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ASSOCIATION OF THE CYTOTOXIC EFFECTS OF WATERPIPE AND CIGARETTE SMOKE EXTRACT WITH EPIGENETIC CHANGES IN BREAST CANCER CELLS

by
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ABSTRACT
OF THE THESIS OF

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Major: Pharmacology and Toxicology

Title: Association of the Cytotoxic Effects of Waterpipe and Cigarette Smoke Extract with Epigenetic Changes in Breast Cancer Cells

Background Smoking is one of the preventable leading causes of diseases and premature death worldwide. In Lebanon, smoking is a main public concern as it scored one of the highest smoking rate in the Eastern Mediterranean region reaching a peak of 53.9%. It is a major risk factor for the development of cancer including breast cancer, as according to the WHO in 2020, the incidence of breast cancer new cases ranked number one and became the second leading cause of death in Lebanon. Studies have shown a positive correlation between early and long term exposure to smoking and the incidence of breast cancer. Tobacco smoke was found to be behind the development of epigenetic aberrations that are linked to breast cancer, with the most compelling evidence for *AhRR* hypomethylation and cigarette smoking.

Aim The aim of this study is to determine the genotoxic and possible development of mesenchymal properties upon exposure to cytotoxic concentrations of cigarette and waterpipe smoke in MCF-7 and MDAMB-231 breast cancer cell lines, and to evaluate the differential methylation of the *AhRR* and *MYT1L* regions of interest that were found in peripherial blood samples to be specific for cigarette and waterpipe smoke respectively, in breast cancer cell lines.

Methods MTT assay was first done to determine the IC20 and IC50 for both waterpipe and cigarette smoke, and the concentrations were validated by trypan blue assay. These were then used to determine the genotoxic and cell cycle arrest effects using the ɣH2AX and flow cytometry assays respectively, and to determine any potential carcinogenic effect by evaluating the expression of epithelial and mesenchymal markers using the RT-PCR assay, and performing migration assay. Differential methylation of the *AhRR* and *MYT1L* regions of interest were then analyzed through direct bisulfide sequencing.

Results Exposure to cytotoxic concentrations of waterpipe and cigarette smoke caused DNA damage in MCF-7 and MDAMB-231 with secondary arrest at the S phase; though with the MDAMB-231 cell line exposure to higher concentrations showed less genotoxic damage that was translated into avoiding cell arrest at S phase. Also for both cell lines, the mesenchymal SNAIL marker increased with a trend of decrease in the CDH-1, an epithelial marker, but the results of the migration assay showed a decrease in migration ability of cells compared to the control at 24hrs. Finally, the differential

methylation of the *AhRR* and *MYT1L* region of interests that were specific for peripheral blood samples were not so for breast cancer cell lines.

Conclusion Exposure to cytotoxic concentrations of cigarette and waterpipe smoke caused DNA damage and S phase cell cycle arrest. It also induced an increase in the expression of the *SNAIL* mesenchymal marker. The mesenchymal phenotype was observed under the microscope yet, and at high concentrations, MDAMB-231 cells may have become resistant to genotoxicity hence the highest expression of *SNAIL*. The lesser migration ability at 24hrs can be due to cell death. With respect to the epigenetic changes, results showed no difference in DNA methylation between the exposure and the control and the outcome was different from that of the peripheral blood samples.

TABLE OF CONTENTS

ILLUSTRATIONS

Figure

TABLES

Table

ABBREVIATIONS

AhR	Aryl Hydrocarbon Receptor
AhRR	Aryl Hydrocarbon Receptor Repressor
ANOVA	Analysis of variance
AP	Apurinic/apyrimdinic
APC	Adenomatous polyposis coli
ARNT	AhR nuclear translocator
B[a]P	Benzo[a]pyrene
CO	Carbon monoxide
COPD	Chronic Obstructive Pulmonary Disease
CSC	Cigarette smoke condensate
CSE	Cigarette smoke extract
DMEM	Dulbecco's Modified Eagle Medium
DMR	Differentially Methylated Regions
DNAPK	DNA-dependent protein kinase
DNMT	DNA methyltransferase
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
FAK	Focal adhesion kinase
FBS	Fetal bovin serum
FDA	Food and Drug Administration
GOLD	Global Initiative for Chronic Obstructive Lung Disease
GSK-3β	Glycogen synthase kinase 3 beta
HDL	High density lipoprotein
HER2	Human epidermal growth factor receptor 2
KO	Knock out
LC-MS	Liquid Chromatography-Mass Spectrometry
LDL	Low density lipoprotein
LINE-1	Long interspersed nucleotide elements
LUMA	Luminometric Methylation Assay
MDC1	Mediators of DNA damage checkpoint protein 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MYT1L	Myelin transcription factor 1-like
NFkB	Nuclear factor kappa light chain enhancer of activated B cells
NTC	No template control
PAF	Platelet Activating Factor
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PI	Propidium Iodide
ROI	Region of interest
RT –PCR	Real time Polymerase Chain Reaction
Sat2	Satellite 2
SEM	Standard error mean
TPM	Total Particulate Matter
VLDL	Very low density lipoprotein
WHO	World Health Organization
WPE	Waterpipe extract

CHAPTER I

INTRODUCTION

A. Smoking

1. Smoking epidemiology

Smoking is one of the preventable leading causes of diseases and premature death worldwide [1, 2]. According to the World Health Organization (WHO), 4.9 million people die per year from smoking, and the death rate is expected to increase to 8 million by 2030 [1, 3]. In Lebanon, smoking is a main public concern as it scored one of the highest smoking rate in the Eastern Mediterranean region reaching a peak of 53.9% [1, 4].

2. Reasons behind smoking

The causes behind the increase in smoking consumption are related to low income, advertisement on TV[5], low education, lack of regulations [6], peer pressure [1] and for the relief of pressure [7].

3. Smoking related complications

Smoking is one of the risk factors for the development of cardiovascular diseases, atherosclerosis, chronic obstructive pulmonary disease (COPD), and cancer.

a. Smoking and cardiovascular diseases

According to the WHO, smoking is behind 10% of cardiovascular disease cases. Smoking affects systemic arteries as it lowers the flow mediated dilatation, causing an elevation in central arterial stiffness. Smoking also causes endothelial dysfunction [8].

Many compounds found in tobacco activate several mechanisms involved in the development of cardiovascular diseases. First is nicotine that increases myocardial oxygen demand, as this constituent stimulates the sympathetic nervous system [9, 10]. The second constituent is carbon monoxide as its affinity to hemoglobin binding is much greater than that of oxygen, thus the oxygen supply is reduced [9, 11]. Moreover, particulate matter, heavy metals, and polyaromatic hydrocarbons (PAH) promote the generation of oxidative stress and inflammatory mediators that activate thrombus and inflammatory processes resulting in vasoconstriction [9]. All of these mechanisms play a role in increasing oxygen demand and decreasing oxygen supply, hence leading to the development of myocardial ischemia and infarction.

Craig et al [12] showed that smoking induces a rise in total cholesterol, VLDL, LDL, and triglyceride serum concentrations. On the other hand, it lowers HDL and apolipoprotein A1 in a dose-dependent manner. Upon consumption, tobacco smoke creates a pro-oxidative environment from the released free radicals and oxidants [8, 13]. The released products provide a high level of oxidized lipids taken up by macrophages, resulting in the formation of foam cells and autoantibodies inside the body [14-17]. The deposition of foam cells plays a role in plaque formation [8]. Also, smoking increases the levels of white blood cells inside the body as reported by Lavi et al [18]. In addition, smoking promotes the presence of inflammatory markers, proinflammatory cytokines,

matrix metalloproteinases, and adhesion molecules that are all well-known inflammatory processes involved in the development of atherosclerosis [19-21].

b. Smoking and lung diseases

Tobacco smoke affects the lungs in different mechanisms. According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [22], COPD is defined as "persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases". The diagnosis of the disease is related to a decline in maximum expiratory flow and forced expiratory volume in 1 second [22].

COPD is one of the major complications that arise from tobacco smoking, as 1 out of 5 of chronic smokers develop the disease [23]. Tobacco smoking compromises the role of immune system and exacerbates inflammation which is the pathophysiologic process of COPD. It activates the NFkB pathway that is an important mechanism involved in the development of COPD inflammation [24]. Also, smoking causes deletion of the NRF2 gene, which is responsible for lowering the incidence of emphysema formation, thereby tobacco induces lung emphysema [25]. Moreover, smoking increases mucus production making the lung surface a site that is more prone for the accumulation of infectious agents.

Tobacco smoking also attacks several lines of defense in the lung. Epithelial cells, which are the first line of defense, produce more surfactant protein A/D and beta-defensin during smoking [26]. Furthermore, smoking affects the mucus removal from the airway as it impairs the mucociliary function [25]. Concerning innate immunity, tobacco smoking modifies the function of neutrophils and macrophages. For example,

and upon long term exposure to tobacco constituents, innate immune cells loose the anti-apoptotic markers, decrease the ability to remove any defective cells in the body, and increase the secretion of cytokines and matrix metalloproteases [25, 27, 28].

c. Smoking and cancer

Tobacco smoke consists of addictive and non-addictive constituents, both of which contribute to cancer development. **Figure 1** illustrates the specific and nonspecific pathways whereby tobacco smoke constituents cause cancer [29]. This process occurs among all types of cancer. Nicotine, an addictive constituent in tobacco, is the main reason behind continuous smoking and hence prolonged exposure to the carcinogens.

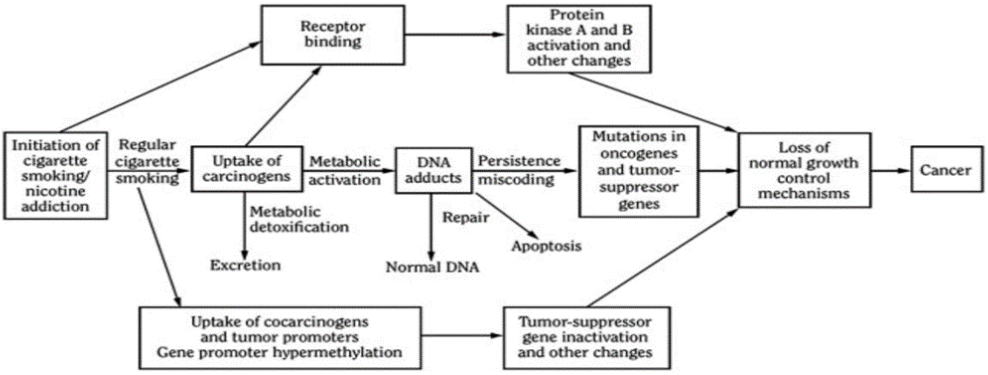


Figure 1 Specific and nonspecific pathways whereby tobacco smoke constituents cause cancer [29].

Carcinogenic compounds and reactive intermediates, produced during smoking, bind covalently to nucleosides forming DNA adducts [30]. The formed DNA adducts then lead to miscoding and mutations of the genes during replication [31]. The growth and repair mechanisms of stem cells are then affected if mutations occurred in positions related to oncogenes, tumor suppressor genes, and repair genes, resulting in

uncontrolled proliferation, evaded apoptosis, more mutations, and ultimately cancer [31, 32]. In addition to DNA adducts, binding of carcinogens to receptors activates protein kinases and signaling pathways that promote carcinogenesis [33].

4. Types of tobacco smoke

According to the food and drug administration (FDA), companies usually update their tobacco products to attract users. As for today, there are many types of tobacco smoking products such as regular and electronic cigarettes, cigars, little cigars, cigarillos, dissolvable products, waterpipe, and traditional smokeless tobacco products [34]. In this study, the focus is on waterpipe and the classical cigarette.

a. Waterpipe tobacco product

Waterpipe, also known as "hookah, shisha, narghile, or argileh", has become a traditional form of smoking worldwide, especially in Middle Eastern countries, and its use is expanding to become a global epidemic [9, 35]. Waterpipe use has been expanding due to many factors including the misconception among people due to advertisement that it is less harmful, the fact that it is made up of many flavors, and the lack of restrictive regulations in cafes [36]. The waterpipe is made up of a tobacco head, body, water bowl, hose, and a mouthpiece [6]. Waterpipe smoking works through the administration of the tobacco content found in the bowl that is covered by burning charcoal, so that as the smoker inhales, the air passes through the charcoal carrying its combustion products with tobacco contents. Then, the contents pass through water and are carried by the hose until they reach the smoker's mouth and ultimately lungs [9, 37,

38]. Waterpipe is the only method of tobacco delivery that applies burning charcoal as a heat source.

b. Cigarette tobacco product

Cigarettes are made up of tobacco, wrapping paper, and a filter. Smokers usually consume this type of product for the sake of the pleasure felt from the addictive constituent nicotine. But at the same time, when the cigarette is burned, smokers are also exposed to toxic and carcinogenic compounds produced from tobacco [34].

c. Waterpipe and cigarette smoke constituents

Several studies have been done to compare the toxicants' content found in waterpipe vs. cigarette smoke, and in order to do that many compared the constituents yield between a waterpipe session and a single cigarette. **Table 1** summarizes findings in the studies regarding the constituents and concentrations generated from a waterpipe session vs. that from inhalation of a single cigarette [39].

Chemical constituents differ between waterpipe and cigarette smoke due to several factors. The first factor is related to the temperature difference between waterpipe and cigarette smoke delivery systems. For instance, the lower temperature in waterpipe smoke (450 degrees Celsius) causes incomplete combustion, thus the generation of more phenolic compounds when compared to cigarette smoke (900 degrees Celsius) [40, 41]. Waterpipe is the only tobacco product that applies charcoal as a heating source during the smoking session, and studies showed that some compounds originate from charcoal. More specifically, Schubert et al [42] showed that

the burning of charcoal is the reason behind the formation of high levels of benzene and toluene. Also, studies showed that the removal of charcoal and replacing it with an electrical heater reduced CO value by 90%, and the level of PAH from 170 to 9 ng, hence reflecting a 95% difference [43]. Moving on to the second factor, the waterpipe consists of humectants and flavors that, upon heating, produce furanic compounds that are not found in cigarette smoke [39]. The third factor is related to the dilution of the amount of tobacco specific content with humectants and flavor in the waterpipe [39]. Thus, the yields of tobacco-specific nitrosamines is greater from cigarette than that from a waterpipe session.

In addition to some differences in the constituents, there are notable differences in the concentrations and yields of the shared constituents from waterpipe and cigarette smoke. As shown in **Table 1**, although we observe overall higher amounts of extracts from a waterpipe session, one cannot immediately conclude that waterpipe is more toxic than cigarette smoke since this comparison does not address the actual harmful effect of each tobacco product [39]. In addition the duration of a waterpipe session is much longer resulting in higher amounts of constituents delivered, but these are distributed in around 1500mg of particulate matter [44], this is in comparison to a single cigarette that is inhaled over a shorter duration, where the constituents are delivered in around 30mg of particulate matter [44]. Thus the dilution effect is greater in waterpipe compared to that of cigarette smoke. Some constituents yield might differ between both as we mentioned before, thus the effect per unit mass of condensate might differ. In other words, the whole final mixture of the total particulate matter (TPM) produced by each product showed that the waterpipe contains lower amounts of constituents that are biologically active [44]. In addition, the charcoal related toxicants, which are specific to

waterpipe products, are diluted in the final total TPM produced, thus their effects are less significant when we are comparing per unit mass of condensate [44]. This means that the constituents yield from the waterpipe might require higher concentrations to produce its toxic effect, which is explained by the fact that the final mixture is diluted between harmful, humectants, and flavor constituents. Also, the passage of the constituents from the water favors some dilution effect to the constituent's yield.

