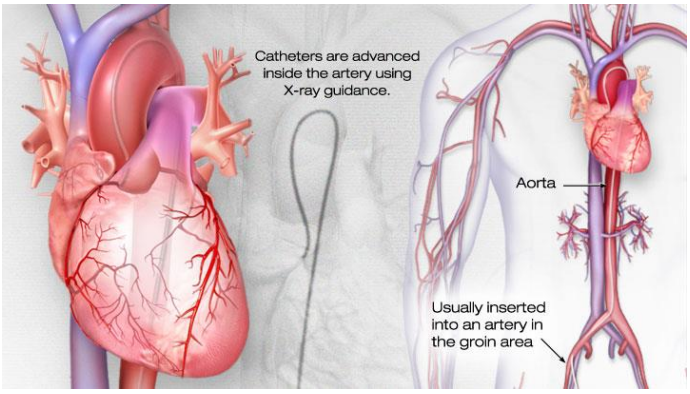


Figure 2 Cardiac catheterization [11].

Second is the Electrocardiogram (ECG) test. This test measures the electrical activity of a single heartbeat. Electrodes are placed on the patient's chest and their readings are recorded on a graphic paper (electrocardiography). A single electrocardiography complex consists of waves, each of which represents an action potential in a heart region. The P wave represents atrial depolarization and the QRS complex represents the action potential propagation to the ventricles and ventricular contraction. After ventricles have ejected blood, another pause occurs before ventricular repolarization, this is represented by ST segment. Finally, the T wave occurs while ventricular cells are repolarizing **(Figure 3)** [13].

ECG can differentiate between ST segment elevation MI (STEMI) and non-ST segment elevation MI (NSTEMI)/UA. In case of STEMI, the ECG shows an elevated ST segment, whereas in case of NSTEMI/UA the ECG shows either ST segment depression or T wave inversion **(Figure 3)**. Increased cardiac biomarkers are used to differentiate between NSEMI and UA. In NSTEMI, cardiac biomarkers are elevated while in UA, they are normal [14].

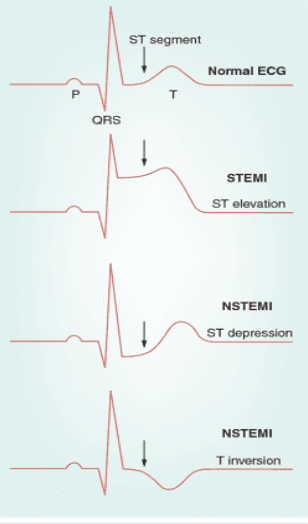


Figure 3 Normal heartbeat, STEMI, NSTEMI, and UA on ECG [13].

Third is the troponin test. Troponin is released in response to cardiac injury that results from inadequate blood supply to cardiomyocytes. There are multiple troponin isoforms among which cardiac troponin I (cTnI) is considered to be the most specific to cardiomyocytes. cTnI test measures the amount of troponin in blood and is implicated in the diagnosis of myocardial infarction (MI) secondary to CAD. It is performed upon patient presentation with chest pain and within 3-6 hours after. Troponin level is considered high when it exceeds 0.04 ng/ml. High-sensitivity cardiac troponin (HScTn) may be used to diagnose early MI as it can detect troponin level as low as 14 ng/L. In comparison to cTnI, HScTn is more sensitive but less specific, possibly giving rise to false positive results. Measuring troponin kinetics every 6 hours (serial troponin), may be needed to exclude other possible conditions behind an MI since the increase and slow consistent decrease in blood troponin level is indicative of CAD [14-16].

Fourth is the creatine phosphokinase (CPK) test. CPK is an enzyme that possesses three isozymes among which CPK MM is predominantly found in cardiac muscle. CPK MM blood levels rise four to six hours following an MI, and then return back to normal level within 24-48 hours. CPK MB is less specific for cardiac injury when compared to troponin. However, unlike troponin which lasts longer in blood, CPK MB can be more useful when re-infarctions are suspected [17, 18].

Fifth is the stress test. In this test, the patient is asked to paddle on a bicycle or to walk on a treadmill to increase cardiac stroke volume to meet body needs, thus increasing cardiac oxygen demand. In parallel, the patient's heartbeats are recorded on an ECG [19, 20]. Cardiac assessment during stress test can also be done using cardiac perfusion scintigraphy. In this technique, patients are injected with radioactive materials, known as tracers, they exercise (as described in stress test) and their blood flow pattern is recorded on a special camera to check for narrowed arteries [21].

Sixth is the calcium score test. This test uses an electron-beam computed tomography to check for calcified coronary arteries. Calcium deposits in the atherosclerotic plaque during atherogenesis but not in healthy coronaries. This test assesses the cardiovascular risk in asymptomatic individuals with CAD risk factors [22].

6. Obstructive vs. non-obstructive

Unlike obstructive CAD, whose pathophysiology is based on the formation of obstructive atherosclerotic plaque [3, 23], ischemic non-obstructive CAD (INOCAD), also known as microvascular angina or cardiac syndrome X, refers to patients who have no more than 50% obstruction in their coronary arteries in the absence of an

atherosclerotic plaque, and presenting with classical CAD symptoms. INOCAD pathophysiology lies behind the imbalance occurring between coronary vasodilators being nitric oxide and prostacyclin, and coronary vasoconstrictors being thromboxane A2 and endothelin, leading to endothelial dysfunction [24, 25].

B. Pollution

1. Types of pollution

There are three main types of pollutions: water, soil, and air pollution.

a. Water pollution

Fresh accessible water constitutes only about 0.036% of whole earth's water content, as the rest is either salty sea water or in the form of icecaps in the poles [26, 27]. However, the quality of fresh water that is used today is decreasing as water pollutants are increasing. Almost fourteen-thousand deaths are attributed yearly to consumption of contaminated water [28]. Water pollution can be classified according to the origin: natural or anthropogenic. Natural sources include salt-water invasion, low-quality surface water bodies, and geothermal fluids, whereas anthropogenic sources are man-made such as industrial activities, domestic sewage and agriculture [26-29].

Several water pollutants have been identified. Nitrates are found in domestic sewage, industrial effluents and fertilizers. High levels of nitrates in water are linked with methemoglobinemia in children and cancers in adults since the binding of nitrates to amides and amines in human body forms nitroso compounds that are carcinogenic [26, 30]. Halogens, such as fluoride, chloride and bromide, may also be found at abnormally higher levels in drinking water due to anthropogenic activities. Higher level

of fluoride in drinking water may cause fluorosis, whereas higher levels of chloride and bromide may lead to cancers in humans and fatalities in aquatic life, respectively [26].

b. Soil pollution

As with water pollution, soil pollution is mainly caused by anthropogenic activities. Industrial wastes contain inorganic species such as heavy metals, and their salts and organic species such as polyaromatic hydrocarbons (PAHs). Lead and mercury are among the most abundant anthropogenic soil pollutants. They are absorbed by plants which are later consumed by animals or humans through the food-chain. When ingested in high doses, lead and mercury can induce central nervous system, urinary and reproductive systems disorders as well as DNA damage [31, 32].

Another way that pollutants are introduced into soil is by agricultural activities. Using pesticides, insecticides, and fertilizers introduce chemicals that are slowly degraded releasing by-products that hinder appropriate plants' growth. It is worth mentioning that soil pollution may lead to water pollution when pollutants reach groundwater, or through contaminated runoffs when reaching oceans and rivers, as well as when volatile soil pollutants, such as ammonia, evaporate and contaminate water [31, 32].

2. Air pollution

a. History

In the 1930's, static weather conditions created a motionless fog in Belgium that killed 60 people in a single day. Investigations revealed high fog pollutants level that came from chimney exhausts [33, 34]. In 1940's, pollutants from industrial activities accumulated over Donora, an industrial city in Pennsylvania, and caused 20 sudden

deaths, in addition to over 5000 residents becoming ill and 400 hospital admissions. All these tragedies pushed scientists to find links between exposure to air pollution and negative health consequences [34].

b. Epidemiology

Outdoor air pollution accounts for over 4.2 millions of premature deaths every year world-wide. in fact, outdoor air pollution occupies the fourth place of risk factors for morbidities and mortalities and it is expected to occupy the first place by 2050 when the number of premature deaths induced by outdoor air pollution will have doubled [2, 35]. In 2016, WHO revealed that 18% of obstructive CAD-induced deaths were attributed to ambient air pollution [36].

c. Types of pollutants

i. Particulate matter (PM)

Particulate matters are particles that are formed in the atmosphere by chemical interactions between pollutants namely, inorganic sulfates and nitrates, in addition to organic components such as dioxins and polyaromatic hydrocarbons, and particulate contaminants such as soot, smoke, and cement dust **(Figure 4)**. PMs originate from natural and anthropogenic sources. These include volcanic eruptions and wind-blown dust and soil, whereas anthropogenic sources come from diesel exhaust, power plants, agriculture and various industrial activities [37]. Thus, PMs are mainly by-products of human activities namely industrial activities, car exhausts, and cigarette smoke [34, 38].

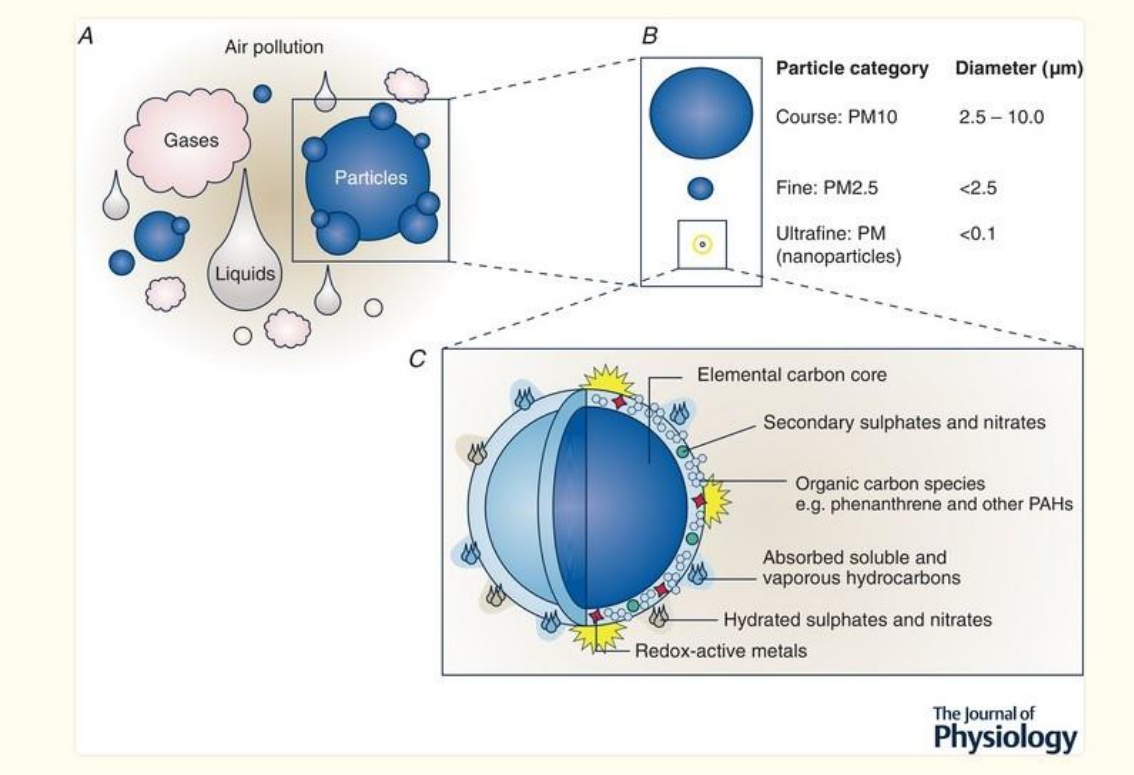


Figure 4 PM composition [39].

PMs differ in size. Some have a diameter of 10 μm, known as PM_{10}, others have a diameter of 2.5 μm, known as $PM_{2,5}$. The severity of health-related issues that PM may cause is dependent on its size. In other words, the smaller the diameter, the deeper the PM can get into the lungs and eventually to the systemic circulation **(Table 1)**. What really makes PMs a matter of concern is that they have long half lives and their tiny dimensions allow them to translocate from the source of emission to neighboring areas in the environment [34, 38]. In Beirut, Lebanon, PM_{10} annual mean concentration is double that of the maximal allowed level set by the WHO (35.24 $\mu g/m^3$ vs. 15 $\mu g/m^3$) [36, 40].

Table 1 Penetrability according to PM size [38].

Particle size	Penetration degree in human respiratory system
>11 μm	Passage into nostrils and upper respiratory tract
7–11 μm	Passage into nasal cavity
4.7–7 μm	Passage into larynx
3.3–4.7 μm	Passage into trachea-bronchial area
2.1–3.3 μm	Secondary bronchial area passage
1.1–2.1 μm	Terminal bronchial area passage
0.65–1.1 μm	Bronchioles penetrability
0.43–0.65 μm	Alveolar penetrability

Many lung disorders are majorly attributed to high PM exposure. For instance, infants exposed to PM showed a reversible decrease in lung function, as measured by peak expiratory flow, and an irreversible retardation in lung development [41]. In addition, PMs have proven to be pro-oncogenic to the lungs by altering gene expression through epigenetic changes, and altering miRNA function on pro-oncogenes **(Figure 5)** [42-44]. Moreover, several cardiovascular diseases have been reported in patients living in areas with high PM levels. As a matter of fact, PMs are key players in atherosclerosis, CAD, MI and blood hypercoagulability [41, 45].