Table 1 Constituents and concentrations produced from smoking after a single waterpipe session compared to inhalation of a single cigarette [39].

Toxicants yields from waterpipes (per use-session) and cigarettes (per cigarette)

Yield per unit smoked	IARC class*	Hoffmann list 'causative agent'	Cigarette	Waterpipe
T/N/CO/NO (mg)				
Tar	—	CVD, chronic obstructive lung disease, lung cancer	1–27	242–2350
Nicotine	—	Tobacco dependence	0.1–3	>0.01–9.29
CO	—	CVD	14–23	5.7–367
Water	—	—		548–1760
NO	—	CVD, chronic obstructive lung disease	0.100–6.00	0.325–0.440
Carbonylic compounds (μg)		Chronic obstructive lung disease, lung/larynx cancer		
Formaldehyde	1		20–100	36–630
Acetaldehyde	2B		400–1400	120–2520
Acetone	—		—	20.2–118
Acrolein	3		60–240	10.1–892
Propionaldehyde	—		48.4†	5.71–403
Methacrolein	—		—	12.2–106
Butyraldehyde	—		—	10.9–70.6
Benzaldehyde	—		—	BLQ (0.339)
Tobacco-specific nitrosamines (ng)		Lung/larynx/oral cavity/oesophageal/bladder cancer		
NAT	3		—	103
NNK	1		80–770	LOD–46.4
NNN	1		120–3700	34.3
NAB	3		—	8.45
Primary aromatic amines (ng)		Urinary bladder cancer		
m-PDA	3		—	6.50
ANL	3		251.6‡	31.3
4,4′-ODA	2B		—	28.0
o-ASD	2B		—	BLQ (3.76)
4-CA	2B		—	BLQ (3.39)
2-ANP	1		1–334	2.84
1-ANP	3		17.0‡	6.20
3,5-DCA	—		—	BLQ (3.77)
2-ABP	—		—	3.33
Furanic compounds (μg)				
HMF	—		—	2420–62 300
FTA	—		—	55.7–552
2-FA	—		—	32.0–401
2-F	—		—	29.6–206
2-FMK	—		—	4.77–12.5
5-M-2-F	—		—	4.62–215

Table 1 (continued)

Polycyclic aromatic hydrocarbons (ng)		**Lung/larynx cancer, oral cavity cancer**		
Naphthalene	2B		360.8†	30–3860
Acenaphtylene	—		71.6†	42–700
Acenaphthene	3		56.8†	25–17 260
Fluorene	3		189.2†	26–437
Phenanthrene	3		138.9†	1277–2650
Anthracene	3		62.3†	133–6280
Fluoranthene	3		52.7†	354–2380
Pyrene	3		44.8†	30–12 950
Benzo[*a*]anthracene	2B		20–70	30–15 190
Chrysene	2B		—	ND–124
Benzo[*k*]fluoranthene	2B		6–12	ND–370
Benzo[*b*]fluoranthene	2B		4–22	ND–170
Benzo[*a*]pyrene	1		20–40	ND–307
Benzo[*g,h,i*]perylene	3		—	ND–140
Dibenz[*a,h*]anthracene	2A		4	ND–147
Indeno[1,2,3-*cd*]pyrene	2B		4–20	ND–183
Heavy metals (ng)		**Cardiovascular, lung/larynx cancer**		
Lead	2B		34–85	200–6870
Copper	—		—	1300–2300
Zinc	—		—	1100–1400
Chromium	1		4–70	250–1340
Nickel	1 or 2B§		ND–600	300–900
Cobalt	2B		0.13–0.2	70–300
Arsenic	1		40–120	165
Boron	—		—	350–1310
Beryllium	1		0.5	65
Volatile organic compounds (μg)				
Isoprene	2B		200–400	4.00
Benzene	1		20–70	271
Toluene	3		5–90	9.92
Ethylbenzene	2B		—	1.00
p-Xylene	3		—	0.929
m-Xylene	3		—	2.47
Pyridine	3		20–200	4.76
o-Xylene	3		—	BLQ
Styrene	2B		10	1.27
Quinoline	—		2–4	BLQ
Phenolic compounds (μg)		**Lung/larynx cancer**		
Hydroquinone	3		30.9†	21.7–110.7
Resorcinol	3		0.474†	1.689–1.87
Catechol	2B		90–2000	166–316.1
Phenol	3		170	3.21–58.03
Guaiacol	—		1.00†	7.00
m-Cresol	—		6.05¶	2.37¶–4.655
p-Cresol	—		—	—¶–5.375
o-Cresol	—		2.09†	2.93–4.409
Others (mg)				
Propylene glycol	—		—	211
Glycerol	—		—	423
Vanillin	—		—	3.192
Ethyl vanillin	—		—	0.616
Benzyl alcohol	—		—	0.232
Biological components				
Ergosterol (ng)	—		—	84.4
LPS (pmol)	—		—	1800

*IARC classification groups: 1=carcinogenic to humans; 2A=probably carcinogenic to humans; 2B=possibly carcinogenic to humans; 3=not classifiable as to its carcinogenicity to humans.

†3R4F reference cigarette.

‡2R4F reference cigarette.

§Depends on its form.

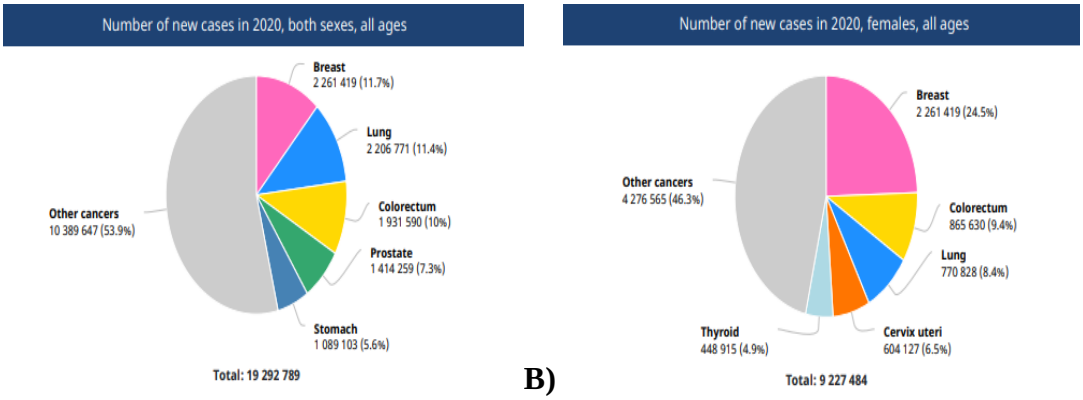
¶Combined values for *m*/*p*-cresol.

1-ANP, 1-naphthylamine; 2-ABP, 2-aminobiphenyl; 2-ANP, 2-naphthylamine; 2-F, 2-furaldehyde; 2-FA, 2-furoic acid; 2-FMK, 2-furyl methyl ketone; 3,5-DCA, 3,5-dichloroaniline; 4,4'-ODA, 4,4'-oxydianiline; 4-CA, *p*-chloroaniline; 5-M-2-F, 5-methyl-2-furaldehyde; ANL, aniline; BLQ, below limit of quantification; CO, carbon monoxide; CVD, cardiovascular disease; FFA, furfuryl alcohol; HMF, 5-(hydroxymethyl)-2-furaldehyde; IARC, International Agency for Research on Cancer; LOD, limit of detection; LPS, lipopolysaccharide; *m*-PDA, *m*-phenylenediamine; ND, not detected; NAB, *N*-nitrosoanabasine; NAT, *N*-nitrosoanatabine; NNK, 4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone; NNN, *N*'-nitrosonornicotine; NO, nitric oxide; *o*-ASD, *o*-anisidine.

B. Breast cancer

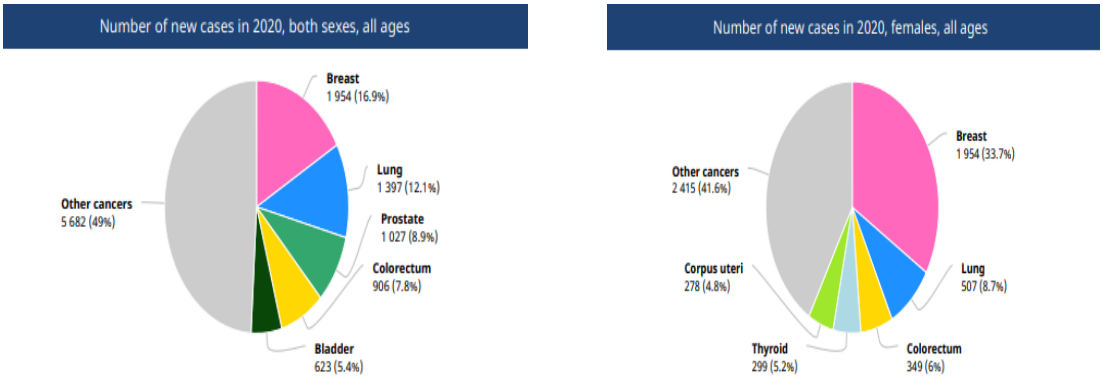
1. Breast cancer epidemiology

According to the most recent data from the WHO in 2020, the incidence of breast cancer new cases ranked number one worldwide and Lebanon [45, 46]. Breast cancer is the fourth leading cause of death worldwide, while it is the second in Lebanon. **Figures 2A and 3A** show the breast cancer data of 2020 worldwide and in Lebanon, with respect to other types of cancer, with incidence among both genders of all ages, while **Figures 2B and 3B** show the data among females only.



A) **B)**

Figure 2 Percentage of breast cancer incidence of new cases for both genders of all ages **(A)**, and percentage of incidence among females only **(B)** worldwide [49].



A) **B)**

Figure 3 Percentage of breast cancer incidence of new cases for both genders of all age **(A)**, and percentage of incidence among females only **(B)** in **Lebanon** [45].

2. Types of breast cancer

Based on the immunohistochemical profile, breast cancer is classified into different subtypes based on estrogen and progesterone receptors and HER2 expression [47]. The hormone receptor positives are subdivided into luminal A, luminal B, and luminal HER2, whereas, the hormone receptor negative is subdivided into HER2 enriched, basal and non-basal like phenotypes. Starting with the luminal A subtype, it is characterized by breast cancer tissue that is positive for estrogen and progesterone receptors with ki-67, that reflects tumor nuclei, being below 14% [47]. Luminal B on the other hand has the same characteristics as luminal A, but with equal or more than 14% of the ki-67. As for luminal HER2, it reflects breast cancer tissue positivity to the three estrogen, progesterone, and HER2 receptors. Moving on to the hormone negative subtypes, the HER2 enriched type entails cancer tissue that is estrogen and progesterone receptors negative, but HER2 positive [47]. Also, the triple negative type is subdivided into basal and non-basal phenotype, the difference is that the basal can be EGFR or CK5/6 positive whereas the non-basal is negative for all [47].

3. Breast cancer risk factors

Breast cancer development is associated with reproductive factors as high serum estrogen levels are directly correlated with breast cancer. More specifically, starting with menarche, the incidence of breast cancer is reduced by 10% with every 2 years delay in the onset of menarche [48]. Also, full term pregnancy at early age, breast-feeding and usual menopausal age reduce the risk of breast cancer especially the hormone positive receptor type [49, 50]. In addition, parity causes a brief rapid proliferation of the breast epithelial cells yet at the long term, the breast epithelial cells

undergo differentiation giving the cell cycle more time for DNA repair hence a lower incidence of cancer [50]. Moreover, any type of exogenous hormonal intake such as oral contraceptives increases the probability of forming breast cancer [50].

Concerning weight, obesity in premenopausal women shows a protective effect against breast tumor, while obesity in postmenopausal woman shows the opposite effect with high probability of hormone positive receptor type of breast tumor. The reason behind this observation is related to the ability of adipose tissue to convert circulating androgen to estrogen by aromatase enzyme; as for premenopause the lower risk is because of decreased levels of estrogen due to obesity [51-53].

As for genetic risk factors, mutations of *BRCA1/BRCA2, P53*, and *PTEN* genes increase the risk of breast cancer [54-59]. And with respect to age, as age increases, the probability of the occurrence of estrogen positive breast cancer increases [60].

Moving on to the lifestyle and dietary related risk factors, alcohol and tobacco consumption are significantly associated with increased incidence of the disease [61-63], while physical activity and soy intake are protective against breast cancer incidence. For instance, studies showed that regular physical activity reduces risk by about 10 to 12%, and a lower incidence was observed in populations with high soy intake [64, 65].

Exposure to high doses of radiation, such as that used to treat Hodgkin lymphoma patients, is also one of the risk factors for the development of breast cancer. The relation is related to how much the woman has been exposed to radiation during her lifetime especially if at younger age [66, 67].

Cigarette smoking constituents were found to affect breast tissue. The carcinogens have a high affinity to lipids making them easily permeable to cross the

alveolar membrane, then be transported by plasma lipoproteins to breast tissue where they are stored in the breast adipose tissue and bioactivated by breast epithelial cells [63, 68]. Also, cigarette smoking was associated with an increased risk of p53 gene mutations [69]. Breast cancer risk is positively correlated with smoking intensity, and duration.

Almost all of the above mentioned risk factors for breast cancer development are high among Lebanese women as the incidence of obesity recently increased to reach 36.5% in 2015 [70] and the mean maternal age increased to around 28.3 years. The fertility rates is also as low as 1.7 per woman, and the prevalence of smoking is as high as 27.2% [71].

4. Smoking and breast cancer

a. Epidemiological association studies

There have been many studies done in order to evaluate whether there is an association between cigarette smoking and the incidence of breast cancer [72]. The studies that showed a positive association were mainly related to smoking at an early age, because the smoker becomes exposed to the carcinogenic constituents for a longer duration, thus a higher chance of breast cancer incidence. The results of the epidemiological studies that were done before the 1980s and 1990s did not show any link between breast cancer incidence and cigarette smoke. The reason behind this observation might be that early age initiation smokers were limited in these studies [72]. After that, more recent epidemiologic studies from, for example, hospital based case control studies concluded that a longer duration of smoking is linked to a higher incidence of breast cancer. One of these studies showed a 70% increase in risk when the

smoking duration is around 40 years and more[73], and another showed a 140% increase in risk when the smoker started smoking before age of 14 [72]. Population based case control studies showed similar results such that a 20 years' smoking duration had a significantly positive association with breast cancer incidence [74-76]. Moreover, cohort studies showed a link between high intensity, long duration of smoking, and the risk of breast cancer [77, 78]. In conclusion, there is strong evidence linking intensity and duration of cigarette smoking to breast cancer risk. Importantly, no such epidemiological data are yet available for waterpipe smoking.

b. Pathophysiology

There is a debate about the effect of cigarette smoke on breast tissue. Some studies suggested that it might have an 'antiestrogenic activity' while others argued that cigarettes contain many harmful constituents that may act as an initiator for the development of cancer [72, 79, 80]. More recently, it has been asserted that cigarette smoke has harmful effects, as studies found smoking related DNA adducts in human breast epithelial tissue [80, 81]. These adducts can be used as a biomarker for tobacco smoke exposure, metabolic activity, and the delivery of the genotoxic metabolites to the DNA of breast epithelial cells. The mechanism behind their formation is that the carcinogens found in tobacco smoke are lipophilic making them prone to be stored in breast adipose tissue [31]. Moreover, the breast epithelial cells express enzymes such as CYP1A1, CYP1B1, and NAT2 that can activate genotoxic carcinogens to cause DNA damage [31]. As a matter of fact, studies showed that women with slow acetylation genotype have higher levels of adducts in breast tissue [82, 83].

C. In vitro studies of the effects of cigarette and waterpipe smoke extracts on different cell lines including the breast

1. Cigarette smoke

Few studies evaluated the genotoxic and cytotoxic effects of tobacco smoke on different cells including the breast **(Tables 2 and 4)**.

a. Genotoxicity

Starting with the genotoxic effect, few studies detected the formation of tobacco related DNA adducts in breast tumors and normal adjacent breast tissue obtained from breast cancer patients [30, 81]. Other markers that were observed in relation to DNA damage and mutations are an increase in Adenomatous polyposis coli (APC), a block in base excision repair, and an increase in apurinic/apyrimdinic (AP) lesions upon exposure of MCF10A to the reference cigarette 1R4F extract [84]. Of note, no previous studies performed a direct assay to determine whether cigarette smoke causes DNA damage in breast cell lines. There is only one study that measured the levels of ɣH2AX, an indicator of double strand breakage, using cigarette smoke but on the human alveolar basal epithelial cell line (A459) [85].

b. Cytotoxicity

The above described genotoxic effects have been associated with cytotoxic effects on breast cancer cells, being cell cycle arrest or apoptosis depending on availability or efficiency of DNA repair mechanisms. Narayan and colleagues [86] showed an increase in the cellular response to DNA damage in MCF10A cells after

treatment with reference cigarette 1R4F, as the *GADD45* gene was up-regulated and the cell growth was decreased reflecting cell cycle arrest at the S-G2/M phase as shown by flow cytometry [86]. Also in that same study, results showed that mRNA levels of *Bcl-xL* and *gadd45* genes, related to cell cycle and apoptosis, increased in a dose dependent manner [86]. Moreover, the levels of PCNA, p53, and p21 increased indicating that there was a genomic damage that stimulated the pathways related to DNA repair [86]. It is important to mention that in a study on SV-HUC-1 bladder cell line, the cigarette smoke extract triggered the progression of cells from G1 to S phase [87]. This observation might be related to the differences in the concentrations of cigarette extracts that were used as different cytotoxic effects were observed at higher concentrations.

Some studies performed MTT and trypan blue assays to determine cell viability on different cell lines (MCF10A, A549, SAEC, and SV-HUC-1) upon exposure to cigarette smoke extracts [86-88]. The results of the assays are mentioned in details in **Tables 2 and 4.** The study that performed MTT assay on MCF10A showed that cigarette smoke decreased the metabolic activity of the cells [86]. It is important to mention that in a study on SV-HUC-1 bladder cell line, the cigarette smoke enhanced cell viability at low doses, but then the cells started to die with higher concentrations [87]. So the higher concentrations of cigarette smoke used on SVH-UC-1 cell line caused cytotoxic effects shown through MTT and flow cytometry assays [87].

c. Epithelial-mesenchymal transition

Cigarette smoke also induced normal breast epithelial cell transformation and promoted epithelial-mesenchymal transition (EMT). For instance, a study showed that upon treating MCF10A and MCF-7 cells with cigarette smoke extract, the expression of

E-cadherin and occludin decreased while vimentin, N-cadherin, and fibronectin increased [89]. These observations indicate that cigarette smoke activates the mitogenic-related signaling pathways that promote EMT in breast cell lines. In addition, the phenotypical changes related to EMT were observed under the microscope for MCF10A, MCF12A, and MCF-7 [89]. As for the nontumorogenic MCF10A and MCF12A cell lines, upon treatment to CSE, they acquired spindle shape and fibroblast-like morphology. Concerning the tumorigenic MCF-7 cells upon exposure to CSE, they formed more spindle shapes that are elongated.

d. Motility, migration, and invasion

After exposure to cigarette smoke extracts, some cells became more motile and gained the ability to migrate and invade. A study done on MDAMB-231, a triple negative breast cancer cell line, showed a significant increase in cell motility when exposed to CSE compared to MDAMB-231 cells cultured with media only and other breast cell lines (MCF-7, MCF10A) [90]. Another study was done to determine the migration ability of MCF10A and MCF12A, and the invasion ability of MCF-7 [89]. Results showed that MCF10A had an increase in migration ability after 37 and 72 weeks, with 3.1 fold increase for 0.5% CSE, and 3.6 fold for 1% CSE. As for the MCF12A cell line, it had an increase in migration ability after 18 weeks of treatment with 10 and 25 μg/ml CSC. With respect to the invasion capability of MCF-7 cell line, it increased after 9 weeks exposure to 0.25%, and 0.50% CSE.

e. Inflammatory response

Beside DNA damage, cell cycle arrest, and epithelial-mesenchymal transition, studies showed that cigarette smoke leads to an inflammatory process that may initiate invasion and metastasis and help in the development of aggressive cancer cell types [91]. For instance, studies showed that exposure of MCF-7 and MDAMB-231 breast cancer cell lines to certain cigarette carcinogens caused stimulation and up-regulation of cycloxygenase2, prostaglandin E2, platelet activating factor (PAF), and TNF-alfa [86, 90, 92, 93].

Table 2 Summary of in-vitro studies of different cell lines exposed to cigarette smoke extracts.

Cell lines	Exposure (concentrations, duration)	Assay performed	Results	Ref.
MCF10A	-Reference cigarette 1R4F. -Concentrations of CSC: 0.25,0.5,1,2,5,10,25 ,50 µg/ml -Duration: 72hr	1-MTT 2-Flow cytometry 3-Western blot 4-Anchorage independent growth	1-Decreased metabolic activity. 2-Arrest at S-G2/M phase 3-Significant increase in expression of: PCNA, gadd45, and Bcl-xL proteins, and moderately increase in: p53, p21, and Bcl-2 protein levels. 4-Increase in number of colonies with 10 to 25 µg/ml in concentration.	[86]
MCF10A MCF12A MCF-7	-Reference cigarette 1R3F. -Concentrations: CSE 0.25%, 0.5%, 1%. CSC: 10 or 25 µg/ml -Duration: 9, 18, 21, 37, and 72 weeks (depending on the assay).	1-Anchorage independent cell growth 2-Migration 3-Invasion 4-Western blot	1-MCF10A: increase in colony formation after 37 weeks. MCF12A: increase in colony formation 4 to 5 folds after 18 weeks. MCF-7: increase in colony formation rapidly (after 9 weeks) with 0.5% CSE. 2-MCF10A: increase in migration ability after 37 and 72 weeks. With 3.1 fold increase for 0.5% CSE, and 3.6 fold for 1% CSE. MCF12A: increase in migration ability after 18 week of treatment with 10 and 25 µg/ml CSC.	[89]

			3-MCF-7: increase in invasive capability after 9 week exposure to 0.25%, 0.50% CSE. 4-Decrease in E-cadherin expression and increase in vimenten expression after 21 weeks treatment of MCF10A and MCF-7 cells.	
MCF10A	-Reference cigarette:1R4F -Concentrations of CSC: 10, 25, 50 μg/ml -Duration: 30hrs, 72 hrs	1-Western blot 2-AP lesion assay	1-Overexpression of APC 2-Accumulation of AP lesions	[84]
MDAMB-231 MCF-7	- CSE from Murty Pharmaceuticals -Concentration: 20 μg/ml -Duration: 24, 48 hr	1-Cell motility	1-Increased motility in MDAMB-231 breast cancer cell line.	[90]
Breast tumor, and their histologically normal adjacent tissues (obtained from cancer patients)	-Tissue obtained from smoker patients	1-^{32}P-postlabeling assay	1-Detection of tobacco related DNA adducts.	[30, 81]
A549 SAEC	-Marlboro red cigarette -Concentrations: 2.5, 5, 10, 25% for A549, and 0.5, 2.5% for SAEC cell line. -Duration: 12, 24hrs	1-MTT 2-Trypan blue	1-Decreased metabolic activity 2-Decrease cell viability	[88]
A459	-Reference cigarette: 2R4F -Amount used: 8.9mg TPM per cigarette -Duration: time interval between 5 and 20min.	1-Immunocytochemical detection of ɣH2AX.	1-Increase in the expression of ɣH2AX	[85]
SV-HUC-1	-Reference cigarette: 3R4F	1- MTT 2-Flow cytometry	1-Increase in metabolic activity from 0.05 to 0.5% concentration,	[87]

	-Concentrations of CSE 0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 4% -Duration: 7 days		then a decrease in the viability was observed. 2-Trigger cell cycle progression from G1 to S phase at 0.05 to 0.5% concentrations.	

Abbreviations:

CSC: cigarette smoke condensate

CSE: cigarette smoke extract

APC: adenomatous polyposis coli

AP: apurinic/ apyrimidinic

2. Waterpipe smoke

In comparison to cigarette smoke, less studies were conducted with waterpipe smoke, the majority being with lung cells, and only one with breast cells **(Tables 3 and 4)**.

a. Genotoxicity

A study performed a western blot assay to determine the levels of phosphorylated histone 3 in A549 and ECV-304 cell lines [44], while another used 53BPI/ɣH2AX staining assay on A549, H460, and BEAS-2B cell lines to determine the levels of 53BPI and ɣH2AX [94]. Both showed an increase in the expression of phosphorylated histone 3, 53BPI, and ɣH2AX all of which are biomarkers of DNA damage.

b. Cytotoxicity

Few studies were done on lung epithelial cell lines to determine the cytotoxic effect of waterpipe smoke with results showing that waterpipe smoke induces growth inhibition and decreases cell viability. These results were obtained from trypan blue assay on A549, ECV-304, H460, and BEAS-2B cell lines [44, 94, 95]. Another marker that determines the cytotoxic effect of waterpipe smoke is the 'cell cycle progression'. As such, two studies have performed flow cytometry assay on A549 and observed, after exposure of cells to waterpipe extracts, a cell cycle arrest at G0/G1 phase [44, 95]

c. Epithelial mesenchymal transition

A study showed that waterpipe smoke promotes epithelial mesenchymal transition by downregulating the expression of E-cadherin and upregulating that of FAK in BT20 and MCF-7 cell lines, hence indicating that waterpipe smoke stimulates the invasion and migration ability of these cells. This study also concluded that the events are mostly related to the activation of ERK1/2 signaling pathways [96].

d. Migration and invasion

A study was conducted to determine the effect of waterpipe smoke on the ability of the breast cells (MCF-7 and BT20) to migrate and invade, through matrigel invasion and wound healing assays. Results showed that waterpipe smoke stimulate the ability of the cells to invade and migrate compared to untreated control cells [96].

Table 3 Summary of in-vitro studies of different cell lines exposed to waterpipe smoke extract.

Cell lines	Exposure (concentrations, duration)	Assay performed	Results	Ref.
MCF-7 BT20	-Waterpipe smoke: Aleppo Method -Final concentration: 200 µg/ml -Duration: 48hr, 3, and 8 days (depending on the assay)	1- Invasion and migration assay 2-Clonogenic cell assay 3- Western blot	1- Increase cell invasion and migration ability in both cell lines. 2-Enhancement of colony formation. 3- Downregulation of E-cadherin expression, upregulation of FAK expression, and increase in the Erk1/Erk2 phosphorylated form.	[96]
A549 ECV-304	-Waterpipe smoke: Beirut Method -Concentration: 0.5, 1, 3, 4, 6, 8mg/ml -Duration: 24hr, or 3 consecutive days (repeated exposure)	1- Trypan blue 2-Flow cytometry 3-Western blot	1-Decrease in viability of A549 cells 2-Cell cycle arrest at G0/G1 for A549 and ECV-304 3-Expression of phosphorylated histone 3 on single exposure for A549, and increase in expression of p53, phosphor-p53, p21.	[44]
A549	-Waterpipe smoke: Puff topography -Concentration: 4mg/ml -Duration: 3 consecutive days (repeated exposure)	1-Trypan blue 2-Flow cytometry	1-Decreased cell viability 2-Cell cycle arrest at G0/G1	[95]
A549 H460 BEAS-2B	-Waterpipe smoke: was prepared using IREADY LIC smoking machine that stimulates human smoking. Briefly, they used 17.5g double apple flavor tobacco (mou'assal), two pieces of quick lighting charcoal briquettes, 5s puff period, and 15s inter-puff interval for 80 puff cycles. -Concentration: 0.5, 1, 2% -Duration: 7 days	1-Trypan blue 2-53BPI/ɣH2AX staining 3-Western blot 4-Confocal microscopy	1-Decreased cell viability 2-Increase in 53BPI and ɣH2AX expression 3-Increase in p21 expression 4-Accumulation of p21 and p53 in the nuclei	[94]

Table 4 Summary of in-vitro studies of different cell lines exposed to waterpipe or cigarette smoke extracts.

Cell lines	Exposure	Concentrations and duration	Cell viability	Cell cycle arrest	Apoptosis	Colony formation	Migration	Invasion	Genotoxicity	Ref.
MCF 10A	Reference cigarette 1R4F. CSC	Concentrations: 0.25,0.5,1, 2,5,10,25,50 μg/ml -Duration: 72hr	↓	↑ S-G2/M phase		↑				[86]
	Reference cigarette 1R3F. CSC and CSE	Concentrations: CSE 0.25%, 0.5%, 1%. CSC: 10 or 25 μg/ml -Duration: 9, 18, 21, 37, and 72 weeks (depending on the assay).				↑	↑			[89]
	Reference cigarette :1R4F CSC	Concentrations: 10, 25, 50 μg/ml Duration: 30hrs, 72 hrs							↑	[84]
MCF -7	Reference cigarette 1R3F. CSE	Concentrations: CSE 0.25%, 0.5%, 1%. CSC: 10 or 25 μg/ml Duration: 9, 18, 21, 37, and 72 weeks (depending on the assay).				↑		↑		[89]
	CSE from Murty Pharmaceuticals	Concentration: 20 μg/ml Duration: 24, 48 hrs					No change			[90]

	Water-pipe smoke: Aleppo Method	Final concentrat-ion: 200 μg/ml Duration: 48hr, 3, and 8 days (depending on the assay)				↑	↑	↑		[96]
MCF 12A	Ref-erence cigarette 1R3F. CSC and CSE	Concentrat-ions: CSE 0.25%, 0.5%, 1%. CSC: 10 or 25 μg/ml Duration: 9, 18, 21, 37, and 72 weeks (depending on the assay).				↑	↑			[89]
MD AMB -231	CSE from Murty Pharmac euticals	Concentrat-ion: 20 μg/ml Duration: 24, 48 hr					↑			[90]
A549	Marl-boro red cigarette	Concentrat-ions: 2.5, 5, 10, 25% - Duration: 12, 24hrs	↓		↑					[88]
	Ref-erence cigarette : 2R4F	Amount used: 8.9mg TPM per cigarette Duration: time interval between 5 and 20min.							↑	[85]
	Water-pipe smoke: Beirut Method	Concentrat-ion: 0.5, 1, 3, 4, 6, 8mg/ml Duration: 24hr, or 3 consecutive days	↓	↑ G0/ G1 phase	No chan-ge					[44]

		(repeated exposure)								
	Water-pipe smoke: Puff topography	Concentration: 4mg/ml Duration: 3 consecutive days (repeated exposure)	↓	↑ G0/ G1 phase						[95]
	Water-pipe smoke	Concentration: 0.5, 1, 2% Duration: 7 days	↓						↑	[94]
SAEC	Marlboro red cigarette	Concentrations: 0.5, 2.5% - Duration: 12, 24hrs	↓		↑					[88]
SV-HUC-1	Reference cigarette: 3R4F CSE	Concentrations: 0.05, 0.1, 0.25, 0.5% Duration: 7 days	↑	↓ G1 to S phase						[87]
		Concentrations of CSE 0.75, 1, 2, 4% Duration: 7 days	↓							
BT20	Water-pipe smoke: Aleppo Method	Final concentration: 200 μg/ml Duration: 48hr, 3, and 8 days (depending on the assay)				↑	↑	↑		[96]
ECV-304	Water-pipe smoke: Beirut Method	Concentration: 0.5, 1, 3, 4, 6, 8mg/ml Duration: 24hr, or 3 consecutive days (repeated exposure)	↓							[44]

H460	Water-pipe smoke	Concentrat-ion: 0.5, 1, 2% Duration: 7 days	↓						↑	[94]
BEA S-2B	Water-pipe smoke	Concentrat-ion: 0.5, 1, 2% Duration: 7 days	↓							[94]

D. DNA methylation

The word epigenetics means “addition to changes in genetic sequence”, and can be described as a modification in the DNA that alters gene activity without affecting its’ sequence [97]. The mechanisms of epigenetics involve DNA methylation, histone acetylation, and non-coding RNA [97]. These mechanisms naturally occur in cells and are sometimes required for organism’s functions; however, any improper change can result in illness and serious diseases. Epigenetic mechanisms have been linked to many diseases and behaviors including cancer, respiratory and cardiovascular diseases, cognitive dysfunction, neurobehavioral illness and so much more [97]. One of the causes behind the development of epigenetic aberrations are related to exposure to different toxic substances such as hormones, radioactivity, heavy metals, bacteria, viruses, and most notably tobacco smoke including PAH compounds [97].