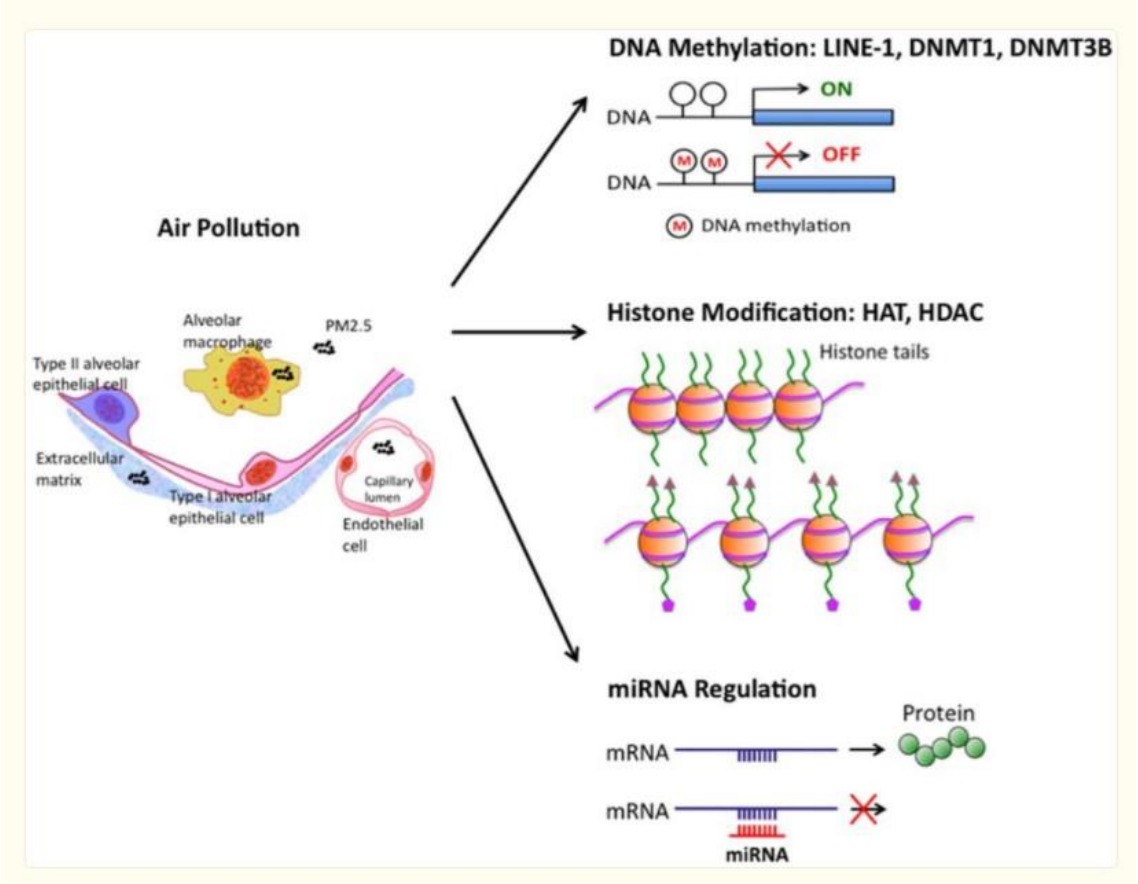


Figure 5 PM-induced epigenetic changes in the lung [43].

ii. Ground-level Ozone (O_3)

Ground-level O_3 is formed when nitrogen oxides react with volatile organic compounds, emitted from vehicles, power plants, and gas stations, in the presence of sunlight [46]. Ground-level O_3 can cause respiratory diseases mainly Chronic Obstructive Pulmonary Disease (COPD), and can trigger asthma attacks. Furthermore, O_3, being an oxidizing gas, is able to induce oxidative stress in the lung cells followed by immune reactions and the release of inflammatory cytokines and chemokines [47].

iii. Carbon monoxide (CO)

CO is mainly emitted from cigarette smoking and car exhausts. Health effects provoked by exposure to CO vary from mild in low exposure concentration being fatigue to fatalities in high exposure concentrations as CO displaces oxygen from hemoglobin leading to impairment of body oxygen supply [48].

iv. Nitrogen dioxide (NO_2)

Nitrogen dioxide is mainly emitted from combustion sources being power generating factories and vehicular emissions [36]. NO_2 is referred to as one of the greenhouse gases that contribute to global warming [49]. As a matter of fact, annual mean NO_2 concentration in Lebanon surpasses the maximal allowed level set by the WHO by 4 folds (40.3 $\mu g/m^3$ vs 10 $\mu g/m^3$ respectively) [36, 40]. Reduced lung function and growth, asthma attacks and bronchitis have been reported with long-term NO_2 exposure [36].

v. Sulfur dioxide (SO_2)

SO_2 is generated from burning of sulfur-containing fossil fuels such as coal and oil and motor vehicles [36]. The WHO has warned that SO_2 exposure can trigger asthma attacks and COPD [36].

C. Polyaromatic hydrocarbons

1. Sources

There are three main sources of PAHs: pyrogenic (or pyrolytic), petrogenic and biological. Pyrogenic PAHs are generated when organic molecules are exposed to high

temperatures under low oxygen availability. In other words, when organic matters undergo incomplete combustion such as in case of open waste burning, cigarette smoke, broiled and smoked foods, and incomplete combustion of motor fuels in cars and forest fires. Petrogenic PAHs, however, are found in crude oils that have been formed over millions of years. Biological PAHs come from biological processes that occur in bacteria and algae. As a result, PAHs are ubiquitously distributed organic compounds [39, 50, 51].

Exposure to PAHs takes place via inhalation of contaminated air, ingestion of contaminated foods, and dermal contact with contaminated soil or dust [2].

Since the available data for our study consisted of four PAHs namely naphthalene, phenanthrene, pyrene, and fluorene, the sources of these PAHs will be specifically discussed. Naphthalene is emitted from domestic activities, such as wood burning in fireplaces and off-gassing, tobacco smoke and largely by gasoline evaporation and its exhaust [52, 53]. Phenanthrene occurs in fossil fuels and is present in products of incomplete combustion such as vehicular emissions, coal and oil burning, wood combustion, municipal incinerators, and tobacco smoke [54]. Pyrene's emission comes from coal tars, cigarette smoke, and roasted food [55]. Fluorene is the most abundant PAH emitted from the vapor phase of PAH sources such as car fume exhausts, cigarette smoke as well as petroleum refineries [56].

2. Chemistry

PAHs are organic compounds that consist of two or more fused aromatic rings. Small PAHs are those having a maximum of six rings whereas large PAHs are those having more than six rings. PAHs have low aqueous solubility, low vapor pressure, as

well as high melting and boiling points. Therefore, the physical and chemical characteristics vary according to their molecular weight. For instance, as the molecular weight of such compounds increases, the aqueous solubility and the vapor pressure tend to decrease while resistance to oxidation and reduction increases. Another method to distinguish PAHs, specifically pyrogenic from petrogenic PAHs, is to check for the five membered rings which are more abundant in the pyrogenic PAHs [50]. The chemical structures of the four PAHs available to us are represented in **Figure 6** [57].

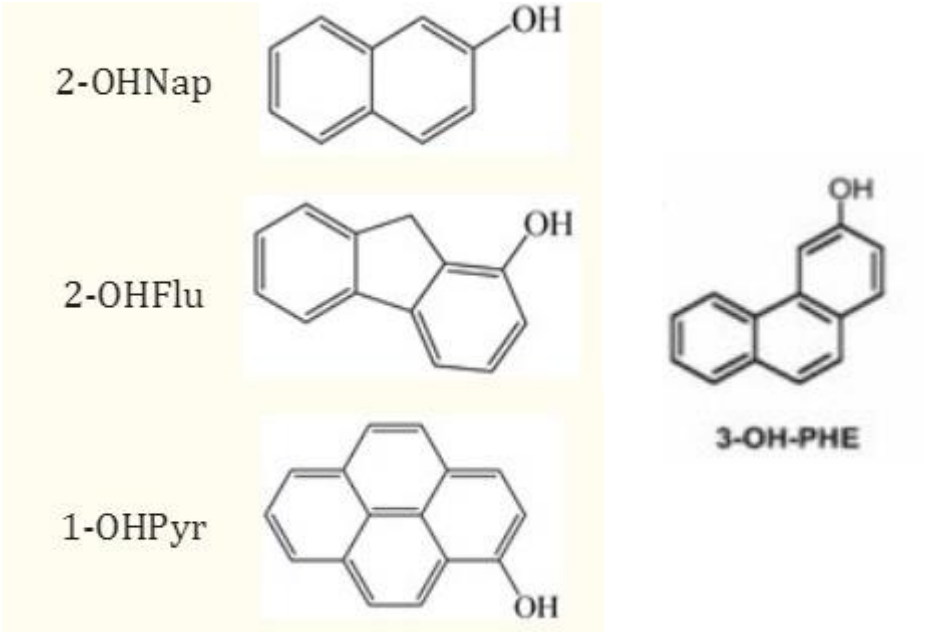


Figure 6 Structure of 2-OHNap, 2-OHFlu, 3-OHPhe, and 1-OHPyr [57, 58].

3. Metabolism

Metabolism of PAHs follows two phases. Phase 1 metabolism (oxidative phase) transforms the parent compounds to oxidized products followed by reduced or hydroxylated metabolites (OH-PAHs). Phase 2 metabolism (conjugation phase) is the process of addition of glucuronic acid or sulfate to OH-PAHs that are now readily

excreted either renally or by bile (depending on molecular weight, as high molecular weight PAHs, like benzo(a)pyrene, are mostly excreted by bile, while low molecular weight PAHs such as OH-NAP are excreted renally). Moreover, Phase 2 metabolism deactivates the carcinogenic reactive PAHs that were initially activated by Phase 1. Urinary PAHs and their metabolites have a short half-life ranging from 2.5h to 6.1h [57, 59-62].

Pyrene's major urinary metabolite, 1-OHPYR, reflects total environmental PAHs exposure [63].

4. Health impact

a. Cancers

CYP1, 2 and 3 enzymes are responsible for metabolic activation of pro-carcinogens to carcinogens with CYP1A1 and CYP1B1 being the most common enzymes involved in the metabolic activation of PAHs. First, PAHs enter the body and stimulate Arylhydrocarbon receptor (AhR). AhR enhances the transcription of several CYP enzymes including CYP1A1 and CYP1B1. PAHs are then converted by CYP enzymes and hydroxylases to diol-epoxides which are highly reactive towards DNA **(Figure 7)** [64, 65].

Benzo[a]pyrene (BaP) is considered the prototype of carcinogenic PAHs [50, 65].

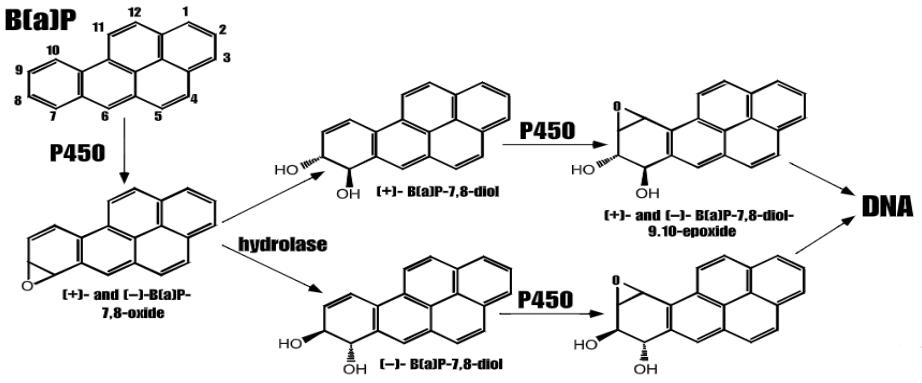


Figure 7 Metabolic activation of Benzo[a]pyrene to diol-epoxide derivative of CYP450 and hydrolase enzymes [64].

b. Immunosuppression

PAHs have been shown to suppress immunocytes. By intercalating within DNA strands, PAHs inhibit B and T lymphocytes. Moreover, they decrease lysosomes within macrophages and induce immunocytes apoptosis [66]. Two mechanisms have been suggested behind PAHs-induced immunotoxicity. The first mechanism is thought to involve AhR-mediated PAHs activation to reactive oxygen species (ROS) that intercalate within immunocytes' DNA and inhibit their proliferation [67]. The second mechanism is by increasing intracellular calcium thus increasing mitochondrial activity and ROS generation followed by increased immunocyte apoptosis [66, 68].

c. Reproductive disorders

As a result of PAHs-DNA adducts, PAHs have been shown to impair meiosis in males manifested as decreased sperm count or quality. In addition, it has been shown that exposing male fetuses to PAHs from smoking mothers results in reproductive abnormalities in male adulthood characterized by decreased testicular size and sperm

count [69]. In female reproductive system, PAHs have been shown to decrease implantation and endometrial receptivity during early pregnancy [70].

5. Association between PAHs and CAD

Several studies have shown a positive association between PAHs exposure and CAD. For example, it was shown that patients with CAD had higher level of PAHs urinary metabolites when compared to non-CAD controls [2, 71]. The mechanism by which PAHs can precipitate CAD falls within endothelial cell injury, thereby inducing oxidative stress and apoptosis through activation of phospholipase A2 and subsequent activation of arachidonic acid metabolism toward the CYP and COX pathways [39, 72]. The impaired integrity of the endothelium will allow leakage of immunocytes and plasma lipids to accumulate within the vascular wall which constitutes the basis of atherosclerosis pathophysiology [73].

6. Exposure detection methods

The need for specific and sensitive methods to monitor exposure to PAHs has emerged because of the significant health drawbacks that PAHs can induce. Measuring human PAHs exposure by measuring ambient PAHs would not be specific since diet and smoking habit as well as lifestyle can interfere with the results. Therefore, monitoring biomarkers, such as the urinary PAHs metabolites, could provide a better image of the actual exposure to PAHs. In addition, it is worth mentioning that the assessment of a single PAH level in urine does not properly reflect the actual exposure to PAHs since their urinary levels vary according to exposure time. Several methods have been used to detect and assess the level of urinary OH-PAHs levels [51].

a. High performance liquid chromatography (HPLC)

HPLC has been developed in response to the need for an easily automated analytical method. It is applied in the analysis of organic compounds that are too unstable or too volatile in gas chromatography (GC). HPLC system consists of solvent or mobile phase reservoir that is attached to a high-pressure pump that pumps the solvent into the column. Just before the column, there is a sample injector to introduce the sample into the column as well. A detector is attached to the HPLC column to detect the eluates, and each sample component displays a peak on the recorder where data acquisition takes place. The components of HPLC system are shown in **(Figure 6).** Its principle is based on sample constituents' partition between a stationary phase, or a column, and a volatile phase, or the solvent [74].

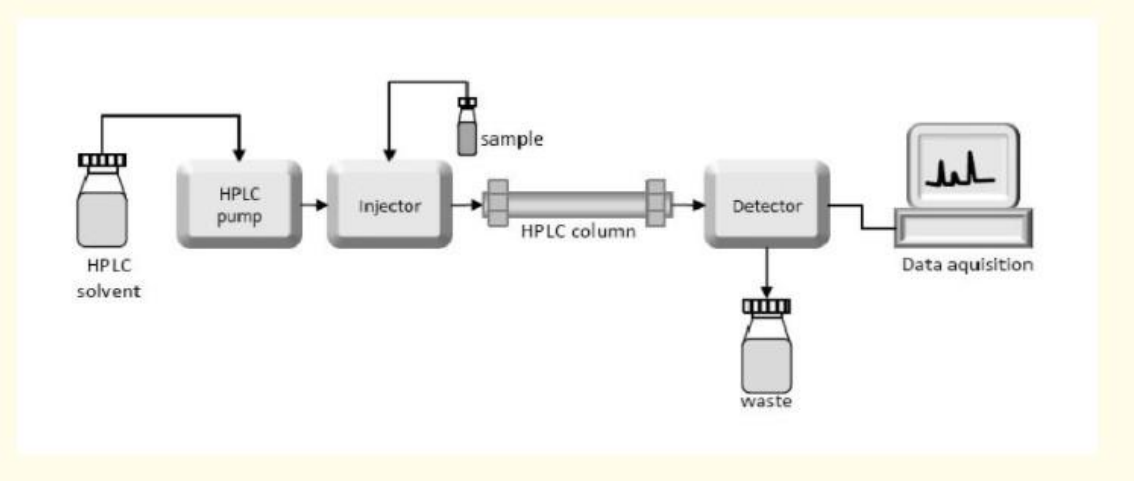


Figure 8 HPLC system components [75].

Two modes of action have been identified: normal phase and reversed phase chromatography. In normal phase chromatography, the stationary phase is polar such as silica or alumina while the mobile phase is non-polar such as hexane. In this mode,

polar compounds are retained more strongly by the polar stationary phase allowing partition of non-polar compounds first. In reversed phase chromatography, however, the stationary phase is non-polar such as hydrocarbons while the mobile phase is polar such as water or methanol. In this mode, non-polar compounds migrate through the column first [74].

The polarity of the solvents in the mobile phase can be changed by using a mixture of solvents. Elution of the compounds is called isocratic when the solvent mixture remains constant throughout the elution step. However, when the composition of the mixture is varied during the elution step, it is referred to as gradient elution [74]. In HPLC method, urinary PAH metabolites are reduced back to their parent PAHs, it has a high sensitivity since it increases the availability of parent compounds but a considerable amount of the PAHs is lost during this process [51]. However, Strickland PT et al. [75] measured urinary pyrene metabolites before conjugation, they found that 1-hydroxypyrene glucuronide was more fluorescent and thus more sensitive than the de-conjugated metabolite.

b. Gas chromatography (GC)

GC is used to separate compounds that are volatile and are not degraded by heat. It consists of a stationary phase, which can be liquid or solid, and a mobile phase, which is an inert gas such as helium. A sample is injected into the system and carried by the mobile phase to reach the column. The column is where separation takes place based on the chemicals' partial pressure as well as their affinity for the stationary phase. Eluates are then detected and data are recorded [76]. GC is often connected to a mass-spectrophotometer (GC-MS) thus, it separates eluates based on their mass [77].