1. DNA methylation definition

The most studied type of epigenetic mechanisms is DNA methylation. DNA methylation involves the addition of a methyl group at cytosine (C5 position) to become 5-methylcytosine; the cytosines being located in CpG islands consisting of repetitive CpG dinucleotides. The added methyl group inhibits the transcription factors from binding to DNA, thus silencing gene expression. DNA methylation is a normal process that plays a key role in human development. As such during development, each differentiated cell obtains a unique DNA methylation pattern resulting in a specific gene transcription for each tissue [98]. Examples of the normal processes whereby DNA methylation occurs include X-chromosome inactivation, suppression of repetitive element transcription, and genome imprinting [99].

2. DNA methylation and enzymes

DNA methylation is catalyzed by DNA methyltransferase (DNMT) enzymes as they covalently add a methyl group at C5 of the cytosine ring [100]. The DNMT family consists of many enzymes including DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. DNMT1 is responsible for the maintenance of DNA methylation sites since, during the DNA replication phase (S phase), the enzyme copies the sites of DNA methylation to daughter strands [101], while the main function of DNMT3A and DNMT3B is related to the de novo methylation during development [102]. Although the function of these enzymes was initially accepted as such, further recent research suggested that each type of enzyme can be involved in both development and maintenance. DNMT3L participates in the de novo DNA methylation; it cooperates with DNMT3A and DNMT3B by increasing their capability to bind to the methyl group of the donor, S-adenosyl-L-methionine, and triggers their activity inside the body [103].

3. DNA methylation and cancer

Any alteration in the DNA methylation process can lead to the development of diseases especially cancer. Cancer has mainly been linked to DNA hypo- or hypermethylation at critical sites, as no evidence was shown related to any deficiency in the DNMT enzyme family [99, 104]. Hypomethylation usually occurs at satellites and retrotransposons sites that are normally heavily methylated [99], thus creating a genomic instability environment with the activation of oncogenes. In contrast, hypermethylation usually occurs at the promoter of tumor suppressor genes [99].

4. DNA methylation and breast cancer

There are several epidemiological studies that determined the differences in global and candidate genes methylation between breast cancer patients and healthy controls.

a. Global DNA methylation

Several studies were conducted to determine the global DNA methylation in breast cancer patients and showed conflicting results. Theses either evaluated the percentage of methylation using the luminometric methylation assay (LUMA) or the concentration of 5-methyldeoxycytosine (5- mdC) using the liquid chromatography-mass spectrometry (LC-MS), or by quantifying the DNA methylation in repetitive elements such as in LINE-1, Alu, and Sat2. The three studies that were done using the LUMA assay obtained different results, as one showed an increase in DNA methylation [105], another showed a decrease [106], and the third showed no significant change in the methylation between normal and breast cancer subjects [107]. Another study was done by Choi JY et al. [108] and showed that 5-mdC levels were significantly lower in breast cancer patients. Regarding LINE-1 methylation, nine studies showed no significant difference between control and breast cancer cases [106, 108-113]. With respect to the other two types of repetitive elements, Alu and Sat2, two studies were done with contradictory results as one showed hypomethylation pattern in both [109], while the other showed no significant difference between control and breast cancer cases [111].

b. Candidate gene DNA methylation

A number of studies evaluated the extent of methylation in breast cancer tissue and peripheral blood in several genes involved in cell cycle regulation (*p16, p14, p15, CCDN2, DAPK*), DNA repair (*MGMT, Hmlh1, BRCA1/2*), transformation (*GSTP1*), adhesion and metastasis (*CDH1, CDH13, e-CADHERIN, TIMP-3*), and signal transduction (*RARbeta2, APC, ER beta*) [114]. Some showed that the hypermethylation of certain tumor suppressive genes is a signature and diagnostic marker for breast cancer development. For example, Hu et al. [115] showed that the hypermethylation of *p16*, which is responsible for cell growth regulation, in the plasma of breast cancer patients was associated with nodal metastasis. Also, others showed that certain genes were found to be hypermethylated in breast cancer as they were involved in DNA repair (*BRCA1, hMLH1, HMSH2*) [116, 117], antiproliferative effect (*RARβ2*) [118], cell adhesion (*E-cadherin*) [119], and inhibition of protease activity (*TIMP-3*) [120], since silencing of the cell adhesion and inhibition of protease activity genes promotes metastasis. In addition, two studies were done to assess the diagnosis of breast cancer through serum DNA methylation of certain genes. For instance, Hoque et al. [121] and Dulaimi el al. [122] showed high sensitivity for the differential DNA methylation of *RAR-β2*, *APC, GSTP1*, *DAPK,* and *RASSF1A* genes in breast cancer patients.

In contrast, other studies showed a hypomethylation pattern of genes associated with breast cancer cell lines such as uPA, and S100A4. The uPA is a serine protease that is responsible for metastasis and invasion; it was found to be highly active in MDAMB-231 breast cancer cell line while methylated (hence inactive) in MCF-7 [123, 124]. Also, the expression of S100A4, a calcium binding protein, was higher in MCF-7

cell line secondary to hypomethylation thus enhancing the ability of the cell to become mobile and invasive [125].

5. DNA methylation and environmental exposure

There are two different ways whereby environmental exposure might lead to epigenetic changes, especially changes in methylation patterns of target genes. The first one illustrated in **Figure 4**, proposes a transcriptional change whereby epigenetic reprogramming leads to either hypo- or hypermethylation of the promoter gene region [126, 127]. For example, and as shown in **Figure 4A,** an environmental exposure causes the activation of transcription factors, as a mechanism of adaptation, and the inhibition of DNMT activity, thus the target gene becomes hypomethylated. The opposite can occur as illustrated in **Figure 4B** whereby environmental exposure inhibits the transcription factors, thus the DNMT enzymes act on the promoter region of the target gene leading to hypermethylation. The other way proposes the idea that a certain environmental exposure can cause DNA damage, and any DNA damage or genetic mutations can lead to epigenetic changes [126].

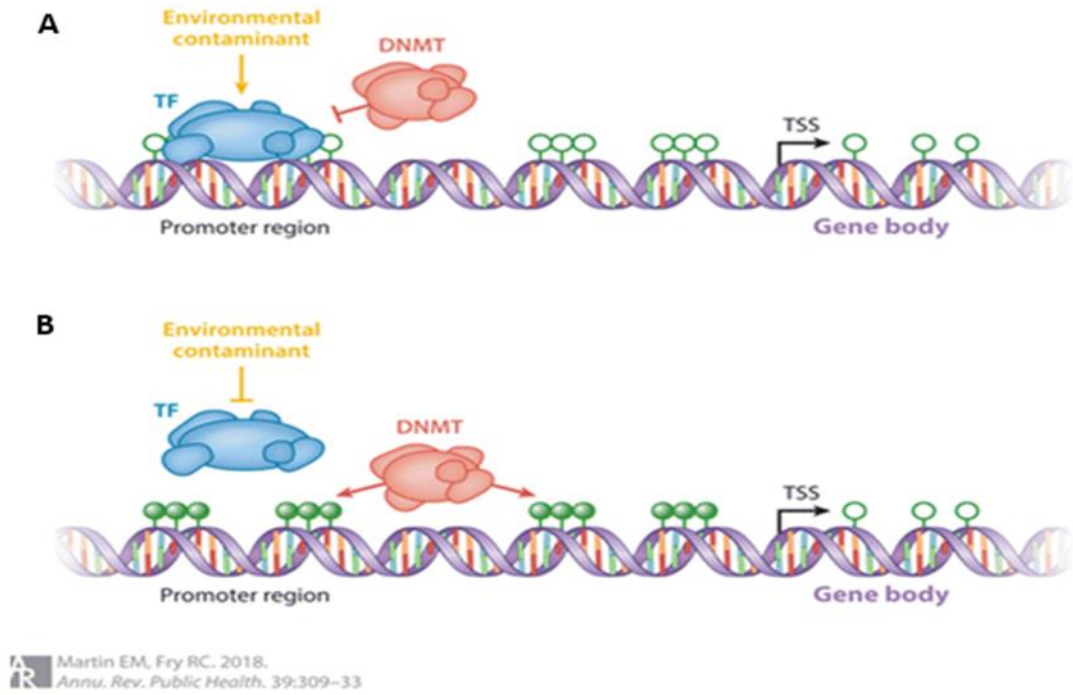


Figure 4 The effect of environmental exposure on DNA methylation, with **(A)** the effect that leads to hypomethylation of the promoter region and **(B)** the hypermethylation pattern [127].

6. DNA methylation and tobacco smoke

Tobacco smoking is one of the exposures that follows the two above described mechanisms of epigenetic induced changes. First, tobacco smoke carcinogens lead to the formation of DNA adducts and mutations and thereby DNA damage, this is accompanied by the development of cancer, as it was illustrated in **Figure 1**. Second they can modify the transcription of several genes with the most compelling evidence for hypomethylation of the aryl hydrocarbon receptor repressor (*AhRR*) gene, thus repressing the aryl hydrocarbon receptor (AhR) pathway [126].

Two very first studies were done on a sample of young African Americans, one with participants with an average age of 19 years and the other with slightly older

participants of 22 years of average age [128, 129]. Both studies indicated that the most significant association with smoking is related to a hypomethylation in a region of interest within the *AhRR* gene with probe ID cg05575921, with a false discovery rate corrected P value of less than 0.002. Interestingly, when comparing both studies, one can conclude that the demethylation of that area increased with longer duration of smoking, since in the 19 year old population the methylation status of the gene changed by 6%, while in the 22 years old population the demethylation increased to 11%.

More recently, a study was conducted on one of the largest European cohorts (European Prospective Investigation into Cancer and Nutrition) to differentiate DNA methylation sites between smokers and nonsmokers to elucidate specific biomarkers for the exposure to tobacco smoke [130]. The study analyzed around 748 CpG sites that were differently methylated between the two groups (smokers and non-smokers). The 748 CpGs were divided into 450 hypomethylated and 298 hypermethylated sites. Five sites were characterized with more than 5% difference (hypo or hyper) between both groups. The five hypomethylated sites were *AhRR* (cg05575921-the same one shown above, and cg23576855), *ALPPL2* (cg21566642), *F2RL3* (cg03636183), and *IER3* (cg06126421), while the five hypermethylated sites were *ZNF385D* (cg03274391, cg23480021, cg23126342), *MYO1G* (cg12803068), and *ZNF385D* (cg15693572). Additionally, 12 noval CpG sites were identified with more than 3% difference between both groups. Five of the 12 were hypomethylated (*ZNF577, LPAR6, PTBP3, WWC3,* and *PRDM1*), whereas 7 out of 12 genes were hypermethylated (*TSPAN4, TMEM136, DPH5, RCAN1*, *MIR5189, MCF2L*, and *SORBS1*). It was shown that the most significant gene with the highest mean methylation difference (17.6%) was the hypomethylation of the *AhRR* (cg05575921) gene shown above.

To our knowledge, no epidemiological blood-based study was performed on waterpipe smoking. We are currently conducting a pilot study in collaboration with the WHO-IARC on previously collected samples and data from the Greater Beirut cohort [131]. The study aims to identify and validate the differentially methylated genes related to cigarette-only vs. waterpipe-only. In the first year of the study, a whole genome DNA methylation was performed on the peripheral blood of three groups of healthy participants: 32 never smokers, 32 cigarette smokers, and 32 waterpipe smokers, whereby healthy controls were included in the study to exclude any bias related to DNA methylation with a certain disease. The obtained differentially methylated regions (DMR) were filtered based on having at least one CpG site of deltaβ value with a minimum of 3%, and for each DMR not less than two-third of the probes having more than or equal to 3% of deltaβ value. With respect to cigarette smokers compared to never smokers, 562 DMRs were obtained based on the criteria, and 321 for waterpipe smokers. Interestingly, only 24 DMRs were common between cigarette and waterpipe smokers.

The focus in the analysis was then done on the top probes with the highest effective size reflecting the most significant changes in the DNA methylation levels for each cigarette and waterpipe smokers. Results showed that the most significant levels of methylation changes in cigarette-only smoke was with cg05575921 of the *AhRR* gene (the same ROI discussed above and hence validated) with -22.58 hypomethylation effect size value. While in the waterpipe-only smokers the cg06201514 (*MYT1L*) was hypermethylated with an effect size of 11.1.

This is the only epigenome study data available to date, and in the current proposal, we will be focusing on cg05575921 of the *AhRR* and cg06201514 of the *MYT1L* gene.

7. DNA methylation, tobacco smoke and breast cancer

To our knowledge there is one epigenome-wide association study that links the changes in the level of methylation of target genes with cigarette smoke in breast cancer and healthy participants, but none is yet available for waterpipe smoke. This study was done on DNA taken from peripheral white blood cells of healthy individuals at the time of the collection, but subsequently developed breast cancer with an average lag time of 4.6 years [132]. Changes in the levels of methylation in relation with the smoking intensity was observed significantly with *AhRR*, 2q37, and 6p21 loci. Interestingly, the most significant ROI associated with smoking and the case-control participants was again, the hypomethylation of the *AhRR* gene with probe ID cg05575921 with as beta value of 0.84 in subjects who never smoked compared to 0.68 in current smokers [132]. Therefore, hypomethylation of cg05575921 in the *AhRR* gene appears to be a molecular biomarker for cigarette smoking exposure, but still needs further investigation on whether it is also associated with breast cancer development.

8. AhRR

AhRR (aryl hydrocarbon receptor repressor) is a transrepression protein that inhibits the AhR (aryl hydrocarbon receptor) pathway [133]. The AhR is a protein that is involved in several cellular responses, as it regulates the expression of proteins that are responsible for cell growth and apoptosis, and the transcription of genes that encode

for drug metabolizing enzymes [133]. AhR is found in the cytoplasm in its inactive form. When stimulated by its exogenous ligands such as PAH, it dimerizes with AhR nuclear translocator (ARNT), and the complex binds to the dioxin-responsive elements (DRE) whereby the transcription of the genes related to the AhR pathways are upregulated [133, 134]. Note that AhR regulates the transcription of its own suppressor the AhRR, hence the negative feedback loop [133]. **Figure 5** represents the AhRR structure [135].The negative feedback loop is referred to the ability of the AhRR protein to compete and dimerize with ARNT and repress the transcription of genes related to the AhR signaling pathways. **Figure 6** illustrates the AhR genomic pathway [135], and **Figure 7** illustrates the physiological and pathological effect of AhR activation [135]. The AhR pathway is responsible for the transcription of detoxifying enzymes, as shown in **Figure 7** under the physiologic effect of the pathway. So when *AhRR* is overexpressed due to hypomethylation, the AhR pathway and the activation of detoxifying enzymes is inhibited, thus leading to toxic carcinogen accumulation in the body which causes the development and progression of cancer.