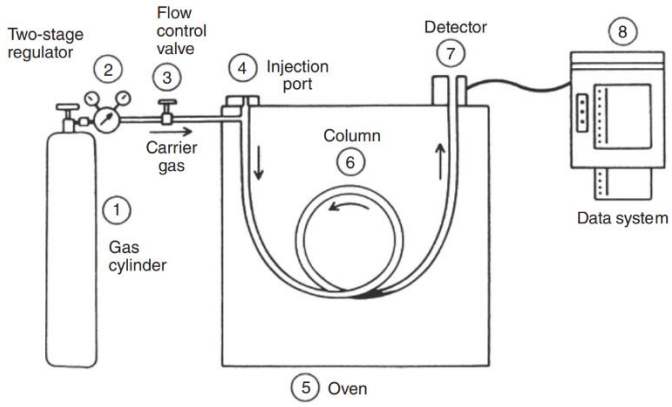


Figure 9 Gas chromatography system components [76].

D. Cigarette smoking

1. Epidemiology

There are currently approximately 1 billion cigarette smokers in the world [78]. In 2002, 4 million people died because of smoking-related morbidities. Estimated smoking-related deaths increased in 2008 to reach 5.4 million, and it is expected to reach 10 million deaths in 2025 with 70% of total world-wide deaths being in developing countries [79, 80]. In Lebanon, A cross-sectional study was done by El-Roueiheb et al [80] and showed that Lebanese in Beirut start smoking cigarettes as early as 12 years of age.

2. Constituents

Cigarettes and cigarette smoke contain thousands of chemicals such as nicotine, formaldehyde, ammonia, benzene, carbon monoxide, and PAHs [79, 81, 82]. In fact, An T. Vu et al. [83] studied PAHs emissions from cigarette smoke and they found that low

molecular weight PAHs, particularly naphthalene, fluorene, and phenanthrene, were the most abundant PAHs.

3. Health impact

Cigarette smoking remains the leading preventable cause of morbidity and mortality world-wide since it can lead to numerous diseases such as cardiovascular disease, cancer, lung disease, and addiction.

a. Cardiovascular disease (CVD)

Around 20% of CVD-related deaths are due to cigarette smoking [84]. The chemicals in the cigarette and its smoke are able to generate free radicals i.e. reactive oxygen species (ROS). These free radicals oxidize LDL to oxLDL and activate immunocytes that release pro-inflammatory mediators, especially NF-KB and TNF-α, in addition to chemokines and cytokines. Furthermore, smoking decreases HDL and raises LDL and total cholesterol [85]. Eventually, these events contribute to the inflammatory atherosclerotic plaque build-up [86].

Moreover, cigarette smoking has been linked to thrombogenic events for its potential to reduce fibrinolysis while promoting thrombogenic factors [87].

b. Cancer

Approximately 30% of cancer-related deaths are attributed to smoking [88]. Lung and colon cancers are among the most common cigarette smoking-induced cancers [89]. On one hand, among the thousands of chemicals found in cigarettes, 70 chemicals are proven to be carcinogens. When inhaled, some of these chemicals are

metabolized, thus activated into reactive epoxides that cause DNA adducts and mutations [89]. On the other hand, in addition to its immunosuppressive effect, cigarette smoking can cause nicotine-mediated disruption of cellular apoptosis thus hindering host defenses against cancer progression [90].

c. Lung disease

Chronic Obstructive Pulmonary Disease (COPD) develops in 50% of smokers, and 80% of COPD-related deaths is attributed to smoking [91, 92]. In fact, smoking releases oxidants and free radicals within the lungs leading to immunocytes activation followed by activation of inflammatory cascades. Upon chronic smoking, the lungs undergo structural remodeling from repeated smoking-induced damages and repairs that do not change even after smoking cessation. These structural remodeling and inflammatory reactions constitute the basis of COPD pathophysiology [92].

d. Addiction

Cigarettes contain nicotine, which resembles acetylcholine in structure. Once inhaled, nicotine is carried to the brain where it stimulates acetylcholine nicotinic receptors thus causing dopamine release, the latter being responsible for the addictive properties of cigarette smoking as it is responsible for rewards circuit since it associates cigarette smoking with pleasure. Addiction is attained once withdrawal symptoms, such as anxiety, irritability and depression, are manifested [78].

4. Exposure detection methods

a. Cotinine test

Cotinine is the most commonly used biomarker for the detection of cigarette smoking exposure. Cotinine is a biproduct of nicotine metabolism. Therefore, cotinine level obtained from serum, urine or saliva reflects the extent of nicotine exposure. Measurement of cotinine levels provides a better profile on smoking exposure than measurement of nicotine levels since 80% of nicotine is converted to cotinine, and cotinine has a longer half-life(17 hours) than that of serum nicotine (3 hours) [93, 94]. In addition, nicotine metabolism and elimination are highly variable in a given population [94].

b. Carbon monoxide (CO) test

CO is one of cigarettes' constituents. Once inhaled, CO binds to hemoglobin to form carboxyhemoglobin (COHb). Respiration represents the major route of elimination for CO. Thus, exhaled CO level reflects COHb. Unlike cotinine, the half-life of COHb is 5-6 hours hence it is more useful for the detection of short-term intermittent smoking behavior [95].

E. Epigenetics

1. Background

Unlike genetics, which is the study of heritable changes in DNA sequence resulting from mutations namely insertion, deletion and point mutations leading to functional changes in gene expression, epigenetics is the study of changes in gene functions and expression without altering the DNA sequence. Even though

hypothetically all cells contain the same genetic information, not all of them, however, express the same genes. [96, 97].

Since its discovery, studies have linked epigenetics to multiple medical conditions namely cancers, cardiovascular, respiratory, neurological as well as immunological and reproductive diseases resulting mainly from environmental exposure to toxicants such as air pollutants [96].

Multiple epigenetic processes have been identified: Methylation, Acetylation, Sumoylation, Phosphorylation and Ubiquitination. These processes are essential for organisms' growth and development but can lead to unwanted behavioral changes and health related effects when they occur improperly [96].

Regardless of the mechanism, epigenetic changes were believed to be nullified in the following generations [96]. However, one study in 2005 by M. Skinner et al. [98] argued against this hypothesis and suggested that the epigenetic changes last for at least 4 generations. For instance, exposing pregnant female mice to high levels of insecticides and fungicides led to decreased sperm count and increased infertility in the male pups secondary to alteration in DNA methylation pattern. Although these male pups had not been exposed to the latter insecticides and fungicides, the observed findings persisted in 90% of the male mice in four subsequent generations [98].

2. DNA methylation

a. Background

Since its discovery from calf thymus in 1948, DNA methylation has been the most studied epigenetic mechanism given the current technology. DNA methylation is

the addition of a methyl group to the fifth carbon of a cytosine nucleotide (5-mC) located in clusters of CpG [97].

It was hypothesized that this methylated cytosine was there to distinguish it from the naked cytosine, the same way uracil differs from thymine (known as methyluracil). Almost 40 years later, scientists discovered the involvement of DNA methylation in regulating gene expression and functions [97].

b. Mechanism

There are three mechanisms that have been proposed for gene silencing mediated by cytosine methylation **(Figure 10)**.

The first mechanism is by direct inhibition of binding of transcription factors to their corresponding promoter regions by methylated cytosines thus inhibiting transcription initiation. The second mechanism proposes that methylated cytosines recruit co-repressors such as Methyl Cytosine Binding Protein 1 and 2 (MeCP1 and MeCP2). Unlike MeCP1, which mainly recognizes symmetrically densely methylated CpG sequences, MeCP2 is able to recognize a single methylated CpG pair [97, 99]. The third mechanism is by altering chromatin structure [97, 99].

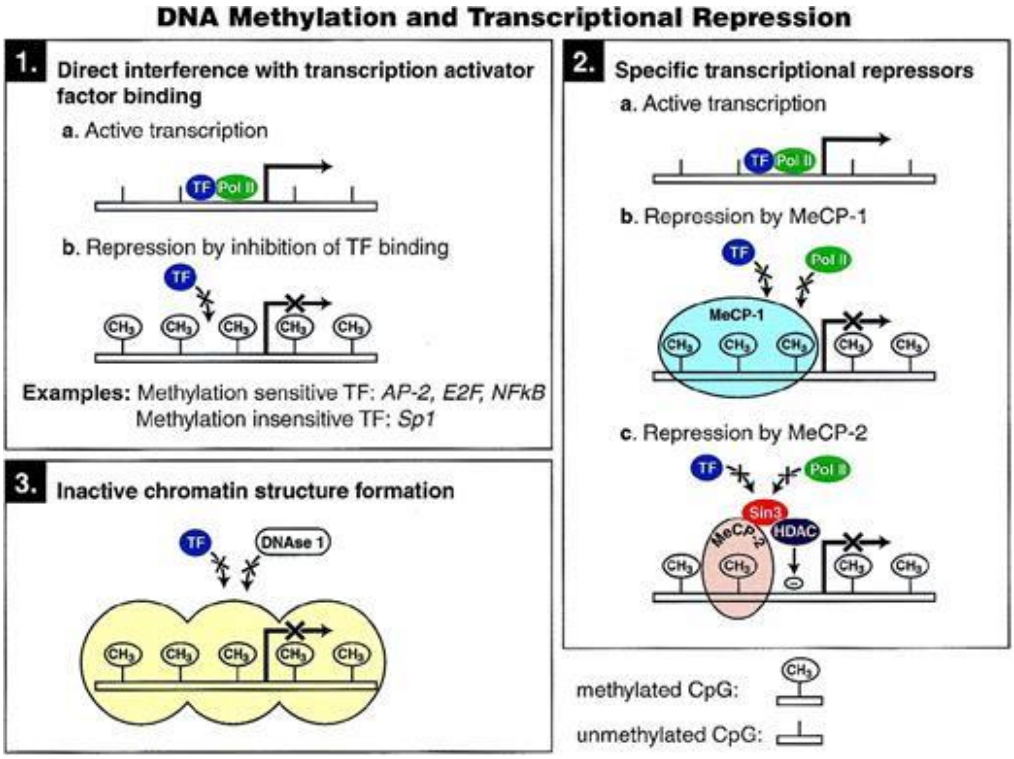


Figure 10 Proposed mechanisms of DNA methylation-mediated gene silencing [99].

c. DNA methylation enzymes

The enzymes involved in adding, removing or mediating DNA methylation are divided into three main categories: writers, erasers and readers.

i. Writers

Writers catalyze the addition of a methyl group to the cytosine's fifth carbon (5-mC). DNA methyl transferases have three main enzymes that directly catalyze this reaction: DNA methyl transferase 1 (dnmt1), and DNA methyl transferase 3a and 3b (dnmt3a, dnmt3b) [97, 99].

Dnmt1 was first to be discovered. It binds to newly synthesized hemi-methylated DNA and methylates cytosine nucleotides, precisely mimicking the methylated cytosine nucleotides in the mother strands. Thus, it is known as the

maintenance dnmt since it maintains the original DNA methylation pattern. However, it has been shown that dnmt1 is also able to perform de novo methylation in vitro in aberrant DNA. In a study done on dnmt1 knockout mice, lethality was observed in the off-springs indicating the possibility in dnmt1 involvement in development [97, 99].

Dnmt3a and dnmt3b are able to methylate new cytosine nucleotides not previously methylated with no preference to hemi-methylated DNA. Thus, they are known as de novo dnmt since they introduce a new methylation pattern onto cytosine nucleotides. Two different characteristics distinguish dnmt3a from dnmt3b. First, dnmt3a is widely distributed, whereas dnmt3b has a limited distribution in differentiated tissues. Second, like dnmt1, dnmt3b knockout mice showed lethality in the off-springs while it took dnmt3a knockout mice's offs-prings about a month before they died. This proves that dnmt3a is involved in differentiation, while dnmt3b is involved in development [97, 99].

ii. Erasers

In mammals, there is no known mechanism that is able to break the covalent carbon-carbon bond to strip cytosine from the methyl group. Instead, this 5mC has to undergo further chemical modifications. For instance, deamination of the 5mC converts it to thymine creating a T/G mismatch which is recognized by the base excision repair (BER) pathway that is going to replace thymine with an unmethylated cytosine [97, 99]. Another mechanism by which demethylation takes place is initiated by the Ten-Eleven Translocator (Tet) enzymes. These enzymes hydroxylate the 5mC to 5mhC which, in turn, is converted back to naked cytosine through a series of reactions. Like methylation, 5hmC is also involved in gene silencing [97, 99].

iii. Readers

Three 5mC-recognizing protein families have been identified: Methyl CpG binding domains (MBD), the ubiquitin-like containing PHD and RING finger domain (UHRF), and the zinc-finger proteins.

MBD family is subdivided to methyl cytosine binding protein 2 (MeCP2), along with MBD1, MBD2, MBD3, and MBD4. MBD4 is involved in DNA mismatch repair while the rest directly bind to methylated cytosine and recruit transcriptional repressors through their transcription repression domain (TRD). MeCP2, as an additional mechanism, can also recruit dnmt1 to hemi-methylated DNA to perform maintenance methylation [97].

Ubiquitin-like containing PHD and RING finger domain does not directly bind to 5mC but rather binds to dnmt1 and, just like MeCP2, recruits it to hemi-methylated DNA to perform maintenance methylation [97]. Proteins with zinc finger domains, like MBD, repress transcription through a methylation-dependent manner [97].

d. DNA methylation in atherosclerosis

There are multiple proposed mechanisms by which DNA methylation plays a role in atherosclerosis. These are Dnmt1 regulation and blood flow disruption, inflammation, oxidation, and TET2 regulation [4].

First is the extent of blood flow disruption. Atherosclerosis is characterized by a disturbed blood flow (DBF) that has been shown to be associated with upregulation of the DNA methylating enzyme, dnmt1. In order to support this hypothesis, Zhang YT. and colleagues [100] proved that the inhibition of dnmt1 resulted in improvement of DBF and therefore, decreased atherosclerotic plaque progression. In order to explain the mechanism behind DBF-induced dnmt1 upregulation, Zhang YT. and colleagues [100]

attempted to better understand the role of mTOR which is involved in signaling pathways that translate mechanical stimuli (in this case DBF) into electrochemical activity. Endothelial cells were treated with the mammalian target of rapamycin (mTOR) blocker, rapamycin, before inducing DBF. Neither mTOR nor dnmt1 were activated in comparison with the control group that was not pre-treated with rapamycin. These results may elucidate the role of mTOR, induced by DBF, in the upregulation of dnmt1 [100].

Second is the level of inflammatory cytokines. The disruption of endothelial cells permits monocytes to infiltrate towards the atherosclerotic lesion to become cytokine secreting macrophages. In a study done by Yu J et al [101], DNA extracted from macrophages in atherosclerotic models showed dnmt1 overexpression, and peroxisome proliferator activated receptor gamma (PPAR-ɤ) was hypermethylated, hence inactivated, by dnmt1. Pharmacological activation of the PPAR-ɤ increased the anti-inflammatory cytokines and decreased atherosclerotic plaque progression [101].

Third is the level of oxidized LDL (oxLDL) which is an important player in the pathophysiology of atherosclerosis. In this context, the role of breast cancer type 1 (BRAC1) gene was studied by Singh KK et al [102]. For instance, in animal models with atherosclerosis, BRAC1 was knocked out in the first group and was over-expressed in the second one. The authors found that mice over-expressing BRAC1, were protected against oxLDL. This result shows that in atherosclerosis, where dnmt1 is over-expressed, BRAC1 is hypermethylated, and therefore inhibited, putting cells at risk of oxLDL [102, 103]. Furthermore, in the context of oxidation, oxidative stress induced by ROS has been linked to changes in DNA methylation pattern and atherosclerosis [104]. In fact, O'Hagan et al. [105] found that exposing cells to

hydrogen peroxide resulted in hypermethylated CpG islands in a dnmt1-dependent mechanism. In atherosclerotic lesions, DNA methylation pattern could be similarly changed by ROS [104, 105]. Moreover, A. Baccarelli and V. Bollati [106] studied the role of superoxide dismutase on smooth muscle cell (SMC) proliferation, and found that methylation of superoxide dismutase gene by dnmt1 increased SMC proliferation, whereas inhibiting dnmt1 with 5-azacytidine restored superoxide dismutase activity and stopped SMC proliferation.

Fourth is TET2 inactivation. Normally, TET2 increases the expression of autophagic flux-related genes by oxidatively demethylating 5meC to 5hmC. Therefore, TET2 decreases atherosclerotic plaque formation through an autophagic process. When TET2 is hypermethylated by dnmt1, thus inactivated, it can no longer activate autophagic genes, and it can no longer decrease the atherosclerotic plaque formation [107].

F- The arylhydrocarbon receptor repressor (*AHRR*)

1. AhR/AHRR pathway

AhR is a transcription factor that belongs to the helix-loop-helix family. In a ligand-dependent manner, it enhances the expression of certain genes while inhibits the expression of others.

Several AhR ligands have been identified, including aromatic hydrocarbons, flavonoids, and endogenous metabolic byproducts [108, 109]. In the absence of a ligand, AhR floats in the cytoplasm while bound to chaperone proteins to guarantee proper folding and recognition of its ligands, as well as other regulatory proteins such as P23 and immunophilin-like protein 2 (XAP2). Once a ligand enters the cytoplasm and

binds to AhR via its PAS-B domain, the AhR-ligand complex translocates to the nucleus. Chaperone proteins disassociate from AhR, exposing its PAS-A domain and allowing AhR to bind to the arylhydrocarbon receptor nuclear translocator (ARNT) to form an active heterodimer. Then, this heterodimer binds to and increases the expression of target genes such as arylhydrocarbon receptor repressor (*AHRR*). AHRR protein inhibits AhR through a negative feedback loop by inhibiting ARNT. The remaining free AhR exits the cytoplasm and is subjected to proteosomal degradation. This is known as AhR genomic pathway **(Figure 11)** [108, 110, 111].

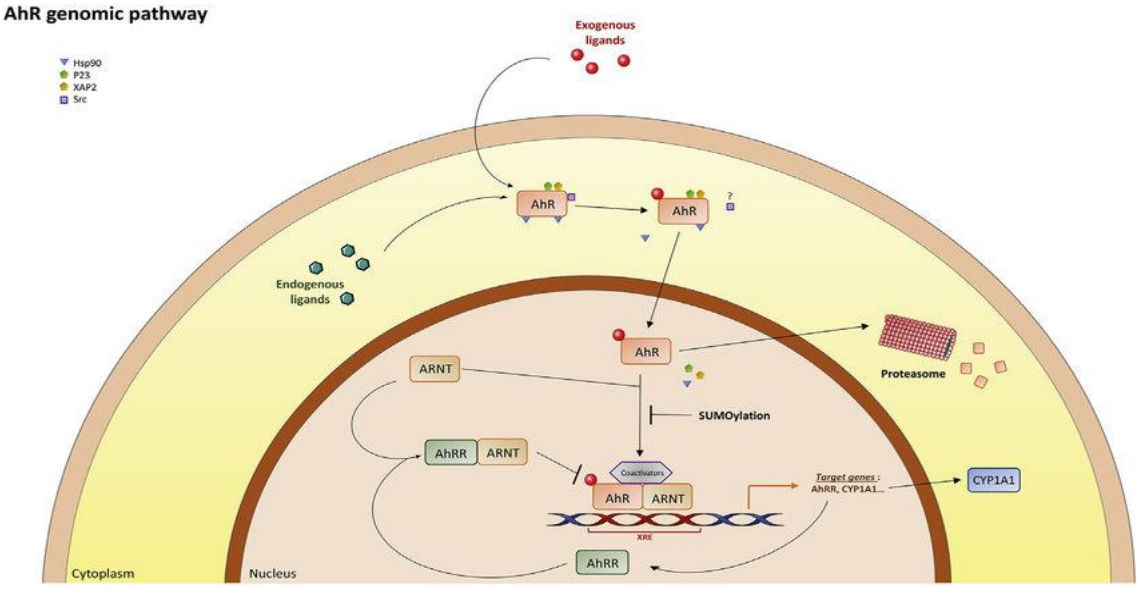


Figure 11 AhR genomic pathway [108].

2. AhR regulatory functions

AhR has multiple regulatory functions.

First is organism detoxification. When foreign chemicals enter the body (e.g., PAHs), AhR is activated, and the AhR-ARNT complex eventually binds to Xenobiotic-responsive element, also known as Dioxin-responsive element, and activates

xenobiotic-metabolizing enzymes such as microsomal cytochrome P4501A1 (CYP1A1), which detoxifies PAHs and dioxins, among others [108, 112].

Second, AhR's pro- and anti-proliferative effects which depend on the cell type and cell cycle phase and determine whether AhR acts as an oncogene or tumor suppressor. For example, AhR promotes the transcription of tumor-suppressing genes while inhibiting the action of pro-proliferative factors (E2F). Furthermore, Yunxia Fan et al [113] found that exposing mice to the genotoxic chemical diethylnitrosamine (DEN) induced hepatomas in AhR knockout mice but not in the wild type group [114] thereby regulating DNA damage, inflammatory cytokines, and cell proliferation, and that AhR silencing is associated with cancer progression. In contrast, AhR exhibits its pro-proliferative effect through activation of proto-oncogenes or through stimulation of growth factors and inflammatory interleukins mediated by 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) [108, 113, 115].

Third, AhR is involved in regulating male and female reproductive systems. To begin with, AhR regulates apoptosis and survival of pre-antral and antral follicles in adult female ovaries. A study done by Kimberly R. Barnett et al [116] on AhR regulatory role in adult female mice revealed that AhR knockout mice had a slower follicular growth rate and a decreased estradiol 2 levels compared to wild-type mice. Exogenously administered estradiol 2 restored follicular growth in AhR knockout mice [117]. Moreover, Baba et al. [118] found that AhR increases the expression of P450 aromatase (CYP19), a key enzyme for estrogen synthesis. Ovulation was impaired in AhR knockout female mice compared to wild-type group. Although the LH and FSH levels were similar in both mice groups, estradiol level was reduced in AhR knockout mice which reduced the sensitivity of the ovaries to gonadotropins [118]. At the level of

the uterus, AhR stimulation has been associated with increased transcription of CYP1A1 and CYP1B1. In addition, AhR has shown to regulate uterine epithelial growth as well as secretory pattern [119].

As for the male reproductive system, the AhR is found in male prostate in normal and diseased state. It has been shown that AhR deletion caused a reduced prostate size in embryonic mice as well as prostate cancer in adult mice [120, 121]. Moreover, androgen receptors' transcriptional activity is regulated by AhR. The unliganded sex hormone receptor displayed transcriptional activity that was regulated by AhR despite deleting its ligand-binding site suggesting cross-talk between AhR and androgen receptors [122].

Fourth, AhR regulates the cardiovascular system. AhR is expressed in cardiomyocytes and is essential for differentiation of these cells during embryogenesis. Deletion of AhR gene in experimental mice resulted in delayed cardiac differentiation. In adult heart, however, deletion of AhR induced cardiac hypertrophy accompanied by a decreased pre-load, after-load and cardiac output, and an increased level of plasma angiotensin II and endothelin I [123].

Fifth, AhR mediates toxic effects of pollutants such as PAHs. Although AhR stimulates PAH-detoxifying enzymes, high molecular weight PAHs can be metabolically activated into free radical carcinogens [124].

3. AHRR in diseases

a. *AHRR* in cancer and inflammation

AhR, through its non-genomic pathway, may activate pro-inflammatory and pro-proliferative pathways. In fact, AhR activation is associated with enhanced stimulation of NFκB, epidermal growth factor, and protein kinase A [125].

It is known that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is involved in oncogenesis through AhR whose expression and signaling are enhanced in tumors probably by NFκB and STAT3. A study that was done in mice revealed that CYP1A1 deficient mice had lower mortality rate than controls. However, being an AhR repressor, *AHRR*, may have a tumor-suppressing effect. Indeed, *AHRR* is located on the short arm of chromosome 5, a region that is hypermethylated or deleted in most cancers. In MCF7 breast cancer cells, *AHRR* exerted an inhibitory effect on *ERα* transcription through an AhR-dependent mechanism, suggesting cross-talk between *AHRR* and AhR. In addition to these findings, *AHRR* inhibited the oncogenic effect of AhR/hypoxia-inducible factor-1α (HIF-1α) complex whose activity is enhanced by increased tissue oxygen demands in case of hypoxia or in rapidly growing tumors to ensure appropriate oxygenation [125].

When it comes to skin cancer, *AHRR* acts as tumor-suppressor. Benzo[a]pyrene (BaP) and structurally related PAHs need to undergo CYP1A1 metabolism in order to exert their toxic effects through their active metabolites. Therefore, AhR inhibition by *AHRR* decreases CYP1A1 production and subsequently protects BaP-exposed experimental mice from developing skin cancer. However, long-term carcinogenesis studies revealed that *AHRR* knockout mice had delayed occurrence of BaP-induced skin cancer. Another contradictory role of *AHRR* as a tumor-suppressing gene is its positive

association with lung cancer. Cigarettes contain large amounts of PAHs, those activate AhR on one hand, and cause *AHRR* hypomethylation on the other hand. *AHRR* hypomethylation, however, has been associated with lung cancer. The underlying molecular mechanisms are not well understood, they could involve a cross-talk between *AHRR* and NFκB and EBPβ [125]. Moreover, *AHRR* is involved in regulating the immune response. It prevents exaggerated release of IL-1β and increases ɤ-interferon release by T cells. A study was performed on mice overexpressing *AHRR*, the mice had reduced inflammatory response and an increased toxic response to TCDD compared to controls [125].

b. *AHRR* in atherosclerosis

AHRR hypomethylation has been shown to correlate with atherosclerosis. In fact, Marten A. Siemelink et al [126] have investigated *AHRR* cg05575921 methylation status in carotid plaque lesions in smokers and found significant *AHRR* cg05575921 hypomethylation. Moreover, Robert A. Philibert et al [127] found that *AHRR* cg05575921 methylation status is a predictor of mortality as described by mortality predictors (CAD, stroke, and COPD). Furthermore, Lindsay M. Reynolds et al [128] have found that *AHRR* cg05575921 methylation status decreased significantly in subjects with high carotid plaque score, even after adjusting for smoking status. Another relevant finding proved that *AHRR* when over-expressed, could inhibit anti-inflammatory chemokines which can contribute to atherosclerotic plaque formation [129, 130].

4. Role of smoking in AHRR methylation

Traditionally, smoking status has been based on self-reported data. However, in clinical settings, patients' self-reported status tends to be less reliable. Available tests such as serum or urinary cotinine levels and exhaled carbon monoxide are sensitive and specific biomarkers for smoking. However, given their short half-life, they are unable to detect nascent smokers, those who smoke only periodically, or ex-smokers i.e. they reflect short-term exposure only. Therefore, a more specific test is needed to detect long-term cigarette smoking exposure that goes undetected with the mentioned tests [131, 132].

Several studies have revealed the association of smoking and *AHRR* hypomethylation, specifically in the CpG island in the region of interest (ROI) cg05575921. Therefore, *AHRR* (cg05575921) percent methylation is a newly emerging method to detect smoking status. In fact, *AHRR* has many advantages as a biomarker of smoking status. First, it is highly sensitive, so it can detect low levels of smoking (nascent smokers). Second, it detects second hand smoking. Third, since methylation is a partially reversible process that occurs over a prolonged period of time, it enables *AHRR* (cg05575921) methylation status to detect those who have not smoked in a long time and to provide information on cumulative smoking exposure [131, 132].

Moreover, the smoking-induced *AHRR* hypomethylation increases its expression, leading to increased *AHRR* protein synthesis and extensive ARNT/*AHRR* binding making ARNT less available to bind AhR. This leads to suppression of xenobiotic-metabolizing enzymes synthesis that is normally induced by AhR/ARNT complex signaling. Eventually, this inhibits the metabolism of exogenous dioxin-like compounds [132].

5. Role of pollution in AHRR methylation

In addition to smoking, $PM_{2.5}$ is rich in PAHs and may hence potentially cause *AHRR* cg05575921 hypomethylation. There is, however, a limited number of studies on *AHRR* cg05575921 methylation status as a biomarker of pollution exposure. A study that was done in Taiwan by Disline Manli Tantoh et al [133] showed that Taiwanese adults living in areas with a high level of $PM_{2.5}$ had a lower *AHRR* CpG (cg05575921) methylation status than those living in areas with a lower concentration. Moreover, Tantoh et al [134] showed that $PM_{2,5}$-induced *AHRR* CpG (cg05575921) hypomethylation occurs independently from that of smoking.

As previously mentioned, PMs and tobacco smoke are rich in PAHs. Accordingly, Al Hamdow et al [135] collected urine and blood samples from subjects that had been exposed to creosote and chimney sweeps. On one hand, they found that creosote and chimney sweep-exposed subjects had higher urinary 1-OHPyrene levels than control group who were not exposed. On the other hand, *AHRR* CpG cg05575921 hypomethylation, proven by pyrosequencing, was reported in the study group.

CHAPTER II

SPECIFIC AIMS

Coronary artery disease (CAD) and morbidities from cigarette smoking and pollution are among the leading causes of deaths world-wide. CAD risk factors include pollution exposure and cigarette smoking. Cigarette smoke contains thousands of chemicals like polyaromatic hydrocarbons (PAHs). PAHs are also bound to particulate matter (PM), an air pollutant, as a product of incomplete combustion. Studies have shown that methylation on the CpG site (cg05575921) in the aryl hydrocarbon receptor repressor (*AHRR*) gene is a marker of cigarette smoke exposure as it is hypomethylated among smokers being partially reversible after smoking cessation. Other studies associated this same *AHRR* CpG site hypomethylation in non-smoking subjects exposed to ambient pollution as measured based on geographic distribution. Furthermore, *AHRR* (cg05575921) is hypomethylated in CAD patients. To our knowledge, no studies addressed the association of urinary PAHs metabolites levels, obtained from patients undergoing cardiac catheterization, to *AHRR* CpG site (cg05575921) methylation status.

The aim of this study is to evaluate *AHRR* CpG (cg05575921) methylation status as a sensitive biomarker for ambient PAHs exposure and a risk factor for CAD.

Whole peripheral blood and data of three PAHs urinary metabolites: OH-pyrene, OH-fluorene, and OH-naphthalene are available from subjects admitted to American University of Beirut Medical Center (AUBMC) in the Vascular Medicine Program (VMP) for cardiac catheterization, which is considered the gold standard for CAD assessment. In addition, data on smoking status and CAD status were obtained. DNA was extracted from whole peripheral blood and bisulfite converted. PCR will be performed on bisulfite converted samples followed by melt temperature analysis and

bisulfite sequencing in order to evaluate methylation % and correlate it with urinary OH-PAH levels and CAD.