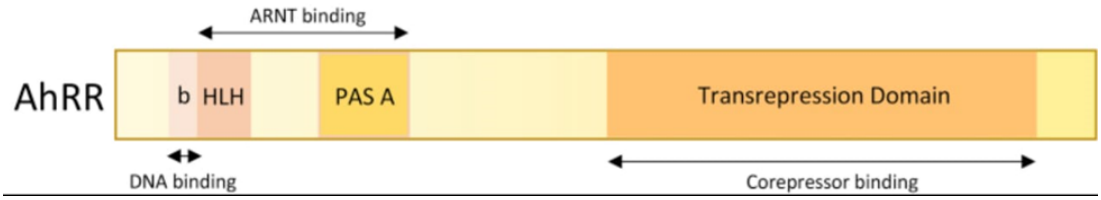


Figure 5 Representation of the *AhRR* gene [135].

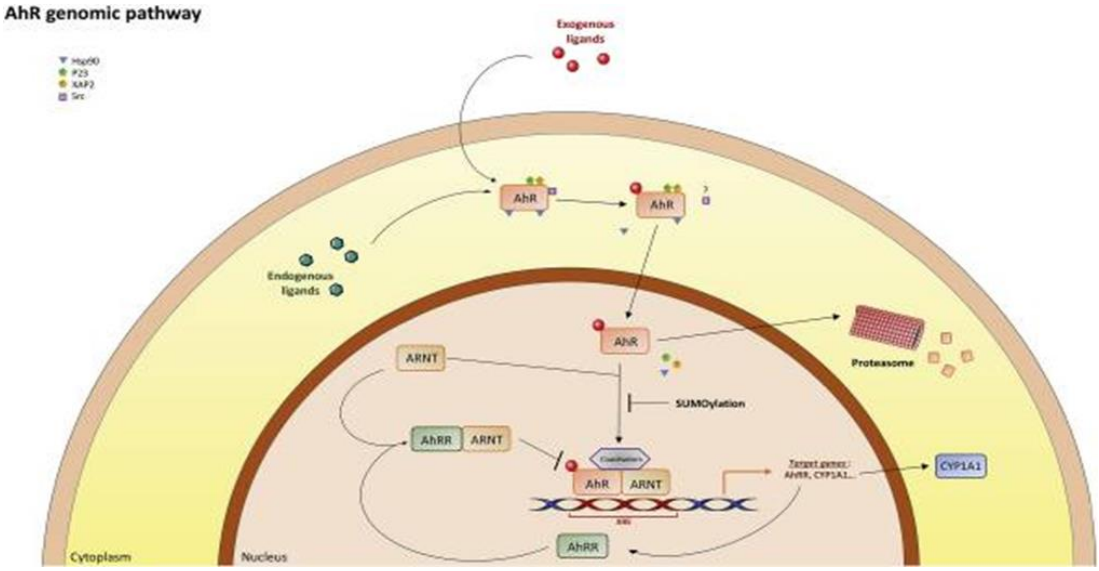


Figure 6 The AhR genomic pathway [135].

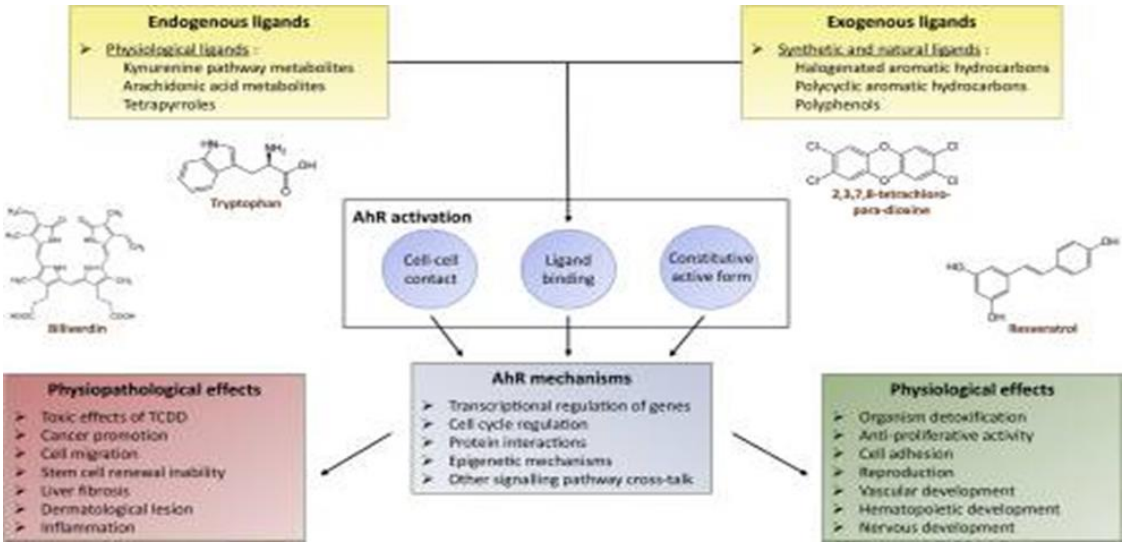


Figure 7 The physiological and pathological effects of AhR activation [135].

AhR can have either proliferative or anti-proliferative effects, and this depends on cell type, timing of the cell cycle with the expression of RelA or pRb (players in cell cycle that interact with AhR), and the developmental period [135]. For example, in AhR knock out (KO) mouse, it was shown that the growth rate in the embryonic fibroblast slows down and the cells arrest at G2/M phase [136]. In addition in human hepatoma cells, the AhR-siRNA resulted in a block of the cell cycle at G1 phase and a decrease in cyclins D1 and E [137]. Moreover in the MCF-7 cell line, the RelA subunit of the NF-kB interacts with AhR and activates the transcription of the c-myc, which is a proto-oncogene [138]. On the other hand, in non-proliferative hepatoma cells and in the fetal thymus, the AhR stimulated the transcription of p27kip1 which is a tumor suppressor gene [139, 140]. Also, the AhR interacted with pRb whereby the complex binds to E2F and stops cell cycle progression [141].

9. MYT1L

MYT1L (myelin transcription factor 1-like) belongs to the zinc finger transcriptional factor protein family which forms two clusters of C2HC zinc fingers [142]. This family is suggested to be involved in the transcriptional activity (both repression and activation), as Myt causes repression of transcription by interacting with Sin3 and histone deacetylase, while MYT1L is generally involved in transcriptional activation of the known consensus site. The St18 (suppressor of tumorigenicity 18) is one of the family members that was found to be a tumor suppressor in breast cancer [143]. MYT1L was found in high levels in the human fetal brain indicating that it has a role in the differentiation and development of neurons [144]. Also, MYT1L was found to be involved in reprogramming fibroblasts and other somatic cells to activate the

neuronal cell program, thus playing a role in converting non-neuronal cells in humans to neurons [145]. These studies hence suggest that the proteins involved in this family might have a role in carcinogenesis and neuronal development.

The MYT1L protein consists of 3 domains separated by two clusters of zinc finger with a third one found near the amino termini, as shown in **Figure 8** [146]. The carboxyl terminal domain is highly conserved in contrast to the amino terminal and the middle domain interacts with the co-repressors. MYT1L binds to the AAAGTTT consensus site, and it binds more strongly when the sequence is found in two copies that are separated by nine nucleotides called the DR9 motif. In an in vitro study done on Hela and A549 cell lines, MYT1L expression lead to activation of DR9-TATA reporter through the N-terminal domain which has the transcriptional activation function in the protein [146].

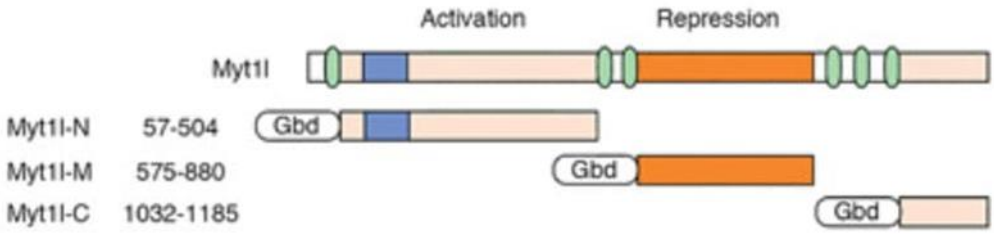


Figure 8 Structure of the MYT1L protein [146].

A2BP1 is a tumor suppressor gene which is involved in controlling the terminal differentiation in neuronal stem cells, and its expression was found to be positively correlated with the MYT1L transcription factor in glioblastoma [147]. As a matter of fact, a study demonstrated that two of the promoter sequences related to *A2BP1* were activated by enforced MYT1L expression, as MYT1L has four distinct binding sites in the two *A2BP1* promoter sequences [147]. Also, MYT1L was found to have binding sites on 258 genes that are responsible for biological functions like cellular assembly

and organization, metabolism, and nervous system development [147]. *MYT1L* was shown to be deleted with *A2BP1* in 5% of glioblastoma samples, this shows that MYT1L-A2BP1 axis is responsible for the terminal differentiation program, and its neutralization led to the development of gliomagenesis [147].

Another in-vitro study was done to determine the role of MYT1L in tumorigenesis [148]. And since the MYT1L transcriptional factor was found to be abundant in the human brain, the human brain tumor cell lines M059K and M059J were used in that study. The M059K cell line consists of normal DNA-dependent protein kinase (DNAPK), which is involved in DNA repair and is overexpressed in human breast cancer as well, in contrast to the M059J cell line that is deprived from DNAPK function. In this study in both cell lines, the miRNA-141 which was previously shown to suppress cell proliferation and induce apoptosis in breast cancer and *MYT1L* expression were tested. In M059K, the ectopic expression of miRNA-141 caused a decrease in the expression of *MYT1L* and cell proliferation, whereas the inhibition of miRNA-141 promoted cell proliferation. In contrast in M059J cell line, miRNA-141 caused inhibition of cell proliferation, as it induced cell cycle arrest at S-phase when it is overexpressed, and at G1 phase when it is inhibited. This suggests that miRNA-141 might exhibit a dual role of tumor suppressor or oncogene. It was also shown in cell line and glioma tissue samples that down-regulation of the miRNA-141 caused up-regulation of *MYT1L* indicating that there is an inverse correlation between them [148].

10. DNA methylation in cell lines upon exposure to smoke extract

Several studies were conducted to determine the DNA methylation changes upon exposure to cigarette smoke on different cell lines and time points of exposure.

Most of the investigations applied the candidate gene ROI approach with only one performing whole methylome analysis.

In one study, the authors analyzed the differential methylation of an area in the candidate gene synuclein-ɣ oncogene between two lung cancer cell lines H292 and A549 [149]. Results showed that exposure of A549 to CSE at different concentrations for 3 days induced a significant concentration dependent increase in the level of synclein-ɣ mRNA, and this was associated with demethylation. Also, a study was done on human oral keratinocytes using CSE exposure to determine the level of methylation changes of the tumor suppressor gene Nischarin (NISCH).Upon exposure to CSE for one week (round 1) at passage 3 and then re-exposure for another week (round 2) after passaging (at passage 4), CSE induced hypermethylation of the NISCH gene [150]. Moreover, studies were done on human bladder and urothelial cells to determine the level of DNA methylation changes. T-24 human bladder cancer cells were exposed for 3 days at different concentrations to detect the changes in the level of methylation for WWOX tumor suppressor gene [151]. Results showed a strong association between the increase in concentration and duration of CSE exposure and decrease in WWOX mRNA expression coupled with an increase in methylation levels of the gene. In addition, treatment of immortalized human urothelial cells with CSE for a long duration resulted in a decrease in the level of methylation for RUNX3 and IGF2-H19 loci [152]. Also another study was done to compare cells (LL29, Hs.888, and NL-20) DNA methylation of several genes (DAPK, ECAD, MGMT, and RASSFIA) upon exposure to low and high concentrations of cigarette smoke [153]. Results showed that fibroblast cell lines (LL29 and Hs888) methylation status did not significantly change; whereas in NL-20 lung cells, ECAD was significantly hypermethylated at both concentrations and

MGMT was hypomethylated only at high concentrations of exposure. In the same study, NL-20 lung cells were also chronically exposed to cigarette smoke, and showed a change of methylation status for RASSF1A from hypomethylated at short term exposure (72hr) to hypermethylated after 28 days of exposure; hence iterating the impact of exposure duration on differential methylation.

As noted above, only one study did genome-wide DNA-methylation at different time points (6, 10, and 15 months) on human bronchial epithelial cells to detect epigenomic changes with chronic exposure to cigarette smoke [154]. Interestingly, different methylation results in different genes appeared at each of the time-points, and *AhRR* hypomethylation did not appear in any of the affected genes.

Therefore, it looks that the epigenetic effects of cigarette smoke are cell line and time dependent. Of note that no such studies were performed with breast cell lines, and all smoke extract exposures entailed cigarette smoke with no waterpipe exposure to date.

CHAPTER II

AIMS

The available epidemiological association studies showed that long term exposure to tobacco smoke is associated with breast cancer. Moreover, several epidemiological studies were done to determine the methylation alterations associated with cigarette, but none with waterpipe smoke. Also, many studies were done using cigarette smoke on different cell lines including breast cell lines, but only one with waterpipe smoke none of which evaluated the methylation effects of the different exposures. Accordingly, we were initially planning on comparing the methylation effects of cigarette smoke to that of waterpipe smoke on breast cancer cell lines MCF-7 and MDAMB-231, upon chronic exposure with low, non-cytotoxic, carcinogenic doses of smoke extract. Nevertheless, and due to the many challenges that we faced regarding the recurrent and extended lockdowns coupled with COVID-19 sicknesses, we decided to start with acute exposure at high, cytotoxic doses with the objective to identify epigenetic markers associated with the cytotoxic and genotoxic effects of waterpipe and cigarette smoke extract in breast cancer cell lines. The following are the two study aims:

Aim 1: To determine the genotoxic and potentially enhanced carcinogenic effects of cytotoxic cigarette and waterpipe smoke in MCF-7 and MDAMB-231 breast cancer cell lines. IC20 and IC50 concentrations will be determined with MTT assay followed by targeted trypan blue assay and cell cycle assay. Genotoxicity will be evaluated with the ɣH2AX assay. Potential for enhanced carcinogenicity will be evaluated by looking at change in morphology, cell migration and EMT markers

Aim 2: To compare the differential methylation of the *AhRR* and *MYT1L* regions of interest in both waterpipe and cigarette smoke in breast cancer cell lines using direct bisulfite sequencing.

In the future, and in collaboration with the WHO IARC, we plan to perform whole methylome analysis to identify novel differentially methylated regions of interest, and to analyze the pathways behind the functional changes related to the gene differential methylation and breast cancer.

CHAPTER III

METHODS

A. Cell lines and media

Two breast cancer cell lines were used: the MCF-7, a hormone positive breast cell type, and MDAMB-231, a triple negative hormone breast cell type. Dulbecco's Modified Eagle Medium (DMEM) was used for both cell lines as media, and completed with 10% fetal bovin serum (FBS), 1% sodium pyruvate, and 1% penicillin with streptomycin.

B. Protocol for smoking

1. Waterpipe extract

Waterpipe constituents were collected according to the standard smoking protocol (Beirut Method) [41], whereby the average of the different smoking parameters was taken according to the mean result from 52 smokers that covered costumers of a café near the American University of Beirut reflecting more the sizeable fraction of young users [41]. The average waterpipe smoking parameters comprise of 530ml puff volume, 2.6s puff duration, 17s interpuff interval, and 171 puff frequency. The tobacco used in the waterpipe consists of 10g two apple flavor the brand used is Nakhla Egypt, and an aluminum foil (of 9*9cm perforated with 18 hole) is placed on top of it separating it from charcoal [40, 41]. A single 33mm cylindrical briquette charcoal three king brand of 5-6g weight was placed on top of the aluminum. At the 105 puff, a new half briquette charcoal was added [40]. The total particulate matter that results after smoking is collected in laboratory filter papers. The filters were weighted before and

after the collection of TPM. The filters were squeezed with DMEM using syringe, collected in a tube. After that, 10% FBS, 1% sodium pyruvate and penicillin/ streptomycin were added to the final volume of extraction, and the tube was stored in the freezer at -20 degree Celsius (not more than one month) until the day of treatment.

2. Cigarette smoke extract

Constituents of the 3R4F cigarette smoke, from University of Kentucky, were collected on filters using the International Organization of Standardization protocol. Briefly, the parameters of the protocol consist of 35ml puff volume, 2s puff duration, 60s puff frequency, and 200ml/min air velocity. Constituents of the filters were collected with the same process as that for waterpipe smoke.

C. Cell metabolic activity assay

The cell metabolic activity was determined using MTT assay. The MTT reagent, obtained from Sigma Aldrich USA, refers to 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, which is a yellow tetrazolium salt that is reduced by NAD(P)H-dependent oxidoreductase enzyme, found in the mitochondria, to become purple formazan crystals reflecting a metabolically active cell.

MCF-7 and MDAMB-231 cell lines were treated with waterpipe and cigarette smoke extracts at different concentrations to determine the IC20 and IC50 of each tobacco product, and media alone was used as a control. Each concentration was used in triplicates for each cell line in 96 well plate for 24, 48, and 72hr. After each endpoint the treatment was removed and MTT reagent was added (25 µl MTT and 100µl media). The cells were incubated with MTT reagent for 4 hours, and then the formed crystals

were dissolved by adding 100 µl DMSO. The absorbance of the colored solution was measured at 595nm. Of note that these data were presented by Miss Carole Abdel Karim, a previous MS candidate in the lab.

D. Cytotoxicity

1. Trypan blue

Trypan blue staining differentiates live from dead cells. Trypan blue stain cannot penetrate an intact membrane, it rather passes through the formed porous membrane of dead cells and enters the cytoplasm, and thus it stains the dead cells blue.

Cells were treated with the 24 hour IC20 and IC50 of each waterpipe and cigarette smoke extract for 24 and 48 hours, as cells were cultured in 6 well plates in duplicates for each condition. After trypsinization and removal of trypsin and media, cells were mixed with 2 ml PBS. A volume of 0.1 µl of cells was removed and mixed with 0.1 µl trypan blue to have 10x dilution factor. Counting at the four squares of the hemacytometer was done (live and dead cells), and the following equation was used to determine the number of live and dead cells: (number of cells counted (live or dead)/number of squares counted) x dilution factor (with PBS) x dilution factor with trypan blue stain. Mean results were calculated from three trials. Of note that these data were presented by Miss Carole Abdel Karim, a previous MS candidate in the lab.

2. Flow cytometry

Cells were harvested, collected in 15ml tube for each treatment, and washed with 1x PBS. Then cells were centrifuged at 15000rpm for 5 min, the supernatant was discarded, and cells were fixed with PBS and ethanol for storage. After that, cells were

centrifuged, washed with PBS, and re-centrifuged. Finally, PBS and PI (Propidium Iodide) were added for sample reading using Guava. The analysis was done using FlowJo software.

E. Genotoxicity

Genotoxicity refers to a direct damage in the DNA. The toxic effect on DNA when exposed to a certain carcinogenic starts with DNA double-strand breaks. This event is followed by the induction of histone H2A phosphorylation on Ser-139 to become ɣH2AX [155]. The formation of ɣH2AX is a marker of DNA damage that might be followed by DNA repair. ɣH2AX plays a critical role in DNA damage response as it stimulates cell cycle checkpoints and repair proteins [156]. For example, ɣH2AX interacts with mediators of DNA damage checkpoint protein 1 (MDC1), 53BP1 and BRCA1 to initiate DNA damage signaling and repair mechanism [157].

The ɣH2AX assay was used to determine the genotoxic effect of waterpipe and cigarette smoke. Briefly, cells were incubated for 24hours with each condition (the 24hrs IC20 and IC50) for waterpipe and cigarette smoke in duplicates in 6 well plates and coverslips with a well for positive control (H_2O_2), and a well for negative control, whereby cells in this well are not exposed to the primary antibody. After treatment, cells were fixed with formaldehyde, washed with PBS (tween can be added depending on cell type like in MCF-7, but not in MDAMB-231), and permeabilized with 0.5% triton. Primary antibody was then added to the cells except for negative control, followed by fluochrome-conjugated secondary antibody. Then, on a coverslip slides the gold antifade reagent was added with DAPI. After that, images were taken using zenn software on confocal microscope. DAPI, primary and secondary antibodies were

obtained from Cell Signaling Technology Company, headquartered in Danvers, Massachusetts, USA.

F. Morphology

We observed under the microscope at 40X magnification any phenotypic changes for each condition and cell line, with both time points: 24 and 48 hours.

G. EMT markers

The mRNA expression of EMT markers was evaluated by real-time PCR. First, RNA extraction from cell pellets was done by adding trizol, chloroform, isopropanol, Dnase buffer, and Dnase enzyme in this order with centrifugation steps as described in the Trizol-based RNA extraction protocol using DNase treatment and removal kit (Invitrogen, USA). Then, isolated RNA samples were read on the nano-drop spectrophotometer for quantity and quality. They were also run on gel for quality ascertainment followed by reverse transcription into cDNA using the high-capacity reverse transcription kit from Applied Biosystems, USA. RT-PCR followed with a starting concentration of 12.5ng/ml. The RT-PCR mix consisted of Sybr green master mix (Bio-Line sensifast, USA) and primers (E-cadherin and CDH-1 as epithelial markers, vimentin and SNAIL as mesenchymal markers and GAPDH as internal control) **(Table 5)**. 8 µl of that mix were added to 2 µl of the samples in triplicates in a 384 well plate. A standard curve was prepared using one of the samples with a serial dilution of 1/5 ratio (starting from 0.5ng/ml to reach 125ng/ml). A melting temperature of 60 degrees Celsius was used for all.

Relative gene expression was estimated using the threshold cycle of the sample and GAPDH with the following equation used: 2^ (Ct of GAPDH)/2^ (Ct of the sample).

Table 5 Forward and Reverse primers for the epithelial and mesenchymal markers.

Marker type	Primer	Sequence	
		Forward	Reverse
Epithelial markers	E-cadherin	F: CAGAAAGTTTTCCACCAAAG	R: AAATGTGAGCAATTCTGCTT
	CDH-1	F: TTCTGCTGCTCTTGCTGTTT	R: TGGCTCAAGTCAAAGTCCT
Mesenchymal markers	Vimentin	F: AGGTGGACCAGCTAACCAAC	R: TCTCCTCCTGCAATTTCTCC
	SNAIL	F: AAGATGCACATCCGAAGCCA	R: CTTCTCGCCAGTGTGGGTC
Internal control	GAPDH	F: GAAGGTGAAGGTCGGAGTC	R: GAAGATGGTGATGGGATTTC

H. Migration

MCF-7 and MDAMB-231 were seeded in a 6 well plate to reach 100% confluency and in duplicates for each condition control, WPE IC20, CSE IC20. Then a scratch was manually done in the well using a white tip. The distance travelled at 8 and 24 hours was measured systematically with horizontal lines and averaged using the perfect screen ruler software.

I. DNA methylation

1. DNA collection and isolation

DNA isolation for MCF-7 and MDAMB-231 cell lines was done for all conditions as duplicates, after cells being cultured for 48hr, harvested with trypsin, washed with PBS, and then centrifuged leaving the pellet to snap freeze using liquid nitrogen. After that cell pellets were stored at -80 degree Celsius. Pellets were treated with FG2 and protease (to break down cell membrane of proteins), FG1 (to dissolve cell membrane), isopropanol (to precipitate DNA), ethanol, and FG3 (to dissolve DNA) (Flexigene DNA kit by Qiagen, Germany). The concentrations of isolated DNAs were read on nano-drop spectrophotometer.

2. Bisulfite conversion

DNA samples were treated with modification reagent, binding buffer, desulfonation and elution buffer (thermoscientific, Lithuana, EpiJET Bisulfite Conversion Kit). By this, the unmethylated cytosine in the DNA sequence was converted into uracil, while the methylated cytosine stayed the same. Bisulfite converted DNA samples were stored at -20 degree Celsius until analysis.

3. Primer design

The genome browser gateway of the human population of the human assembly Feb.2009 (GRCh37/hg19) was used in order to design primers for the two regions of interest: *MYT1L* and *AhRR.* For the *MYT1L* gene the region of interest (cg06201514) is located on chr2:1,817,409bp, so we chose to start with chr2:1,817,351bp with the addition of 500bp upstream and 509bp downstream. The sequence (positive strand),

which was given by the genome browser gateway, was taken to be used for primer design for bisulfite sequence PCR (BSP) using the methprimer application (urogene.org, website). The chosen primers and their characteristics are shown in **Figure 9.**

	Primer name	Sequence (5'-3')	Len	Start	End	C's	Self_any	Self_end	Tm	GC%	Stability	Score
Pair	F1	TATAGGTGGAGGTGGGTAGTG	21	41	61	2	400	0	56.6	52.4	5.7	3.3
	R1	TCCACCTAAAATCACCAAAATTAAC	25	214	190	5	400	200	59.6	32.0	6.0	4.0

Product size	Pair start	Pair end	CpG's	Comp_any	Comp_end	Tm	Tm_diff	Score
174	41	214	14	800	100	75.1	3.0	12.1

Figure 9 The forward and reverse bisulfite sequence primers covering the region of interest of the *MYT1L* gene.

For the *AhRR* gene, 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 site of ROI. The chosen primers and their characteristics are shown in **Figure 10**.

	Primer name	Sequence (5'-3')	Len	Start	End	C's	Self_any	Self_end	Tm	GC%	Stability	Score
Pair	F1	TGTTGGTAGGATATAGGGGTTG	22	122	143	4	400	0	57.9	45.5	8.2	4.3
	R1	CCTACCAAAACCACTCCCAA	20	315	296	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 10 The forward and reverse bisulfite sequence primers covering the region of interest of the *AhRR* gene.

4. Polymerase chain reaction

PCR mixture consisted of HRM master mix (Thermo Fisher Scientific, Lithuana), forward and reverse primers for *AhRR* or *MYT1L* genes as applicable, and nuclease free water. Different 96 well plates were used for each *AhRR* and *MYT1L* PCR reactions, since the melting temperature for *AhRR* was best at 58 degree Celsius and 57

degree Celsius for the *MYT1L*. 2µl of each sample was prepared in 96 well plate as duplicate, and 18 µl of the mix was added to each sample, thus making the total volume in each well 20µl. After that, PCR samples were purified by either gel extraction kit or PCR cleanup kit (Sigma-Aldrich, USA).

The purification method was selected based on the DNA PCR product quality that can be defined by 1) checking the NTC and 2) making sure that the melting curve of the sample is above threshold to reflect a good DNA PCR product. Accordingly, samples that had a melting curve reflecting poor DNA quality product were purified by gel extraction technique in order to double check and see the band reflecting PCR product quality before preparing and sending them for sequencing , while samples with good melting curve (good DNA quality product) were purified using PCR cleanup kit. In the gel extraction technique, chosen samples were loaded on gel using 4µl loading dye at a voltage of 100 for 40 minutes. Then bands were cut, gel solubilizing solution was added, and samples were heated at 55 degree Celsius to dissolve the band. After that, isopropanol was added to each dissolved band tube with a volume equivalent to the band weight of the sample. The samples with isopropanol were then removed to a column tube that was prepared using column preparation solution, and centrifuged. Washing buffer was then added, centrifuged, and finally elution buffer was added to elute the DNA in 1.5ml eppendorf tube and stored at -20 degree Celsius until sequencing.

Concerning the PCR cleanup kit, the column binding tube was prepared using the column preparation solution. The binding solution was added to the PCR product samples and transferred to the column binding tube, then centrifuged. Washing solution

was added, centrifuged, and finally the elution buffer was added to elute the PCR sample DNA that was stored at -20 degree Celsius until sequencing.

5µl of each purified sample was added to 1µl of loading dye and run on agarose gel to see whether there is a dark band reflecting the quality of the DNA.

5. Sanger sequencing

When ready to be sent to the Sanger sequencer, 10 or 14µl (depending on purified product quality) of the *AhRR* and *MYT1L* purified products (control, WPS and CS IC20, IC50 of three trials for the MCF-7 and MDAMB-231 cell lines) were mixed with 2µl of forward primers *AhRR* or *MYT1L* and 4µl of nuclease free water. The samples were stored at -20 degree Celsius until the day of sequencing.

6. ESME analysis

After checking the quality of the Sanger sequencing output, these were entered to the ESME software [158] for analysis, as the software gives the percentages of methylation at each CpG covered by the sequenced band for each sample.

CHAPTER IV

RESULTS

A. Cell metabolic activity

The MTT results of MCF-7 cell line showed that the IC20 was 6.5mg/ml and IC50 9.5mg/ml after 24 hours of exposure for waterpipe smoke, and for the cigarette 0.5mg/ml and 0.7mg/ml respectively. While for the MDAMB-231 the IC20 and IC50 of the waterpipe smoke after 24 hours were 8.5mg/ml and 13mg/ml, respectively. And for the cigarette smoke the IC20 and IC50 were 0.7mg/ml and 1mg/ml, respectively. These effects were also time dependent with lower IC20 and IC50 at 48 and 72 hours (Figure 11 and 12). The rest of the experiments were performed using the IC20 and IC50 values of the 24 hour exposure. Of note that these data were presented by Miss Carole Abdel Karim, a previous MS candidate in the lab.