A schematic representation of CAD, pollution exposure, and cigarette smoking on AHRR methylation status, in addition to the possible involvement of PAH, are illustrated in **Figure 12**.

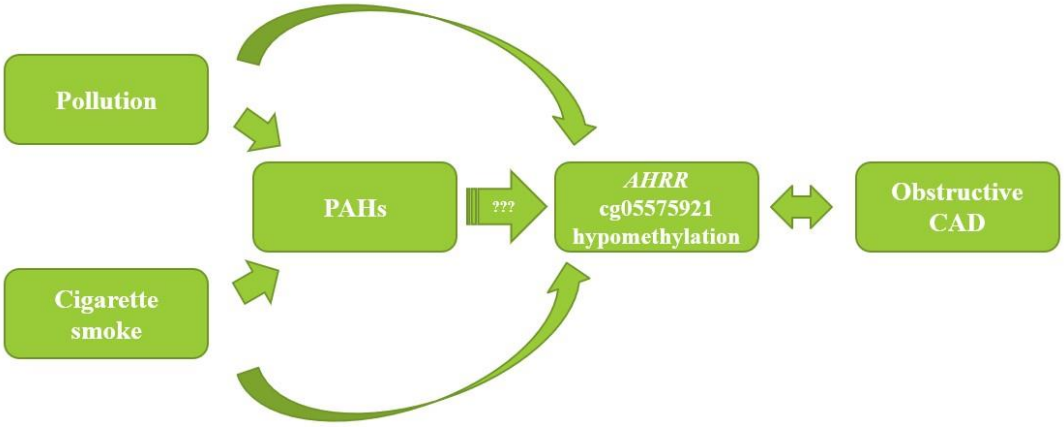


Figure 12 Conceptional framework.

CHAPTER III

METHODS

A. Study participants and clinical data

Of the 1300 recruited subjects at the Vascular Medicine Program (VMP) biorepository, spot urine was available for 315 subjects. Those whose urinary creatinine levels were outside the recommended WHO range of 30-300 mg/dL [136, 137] were excluded. The final sample size was thus 258, among which blood samples for DNA isolation was available for 222 patients.

1. Sample collection

This is a nested case control study. It entails analysis of clinical data and spot urine from subjects who were recruited into a research biorepository by the VMP at the AUBMC upon admission for cardiac catheterization [138]. The biorepository was approved by the institutional review board (IRB) between March 2014 and February 2018. Any subject admitted to the AUBMC for cardiac catheterization was approached. Participants signed an informed consent and agreed that their clinical data and biological samples be used for CVD research. The current study was also reviewed and approved by the IRB.

2. Clinical data

After consent, participants' medical records were reviewed to include age, sex, area of residence, current and past smoking cigarette and waterpipe behavior, and history of comorbid diseases (mainly hypertension, diabetes, and dyslipidemia). In addition, reports of the cardiac catheterization performed on the day of recruitment

were reviewed, and the percentages of obstruction (if present) at each of the coronaries were recorded. Patients with obstructive CAD were defined as those who had at least 50% obstruction in at least one of their coronaries.

Subjects were categorized as never smoker/ever smoker using self-reported questions related to smoking behavior from the medical records.

B. Whole Blood DNA

1. DNA Isolation

DNA was isolated from 1 ml or less, depending on available blood volume, using QIAAmp blood DNA midi kit imported from Qiagen, Germany. Briefly, in order to lyse the cells, blood was mixed with protease and AL buffer. Absolute ethanol was added to ensure lysate binding to the column membrane. Then, two wash buffers were added, AW1 and AW2, to ensure elimination of residual contaminants. Finally, DNA was eluted from the column membrane with buffer AE and its quality and concentration were read on nanodrop spectrophotometer. Isolated DNA was stored at -20°C until bisulfite conversion was performed.

2. Bisulfite Conversion

The extracted DNA samples were treated with multiple reagents for modification, column binding, washing, and elution using Methylamp DNA modification kit imported from Epigentek, USA. The principle of bisulfite conversion entails that unmethylated cytosine is converted to uracil while methylated cytosines remained intact. The converted DNA was stored at -20°C until polymerase chain reaction was performed.

3. Primer design

Primers for bisulfite converted *AHRR* region of interest (ROI) cg05575921 were designed using Methprimer website. The ROI (cg05575921) is located on chr5:373,878bp, and the sequence that was taken for primer design was the addition of 112bp downstream and 237bp upstream from the CpG site. The chosen primers and their characteristics are shown in **Figure 12**.

BSP Pair 1

	Primer name	Sequence (5'-3')	Len	Start	End	Degenerate	CpG's	C's	Self_any	Self_end	Tm	GC%	Stability	Score
Pair	F1	TGTTGGTAGGATATAGGGGTTG	22	122	143	No	0	4	400	0	57.9	45.5	8.2	4.3
	R1	CCTACCAAAACCACTCCCAA	20	315	296	No	0	4	200	0	59.8	50.0	10.0	3.8
	Product size	Pair start	Pair end	CpG's	Comp_any	Comp_end	Tm	Tm_diff			Score			
	194	122	315	7	800	300	72.2	1.9			13.1			

Figure 13 Forward and reverse primers for bisulfite converted *AHRR* ROI.

4. Real-time polymerase chain reaction (PCR)

PCR was performed on CFX96 from Biorad. PCR protocol followed six steps. In step one, samples were subjected to temperature increment until it reached 95°C followed by a plateau for 10 minutes. In step two, temperature remained at 95°C for 15 additional seconds. In step 3, the temperature decreased to reach 58°C and stabilized for one minute. In step 4, step two and three were repeated for thirty-nine cycles. Plate reading was recorded at the end of each cycle. In step 5, melt curve was recorded, with a temperature increase of 0.2°C/0.5 second from 65°C to 95°C. In step 6, the PCR plate was cooled at 4°C for thirty seconds. The PCR mixture consisted of HRM master mix, *AHRR* forward and reverse primers, nuclease-free water, and the bisulfite converted DNA sample. Each PCR well contained 20 µL divided as such: 18 µL from the prepared mixture and 2 µL from each sample with the exception of NTC as it contained 2 µL of nuclease-free water instead.

5. Post-PCR cleanup

In samples not displaying primer-dimer curve on the PCR melt curve, PCR products were taken directly for cleanup using Genelute PCR cleanup kit imported from Sigma, USA. Briefly, samples were treated with binding solution, wash buffer, and eluted with elution solution. The eluate was stored at -20°C until direct bisulfite sequencing was performed.

6. Agarose gel Electrophoresis

In samples displaying primer-dimer curves on the PCR melt curve, samples were taken for gel electrophoresis in order to separate the product of interest from primer-dimer product. Samples were loaded onto 1% agarose gel treated with ethidium bromide and placed in 20% tris-borate ethylenediaminetetraacetic acid. 100 V current was ran for 40 minutes. DNA bands were visualized and cut out of the gel under UV light for purification.

7. Gel purification

DNA bands removed from agarose gel were treated with gel solubilizing solution and heated to dissolve the bands. In addition, the solubilized solution was treated with isopropanol, wash buffer and eluted with water for sequencing using Genelute gel extraction kit imported from Sigma, USA. The eluate was stored at -20°C until direct bisulfite sequencing was performed.

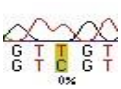
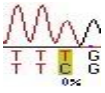
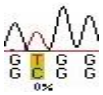
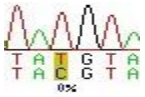
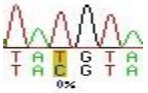
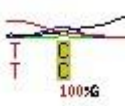
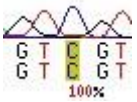
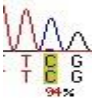
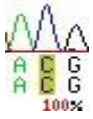
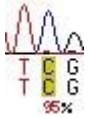
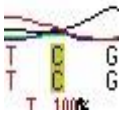
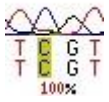
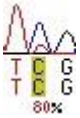
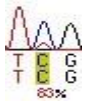
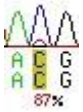
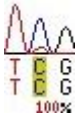
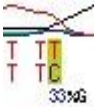
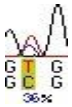
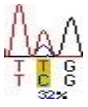
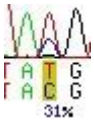
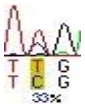
8. Methylation analysis

a. Average methylation and that of position cg05575921

PCR amplicons were sent to Macrogen for direct bisulfite sequencing followed by ESME analysis [139]. The PCR amplicon contains seven CpG sites all of which were analyzed as an average, and position 117 within the PCR amplicon being CpG cg05575921 **(Table 2)**.

Table 2 Representative *AHRR* methylation results.

The CpG site of interest (cg05575921) is highlighted with the red rectangle.

CpG position	**38**	**54**	**92**	**94**	**117**	**137**	**162**
0% methylated control							
100% methylated control							
Never smoker							
Ever smoker							

b. Melting temperature analysis using methylation sensitive high-resolution melting (MS-HRM)

Unfortunately, Precision Melt software was not available at the time of the analysis to draw normalized melt curves. Therefore, melting temperatures from PCR derivative melt peak were recorded and analyzed instead.

C. Statistical analysis

All statistical analyses were carried out using SPSS software version 23.0. Statistical significance was denoted at P value of < 0.05.

Demographic characteristics were described. Continuous variables such as age and average methylation are presented as mean +/- standard deviation (SD), while categorical variables as numbers and percentages.

In order to assess the validity of the melting temperature results with respect to the percent methylation using the most sensitive and specific sequencing-based method, Pearson's correlation was performed to assert correlation among the three methods. In addition, t-test was performed to assert the association of each of the DNA methylation analyses to smoking status.

Then another Pearson correlation was done to evaluate for any potential correlation between each of the PCR melting temperature, average methylation, and that of 117 with three of the studied PAHs: 1-OHPYR, 2-OHNAP, and 2-OHFLU. 3-OHPHE was not included in the analysis due to missing data.

The potential association of each of the melting temperature, methylation at position 117, and average methylation with CAD was performed using Student t-test and non-parametric Mann-Whitney U test after stratification for smoking habits.

CHAPTER IV

RESULTS

A. Demographic characteristics

Whole blood for DNA isolation was available for 222 samples. DNA was extracted from all followed by bisulfite conversion. So far 151 samples were sent to Macrogen for direct bisulfite sequencing. Out of these, sequencing was successful for 77 of them, and *AHRR* MS-HRM PCR melting temperature is available for 119 samples. The below Table shows the baseline demographic characteristics of each cohort **(Table 3)**.

Table 3 Distribution of baseline demographic characteristics in each sub-cohort: those who have *AHRR* MS-HRM PCR melting temperature values and those who have % methylation sequencing values.

Variable	**Values format**	**Those who have MS-HRM PCR melting temperature data (N=119)**	**Those who have methylation % sequencing data (N=77)**
Age (years)	Mean ± SD	62.68 ± 9.51	61.67 ± 9.28
Sex			
Male	N (%)	91 (77.7)	58 (75.3)
Female	N (%)	26 (22.2)	18 (23.4)
Smoking habits			
Never	N (%)	72 (62.1)	49 (65.3)
Ever	N (%)	44 (37.9)	26 (34.7)
Obstructive coronary artery disease			
NO	N (%)	47 (40.2)	33 (43.4)
YES	N (%)	70 (59.8)	43 (56.6)

B. *AHRR* methylation status

As shown in **Figure 13**, **Figure 14**, and **Figure 15**, MS-HRM PCR *AHRR* melting temperature, average methylation %, and that of CpG site cg05575921 all showed statistically significant correlations with that of methylation % at CpG site cg05575921 to average methylation % being the highest.

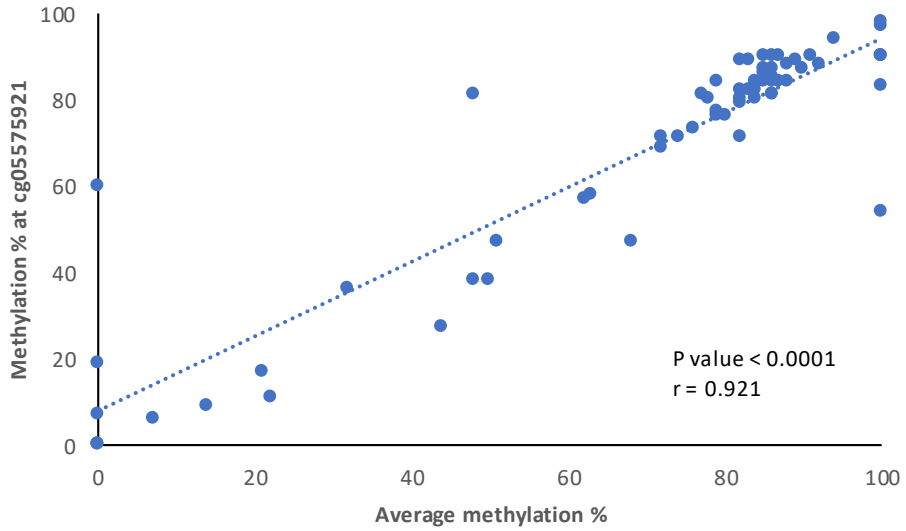


Figure 14 Correlation between *AHRR* methylation % of CpG site cg05575921 and average methylation %.

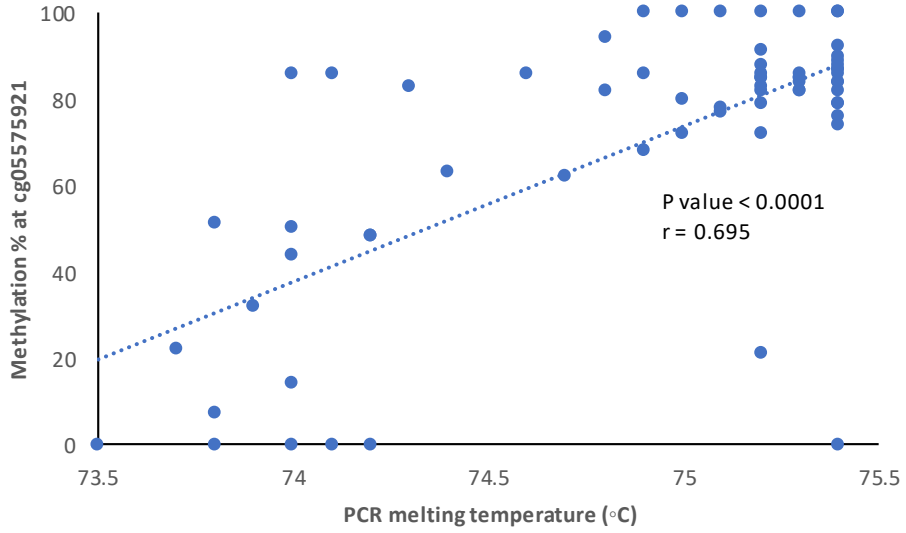


Figure 15 Correlation between *AHRR* methylation % at CpG site cg05575921 and MS-HRM PCR melting temperature.

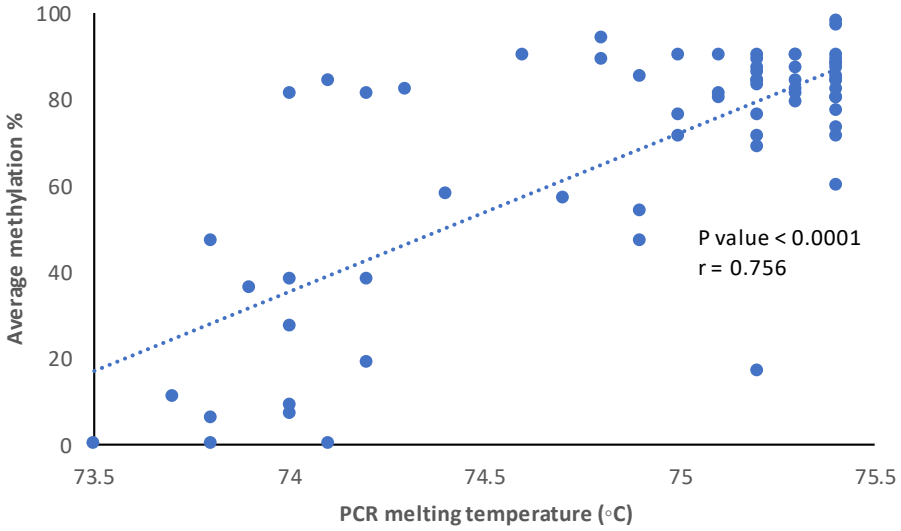


Figure 16 Correlation between *AHRR* average methylation % and MS-HRM PCR melting temperature.

C. Association between urinary OH-PAHs concentration and *AHRR* percent methylation

As shown in **Table 4**, there was no statistically significant correlation between each of MS-HRM PCR *AHRR* melting temperature, % methylation of CpG site cg05575921, nor average methylation % with any of the three studied urinary OH-PAHs in neither of the smoking habits categories.

Table 4 Correlations between each of *AHRR* PCR melting temperature, % methylation of CpG site cg05575921, and average methylation %, and the three studied urinary OH-PAHs metabolites stratified by smoking habits using bivariate Pearson's correlation.

		Never smokers		**Ever smoker**	
		r value	P value	r value	P value
PCR melting temperature with:	2-OHNAP	-0.085	0.495	-0.207	0.183
	2-OHFLU	-0.085	0.495	-0.207	0.183
	1-OHPYR	-0.125	0.368	-0.028	0.864
% methylation at cg05575921 with:	2-OHNAP	0.086	0.570	-0.019	0.927
	2-OHFLU	0.086	0.570	-0.019	0.927
	1-OHPYR	-0.018	0.921	0.218	0.343
Average methylation % with:	2-OHNAP	-0.007	0.962	-0.003	0.991
	2-OHFLU	-0.007	0.962	-0.003	0.991
	1-OHPYR	-0.125	0.480	0.194	0.399

D. Association between *AHRR* methylation and smoking

Methylation results from the three DNA methylation methods' analyses showed statistically significant association with smoking status, whereby ever smokers had significantly lower methylation as compared to never smokers **(Figures 16, 17, 18)**.

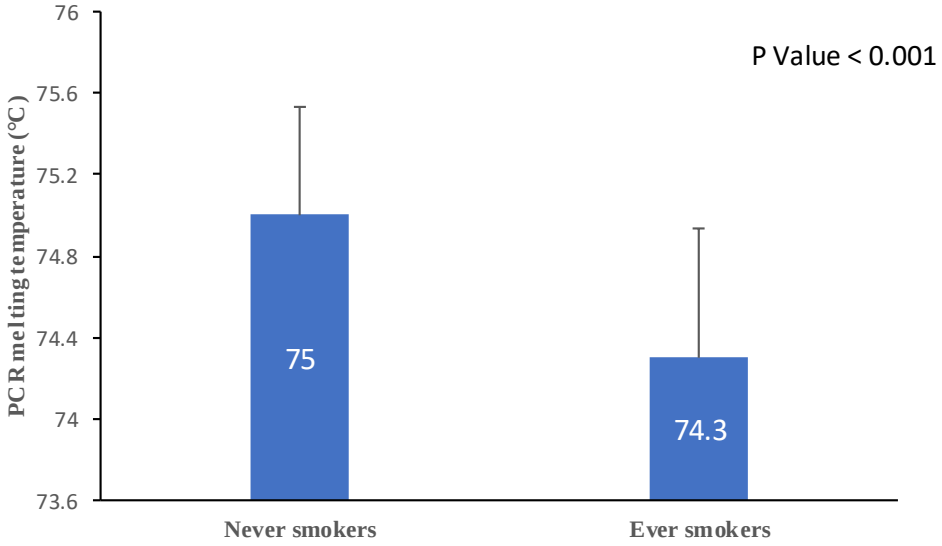


Figure 17 MS-HRM PCR *AHRR* melting temperature by smoking status.

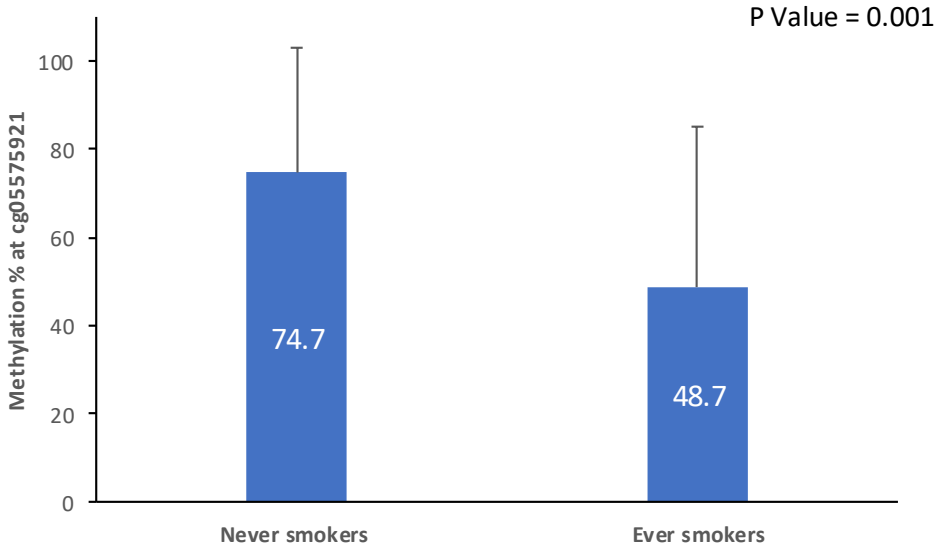


Figure 18 *AHRR* methylation % at cg05575921 by smoking status.

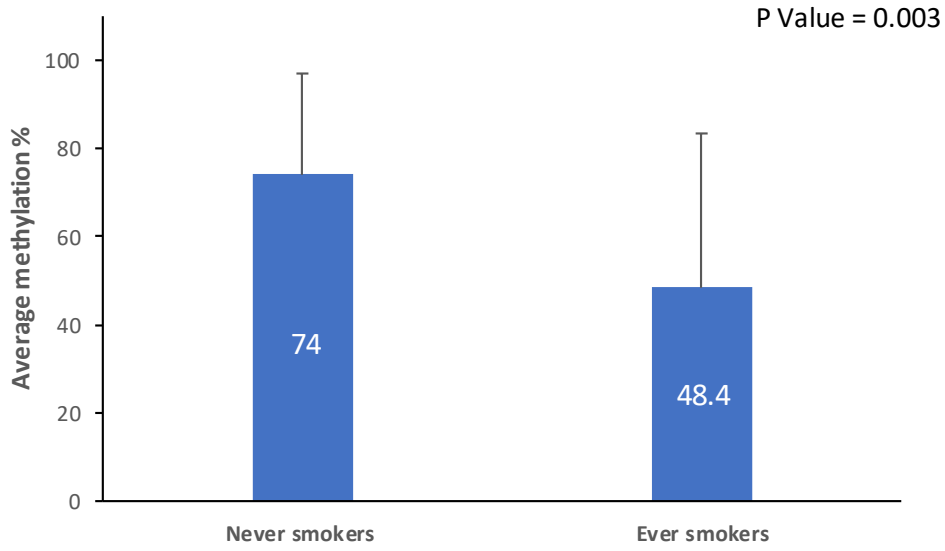


Figure 19 Average *AHRR* methylation % by smoking status.

E. Association between *AHRR* methylation status and CAD

As shown in **Figure 19** and **Figure 20**, though not statistically significant, never smokers had a higher methylation % of CpG site cg05575921 and average methylation % than those of ever smokers. In addition, and within each category, subjects with obstructive CAD had a lower methylation % at CpG cg05575921 and average methylation % than those who have no obstructive CAD.

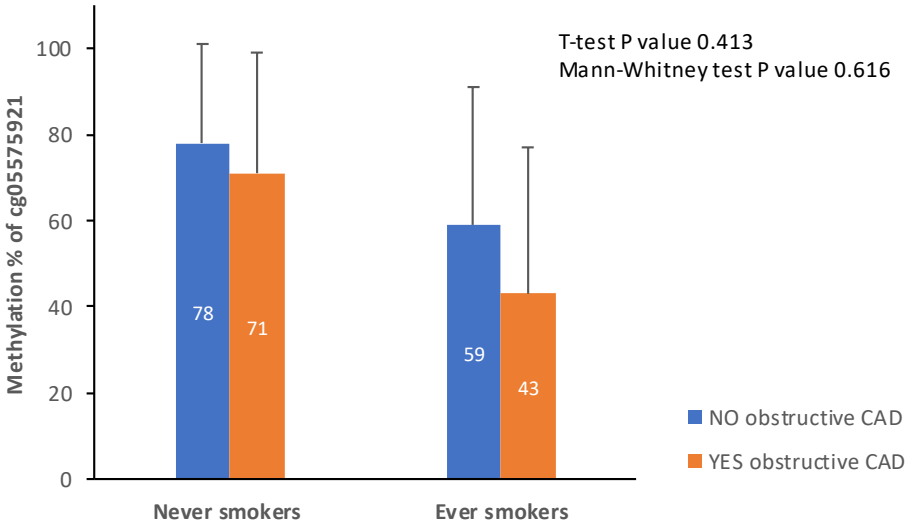


Figure 19 Association of methylation % at CpG site cg05575921 with obstructive coronary artery disease (CAD) status stratified by smoking habits.

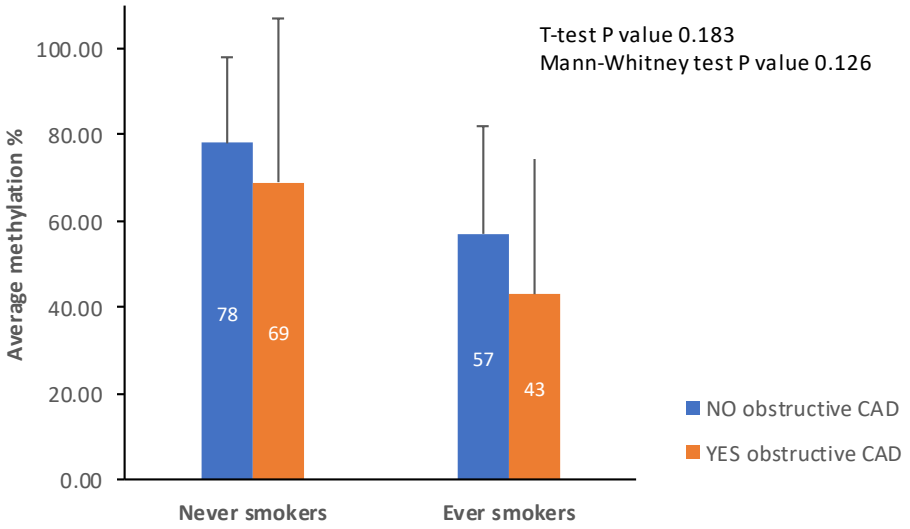


Figure 20 Association of average methylation % with obstructive coronary artery disease (CAD) status stratified by smoking habits.

No similar trends were shown with MS-HRM PCR melting temperature (**Figure 21).**

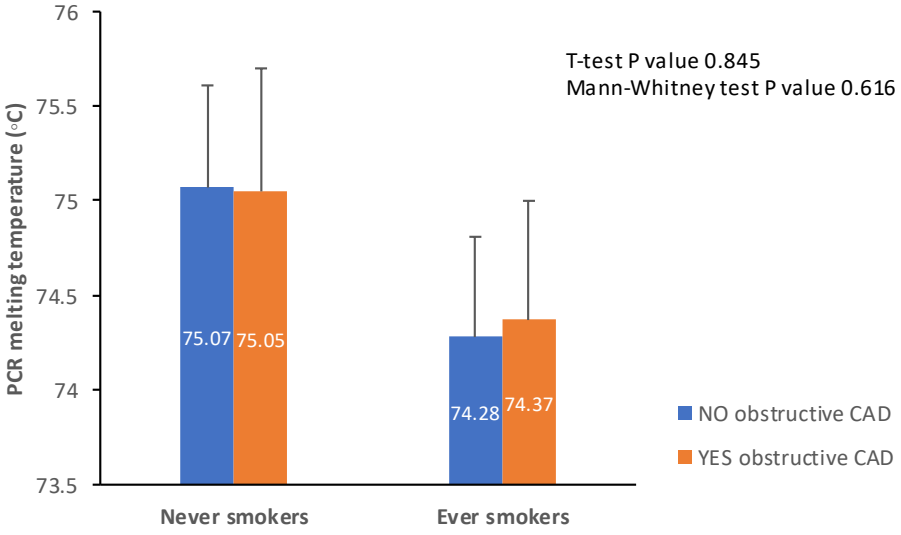


Figure 21 Association of MS-HRM PCR melting temperature with obstructive CAD status stratified by smoking habits.

CHAPTER V

DISCUSSION

Coronary artery disease (CAD) is the leading cause of death world-wide [1]. Its risk factors include cigarette smoke [1] and pollution exposure, as reported in our previous study on the association of urinary OH-PAHs level with CAD [2]. Cigarette smoke contains thousands of chemicals, and PAHs are one example [83]. These PAHs are also found on particulate matter, one of the major air pollutants, as a product of incomplete combustion [41, 43]. Aromatic hydrocarbon receptor repressor (*AHRR*) gene methylation % at CpG site (05575921) is an established sensitive biomarker for cigarette smoke exposure [131, 132] and a potential biomarker for atherosclerotic disease [128, 130]. Few recent studies have also shown an association between *AHRR* CpG site (cg05575921) hypomethylation in non-smoking subjects exposed to ambient air pollution based on demographical distribution [133, 134].

In our study, and as expected, ever smokers showed more *AHRR* cg05575921 hypomethylation when compared to never smokers, which is consistent with other studies [131, 132].

Moreover, methylation % of CpG cg05575921, average methylation % and MS-HRM PCR melting temperature showed significant inter-correlations. Of course, *AHRR* cg05575921 is the most sensitive *AHRR* CpG site to cigarette smoke exposure [140] and reflects average methylation within the sequenced PCR segment, hence its strongest correlation with average % methylation. PCR melting temperature also showed statistically significant correlations with the mentioned methylation % due to the fact that G-C base pairs have more hydrogen bonding than A-T base pairs thus they require higher denaturation temperature in order to break. Therefore, unmethylated bisulfite

converted CpG sites, that were converted to A-T during PCR, have a lower % methylation.

Reynolds et al [128] reported *AHRR* cg05575921 hypomethylation in subjects with carotid plaque lesions even after adjusting for smoking, consistent with our findings where there was a trend of lower *AHRR* cg05575921 % methylation and average % methylation in subjects with obstructive CAD when compared to subjects with no obstructive CAD in the never smoker sub-cohort. This finding was not observed in the case of MS-HRM PCR melting temperature probably because ideally, DNA methylation analysis is based on normalized MS-HRM melt curves obtained from raw fluorescence curves [141], which was not applicable in our case since Precision Melt software was not available at the time of melt curves analysis. Another possible explanation is that PCR melting temperature is not as sensitive and as specific as direct bisulfite sequencing when it comes to DNA methylation analysis. Moreover, PCR melting temperature data can be affected by single nucleotide polymorphisms, which is not the case for sequencing data.