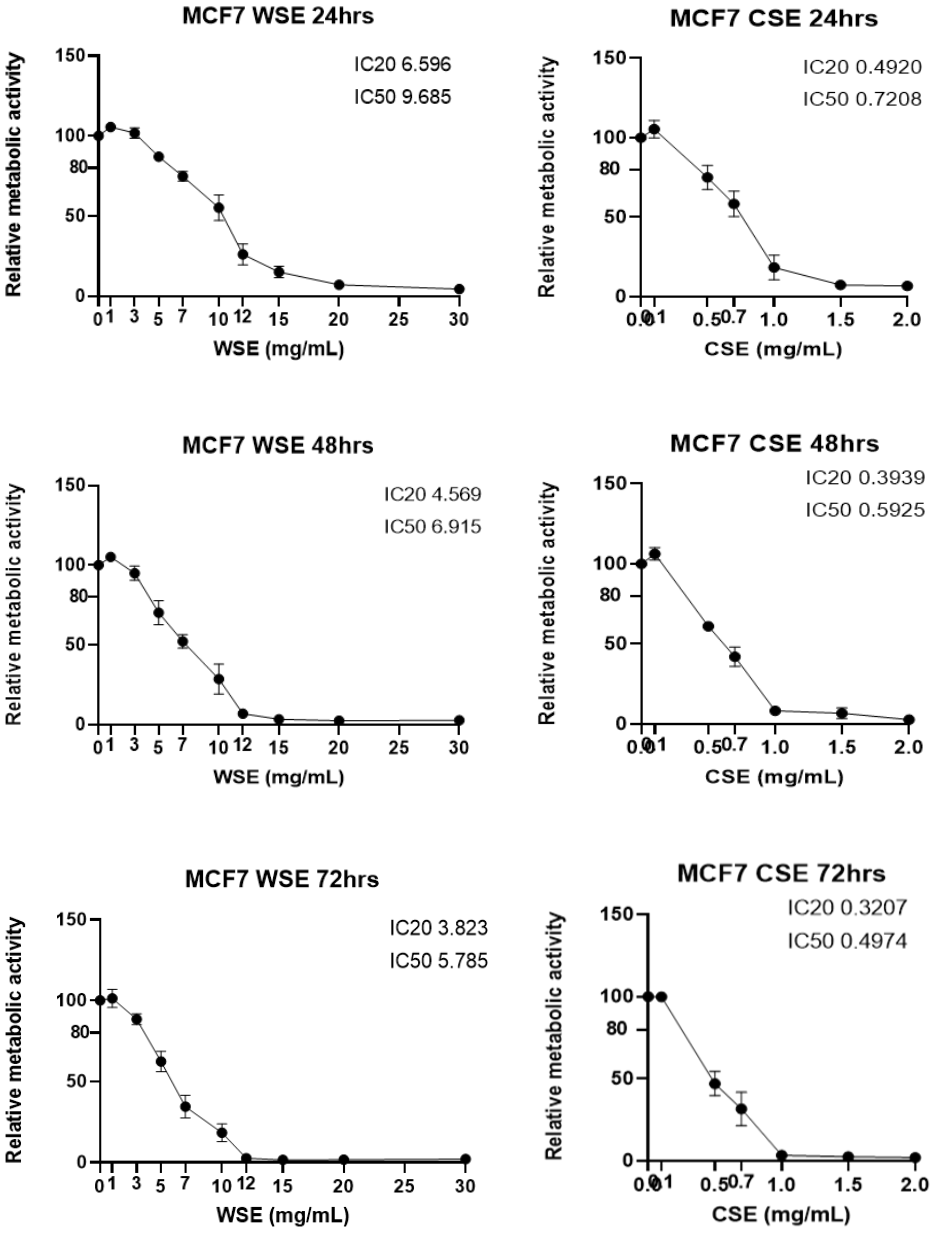
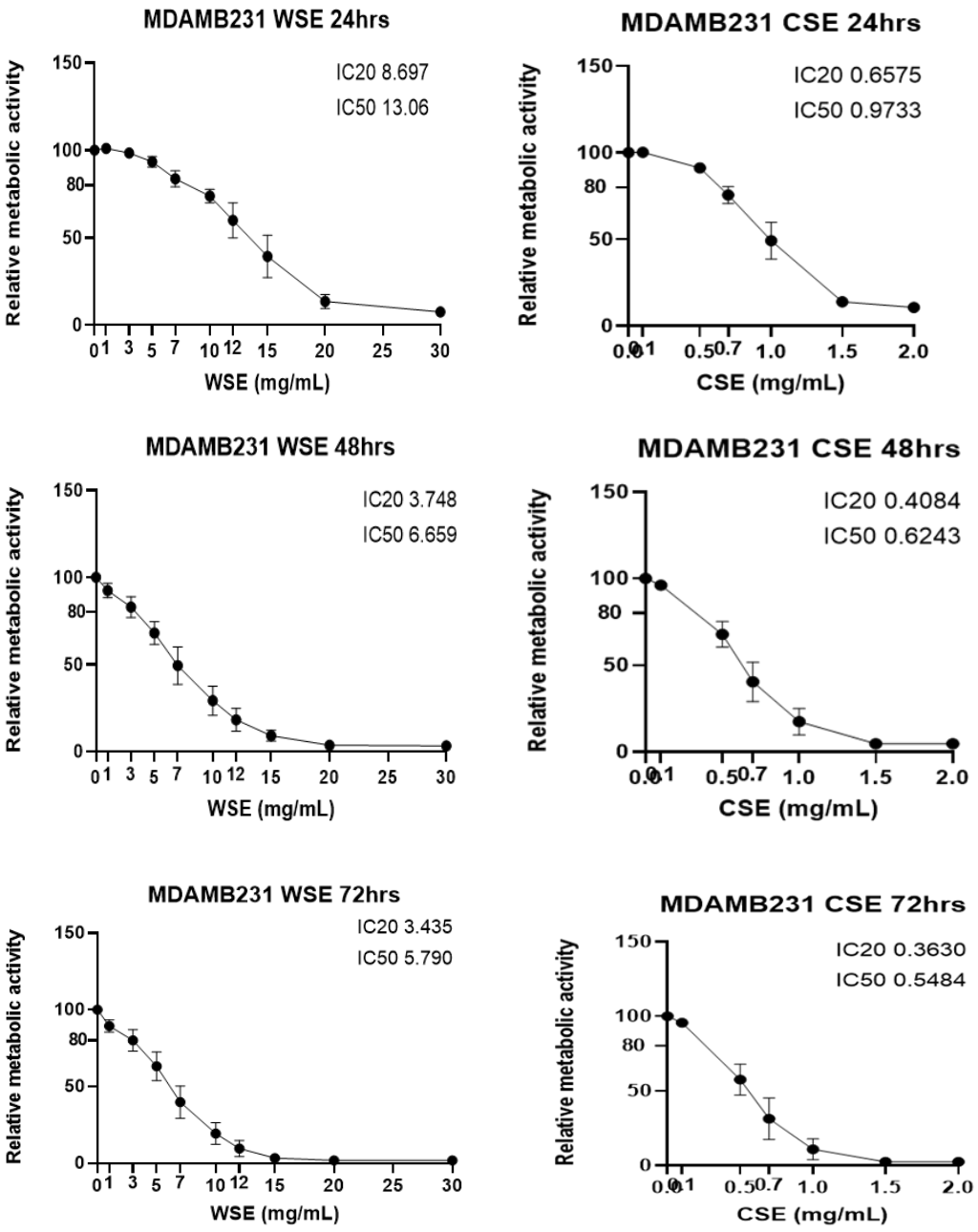


Figure 11 The effect of different concentrations of waterpipe **(A)** and cigarette smoke **(B)** on the MCF-7 cell metabolic activity on 3 consecutive days. Data are means ± SEM of at least three trials.

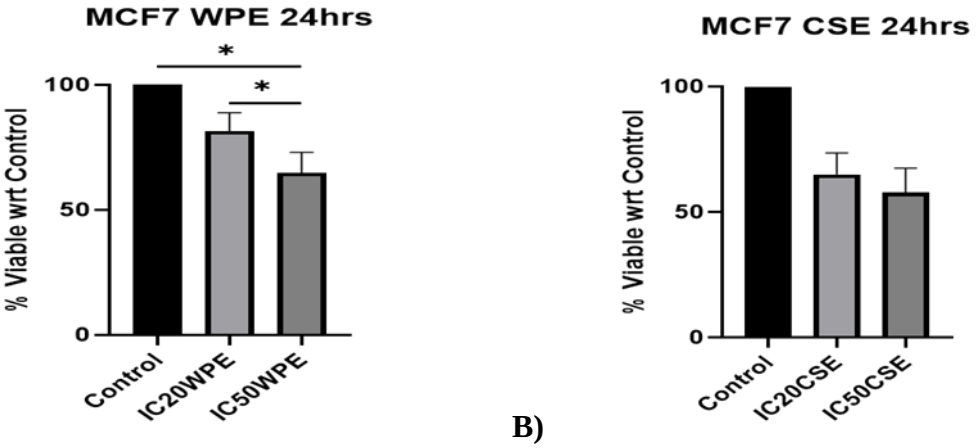


(A) **(B)**

Figure 12 The effect of different concentrations of waterpipe **(A)** and cigarette smoke **(B)** on the MDAMB-231 cell metabolic activity on 3 consecutive days. Data are means ± SEM of at least three trials.

B. Trypan blue

Cell count using trypan blue for the MCF-7 cell line showed that the waterpipe extract caused a significant decrease (P-value< 0.05) in the cell viability after 24 hours of exposure with respect to the control for the IC50 concentration. There was also a statistically significant decrease (P-value< 0.05) between the two exposed concentrations with a higher decrease in cell viability upon exposure to the higher concentration (Figure 13A). With respect to cigarette smoke extract exposure, there was a trend of decrease in cell viability though with no statistical significance (Figure 13B). As for the MDAMB-231 cell line, there was a statistical significant decrease in cell viability after 24 hours of exposure for both IC20 and IC50 concentrations of waterpipe and cigarette smoke extracts with respect to the control, as well as between both concentrations with a more pronounced decrease with the IC50 concentration (Figures 14 A and B).

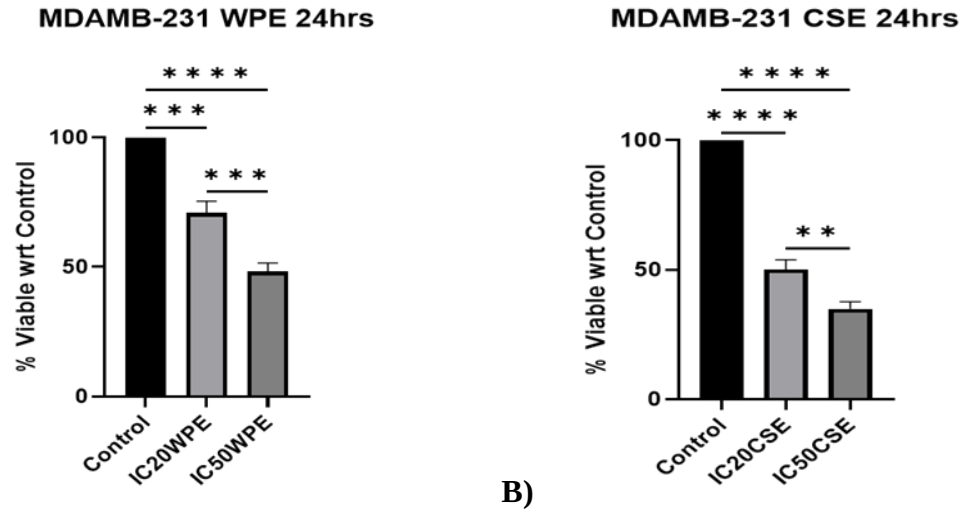


A) **B)**

Figure 13 Effects of different concentrations of waterpipe **(A)** and cigarette **(B)** smoke extract on MCF-7 cell viability and its percentage with respect to control using trypan blue assay.

Data were compared using One-Way ANOVA followed by Tukey posthoc test.

*P<0.05



A) **B)**

Figure 14 Effects of different concentrations of waterpipe **(A)** and cigarette **(B)** smoke extract on MDAMB-231 cell viability and its percentage with respect to control using trypan blue assay.

Data were compared using One-Way ANOVA followed by Tukey posthoc test.

**P<0.05; *** P<0.001; **** P<0.0001

C. Genotoxicity

The images of the genotoxicity assay for MCF-7 cell line showed that the treated cells for 24 hours have more DNA damage relative to the control, as we can observe more green dots that represent expression of ɣH2AX reflecting DNA double strand break. The effect is also concentration dependent. As for the MDAMB-231 cells, images showed that the level of foci increased in exposed cells when compared to controls, but further quantitative measurements should be done to determine the intensity of difference.

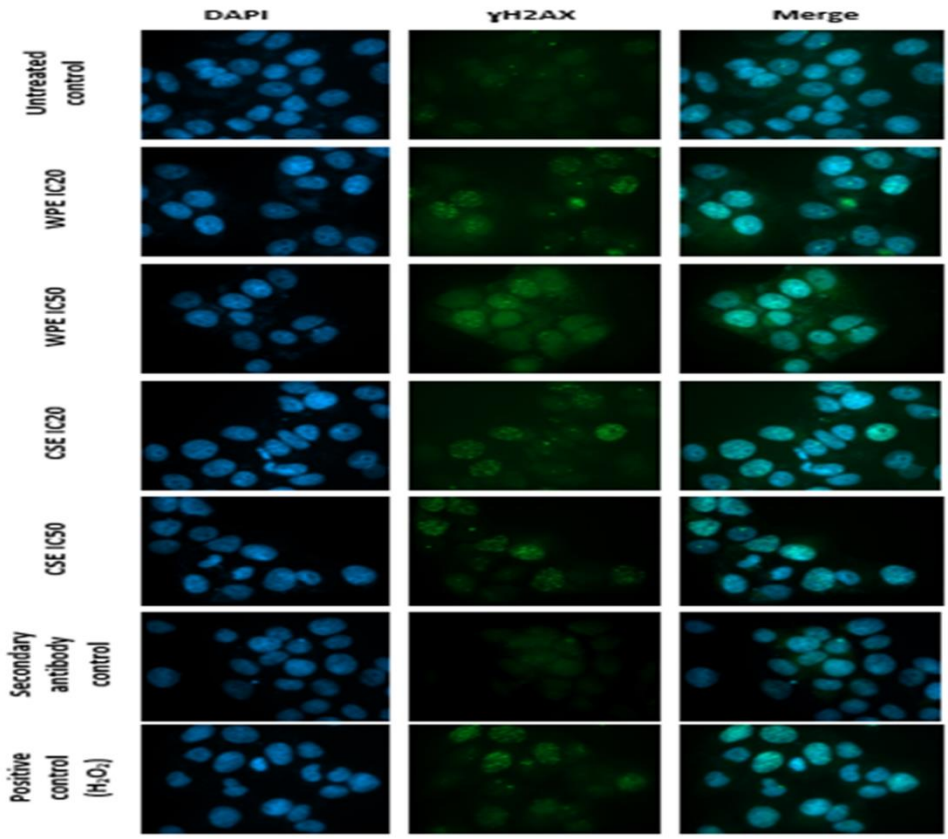


Figure 15 Representative images (100X magnification) showing the effects of different concentrations of waterpipe and cigarette smoke on DNA damage in MCF-7 cell lines using γH2AX assay.

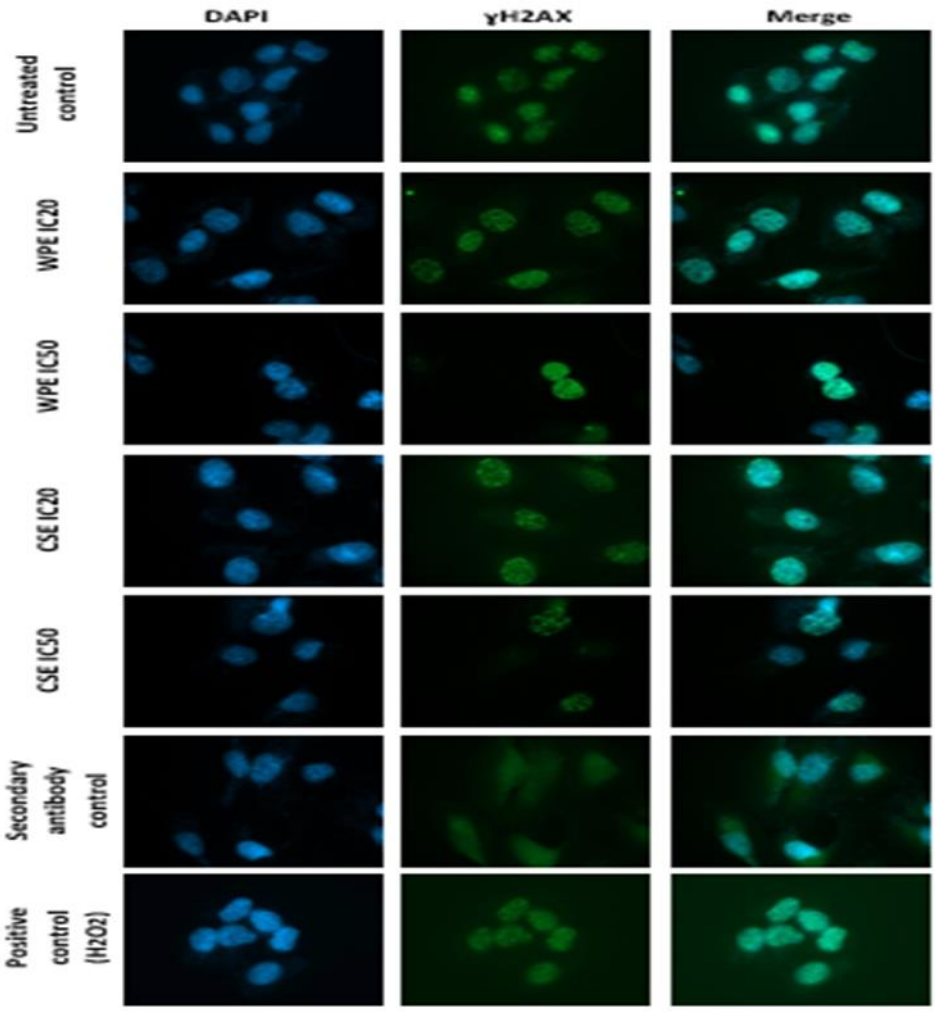


Figure 16 Representative images (100X magnification) showing the effects of different concentrations of waterpipe and cigarette smoke on DNA damage in MDAMB-231 cell lines using ɣH2AX assay.