In addition, never smokers but with obstructive CAD did not show complete % methylation at cg05575921 suggesting that PAHs exposure from a source other than cigarette smoking may have caused this hypomethylation. In our study, however, neither of the three methods of methylation analyses' results showed association with the three studied urinary OH-PAHs and this could be explained by multiple reasons. First, our sample size with available sequencing data was small (77 samples). Second, OH-PAHs have a short half-life (2.5h to 6.1h) so they reflect acute exposure, while *AHRR* methylation status reflects mainly chronic exposure. For instance, a population could be exposed to PAHs two days ago but not at the time of urine collection. Third,

we used direct bisulfite sequencing which is not the gold standard for DNA methylation quantification, unlike pyrosequencing [142]. Nevertheless, Alhamdow et al [135] performed pyrosequencing to assess *AHRR* cg05575921 methylation status and correlated it with urinary 1-OHPYR level in samples collected from workers occupationally exposed to PAHs sources (chimney sweeps and creosote). Similarly, although there was a significant decrease in the *AHRR* cg05575921 methylation in exposed workers, there was no association of *AHRR* cg05575921 methylation status with the level of urinary 1-OHPYR. Therefore, long-term PAHs exposure studies may be needed in order to asses AHRR cg05575921 methylation % in response to chronic PAHs exposure.

CHAPTER VI

CONCLUSION

AHRR cg05575921 is an established biomarker of cigarette smoke exposure and is hypomethylated in pollution exposure. Cigarette smoke and air pollution contain PAHs and are two risk factors of obstructive coronary artery disease (CAD). *AHRR* cg05575921 is also hypomethylated in obstructive CAD as it shows in the trends of hypomethylation in our study. Preliminary data in our study show no correlation between *AHRR* methylation status and PAHs exposure as measured from urinary metabolites. Nevertheless, our results show that never smokers with obstructive CAD do not have complete *AHRR* cg05575921 methylation, suggesting that exposure to PAH (a proven CAD risk factor) had occurred from a source other than smoking. We hope that with further experiments on the rest of the cohort a significant association would be established.

CHAPTER VII

LIMITATIONS

Limitations of our study fall within the fact that urinary OH-PAHs levels were recorded from spot urine samples. Since OH-PAHs are short-lived, their urinary level reflects acute exposure, while *AHRR* epigenetic changes occur over a prolonged period of time. In addition, melting temperature was analyzed based on PCR derivative melt peak temperature rather than analyzing it from MS-HRM normalized melt curve because of the unavailability of the software, and the more specific and sensitive sequencing-based method has been so far successful on a smaller subset of samples (77 samples). Because of the small sample size, we did not perform multivariate analysis to adjust for age, sex, and other CAD risk factors such as hypertension, dyslipidemia, and diabetes mellitus type 2, an analysis that will be performed at a later stage. In addition, data on the research subjects' diet and exercise habits is lacking. Finally, samples were sequenced with direct bisulfite sequencing which is not the gold standard, unlike pyrosequencing.

CHAPTER VIII

FUTURE PLANS

We will continue the experimental work on the rest of the samples of the cohort. As sample size increases, we hope to get a significant association of the studied OH-PAHs with *AHRR* cg05575921 methylation status. In addition, we will be able to perform multivariate analysis to adjust for variables such as sex, age, and other CAD risk factors (hypertension, dyslipidemia, and diabetes mellitus type 2). We hope to perform prospective studies on the association between ambient chronic PAHs exposure and *AHRR* cg05575921 methylation status, and to study the association of other particulate matter contents or other air pollutants, that could also pre-dispose to obstructive CAD, on the methylation status of *AHRR* cg05575921.

BIBLIOGRAPHY

1. Brown, J.C., T.E. Gerhardt, and E. Kwon, *Risk Factors For Coronary Artery Disease*, in *StatPearls*. 2021: Treasure Island (FL).
2. Hajir, S., et al., *The association of urinary metabolites of polycyclic aromatic hydrocarbons with obstructive coronary artery disease: A red alert for action*. Environ Pollut, 2021. **272**: p. 115967.
3. Malakar, A.K., et al., *A review on coronary artery disease, its risk factors, and therapeutics*. J Cell Physiol, 2019. **234**(10): p. 16812-16823.
4. Lee, H.T., et al., *The Key Role of DNA Methylation and Histone Acetylation in Epigenetics of Atherosclerosis*. J Lipid Atheroscler, 2020. **9**(3): p. 419-434.
5. European Society of Cardiology. *2019 Guidelines on Chronic Coronary Syndromes*. 2019; Available from: https://www.escardio.org/Guidelines/Clinical-Practice-Guidelines/Chronic-Coronary-Syndromes.
6. Rafieian-Kopaei, M., et al., *Atherosclerosis: process, indicators, risk factors and new hopes*. Int J Prev Med, 2014. **5**(8): p. 927-46.
7. Association, A.H. *Unstable Angina*. 2015, July 31; Available from: https://www.heart.org/en/health-topics/heart-attack/angina-chest-pain/unstable-angina.
8. Christian W. Hamm, E.B., *A Classification of Unstable Angina Revisited*. American Heart Association, 2000, July 4. **102**(1).
9. Jonathan Abrams, U.T., *Therapy of Stable Angina Pectoris*. American Heart Association, 2005, October 11. **112**.
10. Association, A.H., *Acute Coronary Syndrome*. 2005, July 31.
11. Association, A.H., *Cardiac catheterization*. American Heart Association, 2015, July 31.
12. National Heart, L., and Blood Institute. *Cardiac Catheterization*. Available from: https://www.nhlbi.nih.gov/health-topics/cardiac-catheterization.
13. Sarazan, R., *The QT Interval of the Electrocardiogram*. Elsevier, 2014. **3**: p. 6.
14. Adam Pleister, H.S., Shane M Elton, Terry Elton, *Circulating miRNAs: Novel biomarkers of acute coronary syndrome?* 2013.
15. Blick, D., *Cardiac Troponin Testing*. American Heart Association, 2019.
16. Vinay S. Mahajan, P.J., *How to Interpret Elevated Cardiac Troponin Levels*. American Heart Association, 2011, November 22. **124**.
17. Rahul Kurapati, M.P.S., *CPK-MB*. 2021, April 25.
18. Peter M. Guzy, *Creatine Phosphokinase-MB (CPK-MB) and the Diagnosis of Myocardial Infarction*. The Western Journal of Medicine, 1977: p. 6.
19. Gerald F. Fletcher, P.A.A., Paul Kligfield, Ross Arena, Gary J. Balady, Vera A. Bittner, Lola A. Coke, Jerome L. Fleg, Daniel E. Forman, Thomas C. Gerber, Martha Gulati, Kushal Madan, Jonathan Rhodes, Paul D. Thompson, and Mark A. Williams, *Exercise Standards for Testing and Training*. American Heart Association, 2013.
20. Beckerman, J. *Heart Disease and Stress Tests*. 2020, November 6; Available from: https://www.webmd.com/heart-disease/stress-test#:~:text=The%20exercise%20stress%20test%20%2D%2D,more%20difficult%20as%20you%20go.

21. Association, A.H. *Myocardial Perfusion Imaging (MPI) Test*. 2015, July 31; Available from: https://www.heart.org/en/health-topics/heart-attack/diagnosing-a-heart-attack/myocardial-perfusion-imaging-mpi-test.
22. al., R.A.O.R.e., *American College of Cardiology/American Heart Association Expert Consensus Document on Electron-Beam Computed Tomography for the Diagnosis and Prognosis of Coronary Artery Disease*. 2000, July.
23. Rai Dilawar Shahjehan, B.S.B., *Coronary Artery Disease*. 2021, November 14.
24. Shaw, J. and T. Anderson, *Coronary endothelial dysfunction in non-obstructive coronary artery disease: Risk, pathogenesis, diagnosis and therapy*. Vasc Med, 2016. **21**(2): p. 146-55.
25. al., V.K.e., *An EAPCI Expert Consensus Document on Ischaemia with Non-Obstructive Coronary Arteries in Collaboration with European Society of Cardiology Working Group on Coronary Pathophysiology & Microcirculation Endorsed by Coronary Vasomotor Disorders International Study Group*. European Heart Journal, 2020.
26. Sughosh Madhav, A.A., Amit K. Singh, Jyoti KushawahaJoginder Singh Chauhan, Sanjeev Sharma, Pardeep Singh, *Water Pollutants: Sources and Impact on the Environment and Human Health*. 2019, October 12.
27. Khatun, R., *Water Pollution: Causes, Consequences, Prevention Method and Role of WBPHED with Special Reference from Murshidabad District*. International Journal of Scientific and Research Publications,, 2017, August. **7**(8).
28. Chaudhry FN, M.M., *Factors Affecting Water Pollution: A Review*. Journal of Ecosystem & Ecography, 2017.
29. Mehtab Haseena, M.F.M., Asma Javed, Sidra Arshad, Nayab Asif, Sharon and Z.a.J. Hanif, *Water pollution and human health*. 2017, July 13.
30. Walker, R., *Nitrates, nitrites and N-nitrosocompounds: a review of the occurrence in food and diet and the toxicological implications*. Food Addit Contam, 1990. **7**(6): p. 717-68.
31. Mirsal, I.A., *Soil Pollution Origin, Monitoring & Remediation*. second ed. 2004.
32. Rajesh Kumar Mishra, N.M., N. Roychoudhury *Soil pollution: Causes, effects and control*. 2016, January.
33. Nasser, Z., et al., *Outdoor air pollution and cardiovascular diseases in Lebanon: a case-control study*. J Environ Public Health, 2015. **2015**: p. 810846.
34. Simkhovich, B.Z., M.T. Kleinman, and R.A. Kloner, *Air pollution and cardiovascular injury epidemiology, toxicology, and mechanisms*. J Am Coll Cardiol, 2008. **52**(9): p. 719-26.
35. van der Wall, E.E., *Air pollution: 6.6 million premature deaths in 2050!* Neth Heart J, 2015. **23**(12): p. 557-8.
36. (WHO), W.H.O. *Ambient (outdoor) air pollution*. 2021, September 22; Available from: https://www.who.int/news-room/fact-sheets/detail/ambient-(outdoor)-air-quality-and-health.
37. Rai, P.K., *Multifaceted health impacts of Particulate Matter (PM) and its management: An overview* 2015.
38. Manisalidis, I., et al., *Environmental and Health Impacts of Air Pollution: A Review*. Front Public Health, 2020. **8**: p. 14.
39. Marris, C.R., et al., *Polyaromatic hydrocarbons in pollution: a heart-breaking matter*. J Physiol, 2020. **598**(2): p. 227-247.

40. Farah, W., et al., *Time series analysis of air pollutants in Beirut, Lebanon.* Environ Monit Assess, 2014. **186**(12): p. 8203-13.
41. Kim, K.H., E. Kabir, and S. Kabir, *A review on the human health impact of airborne particulate matter.* Environ Int, 2015. **74**: p. 136-43.
42. Cui, P., et al., *Ambient particulate matter and lung cancer incidence and mortality: a meta-analysis of prospective studies.* Eur J Public Health, 2015. **25**(2): p. 324-9.
43. Li, J., et al., *Particulate matter-induced epigenetic changes and lung cancer.* Clin Respir J, 2017. **11**(5): p. 539-546.
44. Li, R., R. Zhou, and J. Zhang, *Function of PM2.5 in the pathogenesis of lung cancer and chronic airway inflammatory diseases.* Oncol Lett, 2018. **15**(5): p. 7506-7514.
45. Suwa, T., et al., *Particulate air pollution induces progression of atherosclerosis.* J Am Coll Cardiol, 2002. **39**(6): p. 935-42.
46. Association, A.L. *Ozone*. 2020, April 20; Available from: https://www.lung.org/clean-air/outdoors/what-makes-air-unhealthy/ozone.
47. Zhang, J.J., Y. Wei, and Z. Fang, *Ozone Pollution: A Major Health Hazard Worldwide*. Front Immunol, 2019. **10**: p. 2518.
48. Agency, U.E.P. *Carbon Monoxide's Impact on Indoor Air Quality*. Available from: https://www.epa.gov/indoor-air-quality-iaq/carbon-monoxides-impact-indoor-air-quality.
49. Agency, U.E.P. *Overview of Greenhouse Gases*. Available from: https://www.epa.gov/ghgemissions/overview-greenhouse-gases.
50. Hussein I. Abdel-shafy, M.S.M.M., *A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation,*. Egyptian Journal of Petroleum,, 2015, December 18.
51. Kleiböhmer, W., *Polycylic aromatic hydrocarbon (PAH) metabolites. Handbook of Analytical Separations,* . Vol. Volume 3, Environmental Analysis. 2001.
52. Debra A. Kaden, P.D., Kerrie Canavan, Farah Chowdhury, Joshua Gambrell, Greg Yarwood, Ph.D., Allison Glessner, Taylor Roumeliotis, Ph.D., Mike Jammer, Ted Pollock, Ph.D., P. Eng., *Assessment Report on Naphthalene for Developing Ambient Air Quality Objectives 2013 Update*. 2015.
53. Jia, C. and S. Batterman, *A critical review of naphthalene sources and exposures relevant to indoor and outdoor air.* Int J Environ Res Public Health, 2010. **7**(7): p. 2903-39.
54. Gad, S.E., *Phenanthrene*, in *Encyclopedia of Toxicology*. 2014, Elsevier.
55. Agency, U.S.E.P., *Pyrene*.
56. Ding, Y.S., et al., *Determination of 14 polycyclic aromatic hydrocarbons in mainstream smoke from domestic cigarettes.* Environ Sci Technol, 2005. **39**(2): p. 471-8.
57. al., Y.L.e., *Determination of Urinary Hydroxyl PAHs Using Graphene Oxide@Diatomite Based Solid-Phase Extraction and High-Performance Liquid Chromatography.* 2019.
58. al, A.L.e., *Metabolites of the PAH diol epoxide pathway and other urinary biomarkers of phenanthrene and pyrene in workers with and without exposure to bitumen fumes.* 2016.

59. al., B.J.-R.e., *Polycyclic aromatic hydrocarbons and their hydroxylated metabolites in fish bile and sediments from coastal waters of Colombia*. Elsevier, 2008.
60. Ariese, F., J. Beyer, and D. Wells, *Two fish bile reference materials certified for PAH metabolites*. J Environ Monit, 2005. **7**(9): p. 869-76.
61. Buratti, M., et al., *Urinary hydroxylated metabolites of polycyclic aromatic hydrocarbons as biomarkers of exposure in asphalt workers*. Biomarkers, 2007. **12**(3): p. 221-39.
62. Li, Z., et al., *Variability of urinary concentrations of polycyclic aromatic hydrocarbon metabolite in general population and comparison of spot, first-morning, and 24-h void sampling*. J Expo Sci Environ Epidemiol, 2013. **23**(1): p. 109-10.
63. Saengtienchai, A., et al., *Characterization and tissue distribution of conjugated metabolites of pyrene in the rat*. J Vet Med Sci, 2015. **77**(10): p. 1261-7.
64. Shimada, T. and Y. Fujii-Kuriyama, *Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1*. Cancer Sci, 2004. **95**(1): p. 1-6.
65. Moorthy, B., C. Chu, and D.J. Carlin, *Polycyclic aromatic hydrocarbons: from metabolism to lung cancer*. Toxicol Sci, 2015. **145**(1): p. 5-15.
66. Reynaud, S. and P. Deschaux, *The effects of polycyclic aromatic hydrocarbons on the immune system of fish: a review*. Aquat Toxicol, 2006. **77**(2): p. 229-38.
67. Kawabata, T.T. and K.L. White, Jr., *Suppression of the vitro humoral immune response of mouse splenocytes by benzo(a)pyrene metabolites and inhibition of benzo(a)pyrene-induced immunosuppression by alpha-naphthoflavone*. Cancer Res, 1987. **47**(9): p. 2317-22.
68. Gorlach, A., et al., *Calcium and ROS: A mutual interplay*. Redox Biol, 2015. **6**: p. 260-271.
69. Madeen, E.P. and D.E. Williams, *Environmental PAH exposure and male idiopathic infertility: a review on early life exposures and adult diagnosis*. Rev Environ Health, 2017. **32**(1-2): p. 73-81.
70. Zhao, Y., et al., *Exposure of mice to benzo(a)pyrene impairs endometrial receptivity and reduces the number of implantation sites during early pregnancy*. Food Chem Toxicol, 2014. **69**: p. 244-51.
71. Alshaarawy, O., H.A. Elbaz, and M.E. Andrew, *The association of urinary polycyclic aromatic hydrocarbon biomarkers and cardiovascular disease in the US population*. Environ Int, 2016. **89-90**: p. 174-8.
72. He, L., et al., *Nitrated Polycyclic Aromatic Hydrocarbons and Arachidonic Acid Metabolisms Relevant to Cardiovascular Pathophysiology: Findings from a Panel Study in Healthy Adults*. Environ Sci Technol, 2021. **55**(6): p. 3867-3875.
73. Wu, H., et al., *Endothelial barrier dysfunction induced by anthracene and its nitrated or oxygenated derivatives at environmentally relevant levels*. Sci Total Environ, 2022. **802**: p. 149793.
74. Jacqueline M. R. B61anger, J.R.J.P., and Michel Sigouin, *High Performance Liquid Chromatography (HPLC): Principles and Applications* Elsevier 1997.
75. Czaplicki, S., *Chromatography in Bioactivity Analysis of Compounds*. 2013, April.
76. Harold M. McNair, J.M.M., and Nicholas H. Snow, *Basic Gas Chromatography*. 2019.

77. Chauhan, A., *GC-MS*. 2014, November.
78. West, R., *Tobacco smoking: Health impact, prevalence, correlates and interventions*. Psychol Health, 2017. **32**(8): p. 1018-1036.
79. Talhout, R., et al., *Hazardous compounds in tobacco smoke*. Int J Environ Res Public Health, 2011. **8**(2): p. 613-28.
80. Zana El-Roueiheb, H.T., Mayada Kanj, Samer Jabbour, Iman Alayan, Umayya Musharrafieh, *Cigarette and waterpipe smoking among Lebanese adolescents, a cross-sectional study, 2003–2004*. 2008, February. **10**.
81. FDA. *Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke: Established List*. 2012, April; Available from: https://www.fda.gov/tobacco-products/rules-regulations-and-guidance/harmful-and-potentially-harmful-constituents-tobacco-products-and-tobacco-smoke-established-list.
82. Society, A.C. *Harmful Chemicals in Tobacco Products*. 2020, October 28; Available from: https://www.cancer.org/content/dam/CRC/PDF/Public/8344.00.pdf.
83. Vu, A.T., et al., *Polycyclic Aromatic Hydrocarbons in the Mainstream Smoke of Popular U.S. Cigarettes*. Chem Res Toxicol, 2015. **28**(8): p. 1616-26.
84. Organization, W.H. *Tobacco responsible for 20% of deaths from coronary heart disease*. 2020, September 22; Available from: https://www.who.int/news/item/22-09-2020-tobacco-responsible-for-20-of-deaths-from-coronary-heart-disease.
85. Craig, W.Y., G.E. Palomaki, and J.E. Haddow, *Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data*. BMJ, 1989. **298**(6676): p. 784-8.
86. Csiszar, A., et al., *Oxidative stress and accelerated vascular aging: implications for cigarette smoking*. Front Biosci (Landmark Ed), 2009. **14**: p. 3128-44.
87. Gallucci, G., et al., *Cardiovascular risk of smoking and benefits of smoking cessation*. J Thorac Dis, 2020. **12**(7): p. 3866-3876.
88. Onor, I.O., et al., *Clinical Effects of Cigarette Smoking: Epidemiologic Impact and Review of Pharmacotherapy Options*. Int J Environ Res Public Health, 2017. **14**(10).
89. UK, C.R. *How does smoking cause cancer?* 2021, March 19; Available from: https://www.cancerresearchuk.org/about-cancer/causes-of-cancer/smoking-and-cancer/how-does-smoking-cause-cancer#tobaccorefs0.
90. Yamaguchi, N.H., *Smoking, immunity, and DNA damage*. Transl Lung Cancer Res, 2019. **8**(Suppl 1): p. S3-S6.
91. (CDC), C.f.D.C.a.P. *Smoking and COPD*. 2021, February 15; Available from: https://www.cdc.gov/tobacco/campaign/tips/diseases/copd.html.
92. Laniado-Laborin, R., *Smoking and chronic obstructive pulmonary disease (COPD). Parallel epidemics of the 21 century*. Int J Environ Res Public Health, 2009. **6**(1): p. 209-24.
93. Priyamvada Sharma, N.S., Shravanthi D. Anand, P Marimutthu, and Vivek Benegal, *Assessment of cotinine in urine and saliva of smokers, passive smokers, and nonsmokers: Method validation using liquid chromatography and mass spectrometry*. Indian Journal of Psychiatry 2019.
94. Ana Florescu, M., Roberta Ferrence, PhD, Tom Einarson, PhD, Peter Selby, MD, Offie Soldin, PhD, MBA and Gideon Koren, MD, *Methods for*

Quantification of Exposure to Cigarette Smoking and Environmental Tobacco Smoke: Focus on Developmental Toxicology. 2013, May 5.
95. AnnSofi Sandberg, C.M.S., Johan Grunewald, Anders Eklund, Asa M. Wheelock, *Assessing Recent Smoking Status by Measuring Exhaled Carbon Monoxide Levels*. 2011, December 16.
96. Weinhold, B., *Epigenetics: the science of change.* Environ Health Perspect, 2006. **114**(3): p. A160-7.
97. Moore, L.D., T. Le, and G. Fan, *DNA methylation and its basic function.* Neuropsychopharmacology, 2013. **38**(1): p. 23-38.
98. SKINNER, M.D.A.A.S.C.M.U.A.M.K., *Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility*. 2005, June 3.
99. Singal, R. and G.D. Ginder, *DNA methylation*. Blood, 1999. **93**(12): p. 4059-70.
100. Zhang, Y.P., et al., *The Mammalian Target of Rapamycin and DNA methyltransferase 1 axis mediates vascular endothelial dysfunction in response to disturbed flow*. Sci Rep, 2017. **7**(1): p. 14996.
101. Yu, J., et al., *DNMT1-PPARgamma pathway in macrophages regulates chronic inflammation and atherosclerosis development in mice*. Sci Rep, 2016. **6**: p. 30053.
102. Singh KK, S.P., Quan A, Al-Omran M, Lovren F, Pan Y, et al, *BRCA1 is a novel target to improve endothelial dysfunction and retard atherosclerosis.* Elsevier, 2013, February 14.
103. al, G.I.e., *Identification of differentially methylated BRCA1 and CRISP2 DNA regions as blood surrogate markers for cardiovascular disease*. 2017, July 11.
104. Tabaei, S. and S.S. Tabaee, *DNA methylation abnormalities in atherosclerosis.* Artif Cells Nanomed Biotechnol, 2019. **47**(1): p. 2031-2041.
105. O'Hagan, H.M., et al., *Oxidative damage targets complexes containing DNA methyltransferases, SIRT1, and polycomb members to promoter CpG Islands.* Cancer Cell, 2011. **20**(5): p. 606-19.
106. Andrea Baccarelli, V.B., *Baccarelli A, Bollati V. Epigenetics and environmental chemicals*. 2011, February 9.
107. Peng J, Y.Q., Li AF, Li RQ, Wang Z, Liu LS, et a, *Tet methylcytosine dioxygenase 2 inhibits atherosclerosis via upregulation of autophagy in ApoE−/− mice.* 2016, November 22.
108. Larigot, L., et al., *AhR signaling pathways and regulatory functions*. Biochim Open, 2018. **7**: p. 1-9.
109. Calo, M., et al., *Role of AHR, AHRR and ARNT in response to dioxin-like PCBs in Spaurus aurata.* Environ Sci Pollut Res Int, 2014. **21**(24): p. 14226-31.
110. Evans, B.R., et al., *Repression of aryl hydrocarbon receptor (AHR) signaling by AHR repressor: role of DNA binding and competition for AHR nuclear translocator*. Mol Pharmacol, 2008. **73**(2): p. 387-98.
111. Haarmann-Stemmann, T. and J. Abel, *The arylhydrocarbon receptor repressor (AhRR): structure, expression, and function.* Biol Chem, 2006. **387**(9): p. 1195-9.
112. Ma, Q., *Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles.* Curr Drug Metab, 2001. **2**(2): p. 149-64.

113. Yunxia Fan, G.P.B., Erik S. Knudsen, Daniel W. Nebert, Ying Xia, and Alvaro Puga, *The Ah receptor has a tumor suppressor function in liver carcinogenesis*. 2010.
114. Yunxia Fan, G.P.B., Erik S. Knudsen, Daniel W. Nebert, Ying Xia and Alvaro Puga, *The Aryl Hydrocarbon Receptor Functions as a Tumor Suppressor of Liver Carcinogenesis*. American Association for Cancer Research, 2010.
115. Stephen Safe, Y.C., and Un-Ho Jin, *The Aryl Hydrocarbon Receptor (AhR) as a Drug Target for Cancer Chemotherapy*. 2017.
116. Isabel Hernandez-Ochoa, K.R.B.-R., 4 Stacey L. Dehlinger, Rupesh K. Gupta,Traci C. Leslie, Katherine F. Roby, and Jodi A. Flaws, *The Ability of the Aryl Hydrocarbon Receptor to Regulate Ovarian Follicle Growth and Estradiol Biosynthesis in Mice Depends on Stage of Sexual Maturity*. 2010, July 14.
117. Kimberly R. Barnett, D.T., Rupesh K. Gupta, Kimberly P. Miller, Sharon Meachum,Tessie Paulose, and Jodi A. Flaws, *The Aryl Hydrocarbon Receptor Affects Mouse Ovarian Follicle Growth via Mechanisms Involving Estradiol Regulation and Responsiveness*. 2007, February 28.
118. Takashi Baba, J.M., Naohito Nakamura, Nobuhiro Harada, Masayuki Yamamoto, Ken-ichirou Moroha, *Intrinsic Function of the Aryl Hydrocarbon (Dioxin) Receptor as a Key Factor in Female Reproduction.* American Society for Microbiology, 2005, November.
119. Hernandez-Ochoa, I., B.N. Karman, and J.A. Flaws, *The role of the aryl hydrocarbon receptor in the female reproductive system.* Biochem Pharmacol, 2009. **77**(4): p. 547-59.
120. Vezina, C.M., T.M. Lin, and R.E. Peterson, *AHR signaling in prostate growth, morphogenesis, and disease*. Biochem Pharmacol, 2009. **77**(4): p. 566-76.
121. Fritz, W.A., et al., *The aryl hydrocarbon receptor inhibits prostate carcinogenesis in TRAMP mice*. Carcinogenesis, 2007. **28**(2): p. 497-505.
122. Ohtake, F., et al., *Intrinsic AhR function underlies cross-talk of dioxins with sex hormone signalings*. Biochem Biophys Res Commun, 2008. **370**(4): p. 541-6.
123. Yi, T., et al., *Aryl Hydrocarbon Receptor: A New Player of Pathogenesis and Therapy in Cardiovascular Diseases.* Biomed Res Int, 2018. **2018**: p. 6058784.
124. Yanli Liu, H.Z., Huitao Zhang, Yingying Niu, Ye Fu, Jisheng Nie, Aimin Yang, Jinzhu Zhao, Jin Yang *Mediation effect of AhR expression between polycyclic aromatic hydrocarbons exposure and oxidative DNA damage among Chinese occupational workers.* Elsevier, 2018, December.
125. Vogel, C.F.A. and T. Haarmann-Stemmann, *The aryl hydrocarbon receptor repressor - More than a simple feedback inhibitor of AhR signaling: Clues for its role in inflammation and cancer*. Curr Opin Toxicol, 2017. **2**: p. 109-119.
126. al, M.A.S.e., *Smoking is Associated to DNA Methylation in Atherosclerotic Carotid Lesions*. 2018.
127. al, R.A.P.e., *AHRR Methylation is a Significant Predictor of Mortality Risk in Framingham Heart Study*. 2019.
128. Lindsay M. Reynolds, M.W., Jingzhong Ding et al, *DNA Methylation of the Aryl Hydrocarbon Receptor Repressor Associations with Cigarette Smoking and Subclinical Atherosclerosis*. 2015.
129. al, E.G.A.e., *Wood smoke enhances cigarette smoke-induced inflammation by inducing the aryl hydrocarbon receptor repressor in airway epithelial cells*. 2015.

130. Xu, J.W.C.a.H., *Aryl Hydrocarbon Receptor Repressor Methylation: A Link Between Smoking and Atherosclerosis*. 2016.
131. Lee, D.H., et al., *Performance of urine cotinine and hypomethylation of AHRR and F2RL3 as biomarkers for smoking exposure in a population-based cohort.* PLoS One, 2017. **12**(4): p. e0176783.
132. Philibert, R.A., S.R. Beach, and G.H. Brody, *Demethylation of the aryl hydrocarbon receptor repressor as a biomarker for nascent smokers.* Epigenetics, 2012. **7**(11): p. 1331-8.
133. al., D.M.T.e., *Methylation at cg05575921 of a smoking-related gene (AHRR) in non-smoking Taiwanese adults residing in areas with different PM 2.5 concentrations*. 2019, May.
134. Tantoh, D.M., et al., *AHRR cg05575921 methylation in relation to smoking and PM2.5 exposure among Taiwanese men and women*. Clin Epigenetics, 2020. **12**(1): p. 117.
135. Ayman Alhamdow, C.L., Jessika Hagberg, et al., *DNA methylation of the cancer-related genes F2RL3 and AHRR is associated with occupational exposure to polycyclic aromatic hydrocarbons*. 2018, July.
136. WHO. *Biological Monitoring of Chemical Exposure in the Workplace: Guidelines*. 1996; Available from: https://apps.who.int/iris/handle/10665/41856.
137. Barr, D.B., et al., *Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements.* Environ Health Perspect, 2005. **113**(2): p. 192-200.
138. American University of Beirut Medical Center (AUBMC), V.M.P.V., *Faculty of Medicine, Vascular Medicine Program: the Greater Beirut Area Cardiovascular Cohort (GBACC)*. 2014.
139. Lewin, J., et al., *Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates.* Bioinformatics, 2004. **20**(17): p. 3005-12.
140. Robert A. Philibert, * Steven R.H. Beach, and Gene H. Brody, *Demethylation of the aryl hydrocarbon receptor repressor as a biomarker for nascent smokers*. 2012.
141. Tomasz K Wojdacz, T.H.M., et al, *Limitations and advantages ofMS-HRM and bisulfitesequencing for single locusmethylation studies*. 2010.
142. Poulin, M., et al., *Pyrosequencing Methylation Analysis*. Methods Mol Biol, 2018. **1856**: p. 283-296.