D. Flow cytometry

The flow cytometry results for the MCF-7 cell line (Figure 17) showed that, along with the expected increase in cell death (SubG0 and trypan blue results), there is a trend of increase in cell cycle arrest at the S phase being statistically significant upon exposure to WPE and CSE at IC50 (Figure 18). As for MDAMB-231 (Figure 19), cells were significantly arrested in the S phase upon exposure to the CSE IC20 as shown in Figure 20.

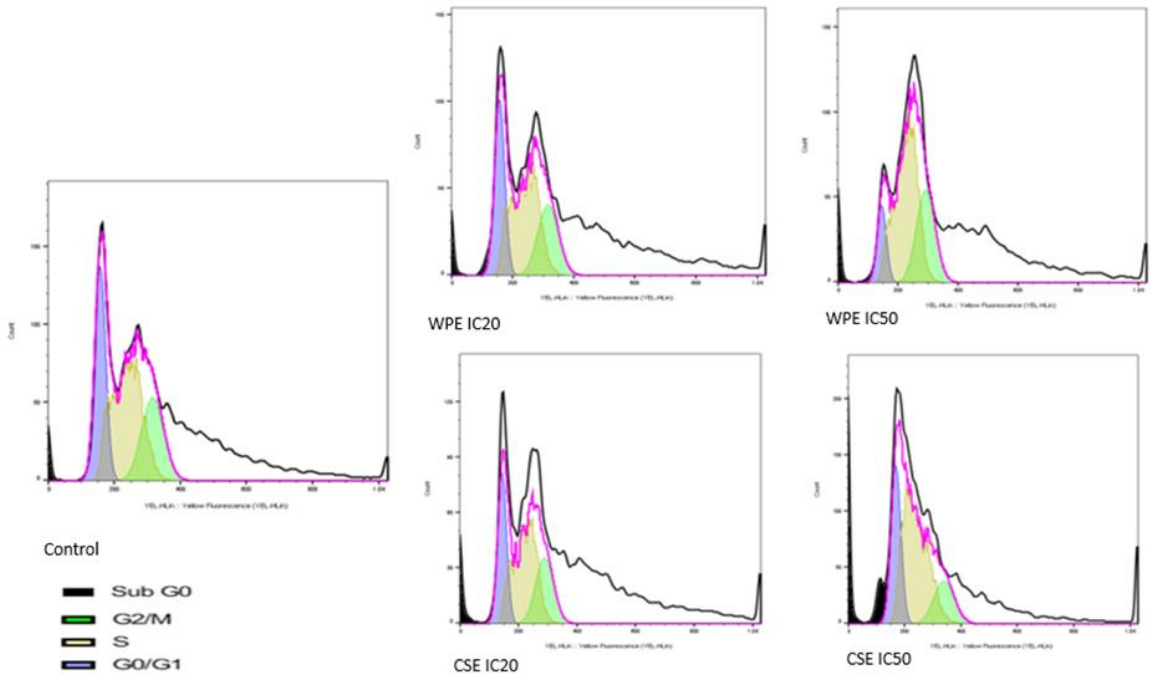


Figure 17 Representative MCF-7 cell line cell cycle upon exposure to different concentrations of WPE and CSE.

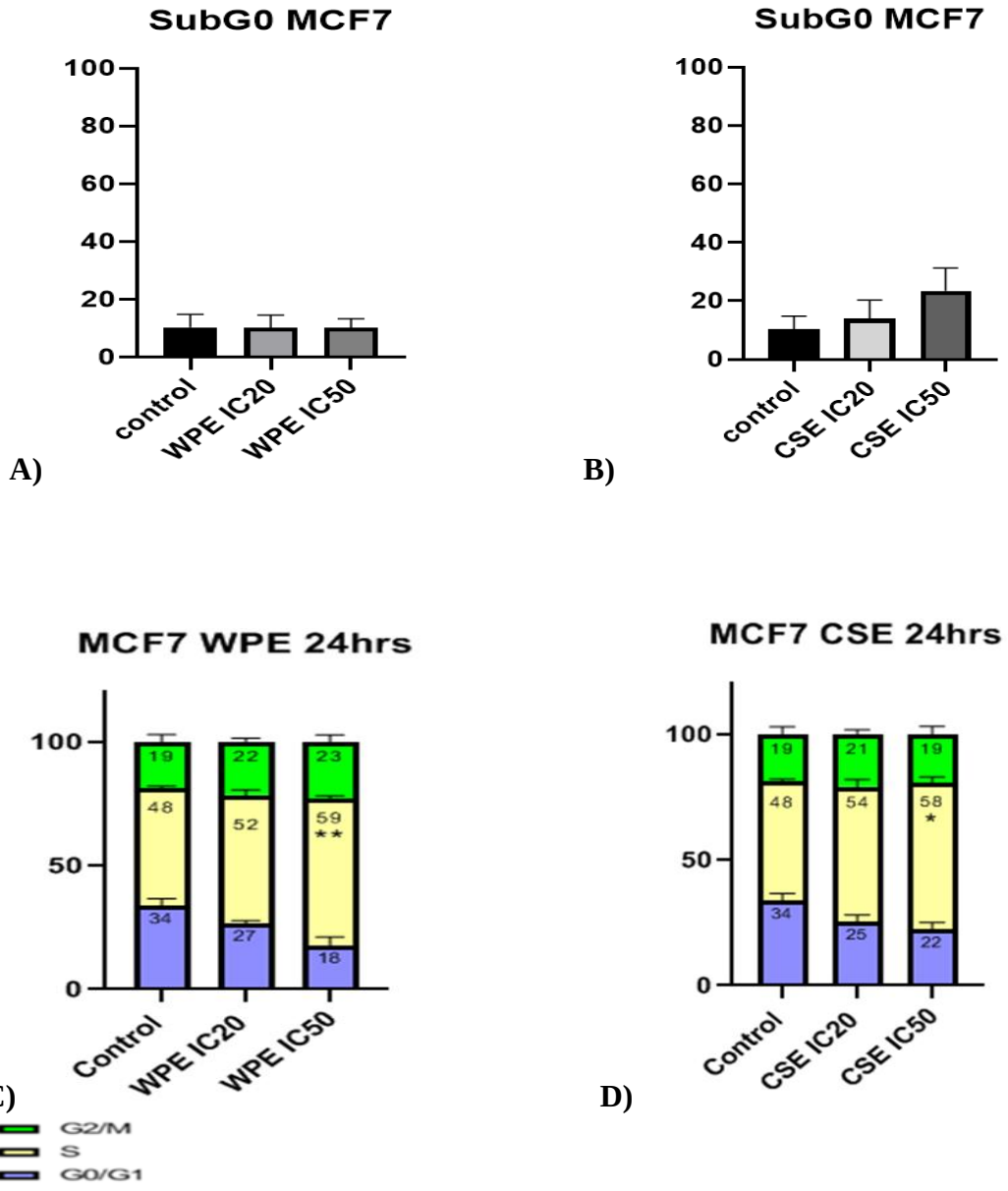


Figure 18 SubG0 phase **(A and B)** and Cell cycle distribution **(C and D)** of MCF-7 cell line upon treatment with different concentrations of waterpipe and cigarette smoke extract respectively.

Data were compared using the Chi square and One-way ANOVA as applicable.

*P<0.05; **<0.001

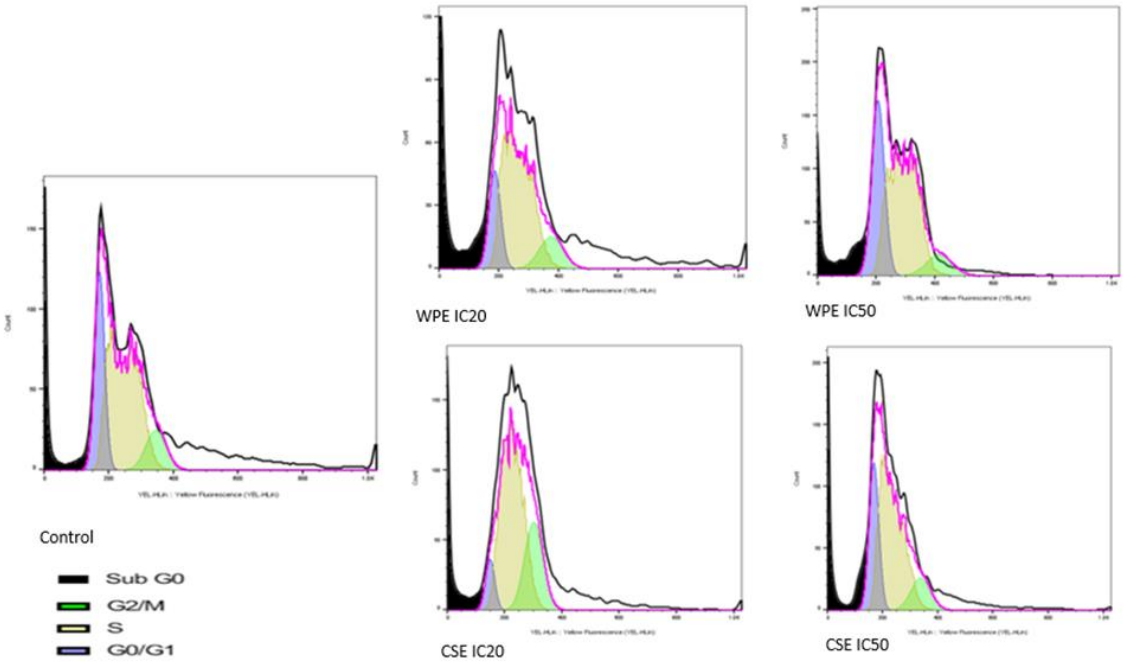


Figure 19 Representative MDAMB-231 cell line cell cycle upon exposure to different concentrations of WPE and CSE.

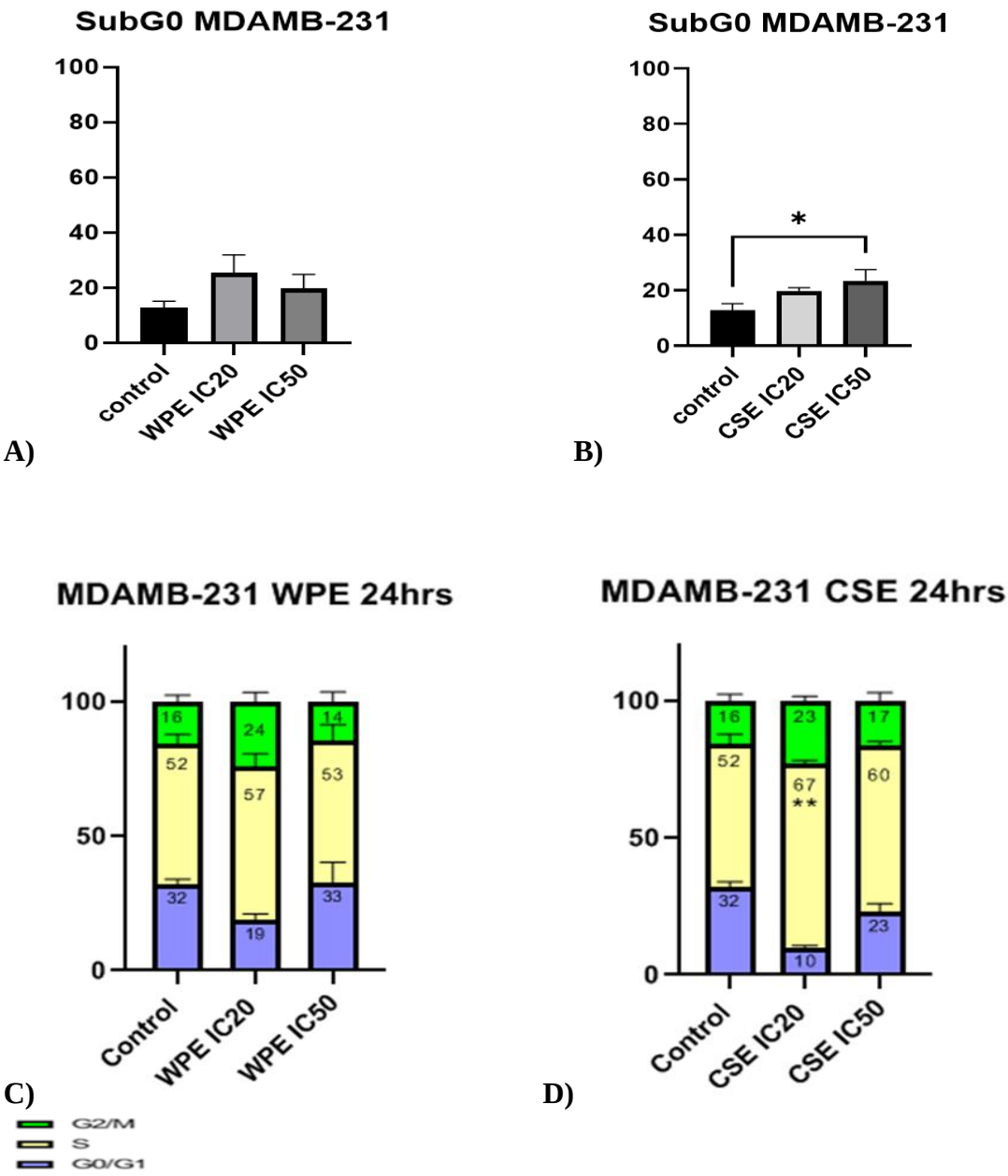


Figure 20 SubG0 phase **(A and B)** and Cell cycle distribution **(C and D)** of MDAMB-231 cell line upon treatment with different concentrations of waterpipe and cigarette smoke extract respectively.

Data were compared using the Chi square and One-way ANOVA as applicable.

*P<0.05; **<0.001

E. Morphology

Microscopic images of the MCF-7 cell line showed that, upon exposure to IC20 of waterpipe and cigarette smoke extracts, the cells were separating away from each other and started to gain more enlarged cell size property. As for the higher concentration (IC50), the number of enlarged cells increased with detachment between the cells, as shown in **Figure 21**.

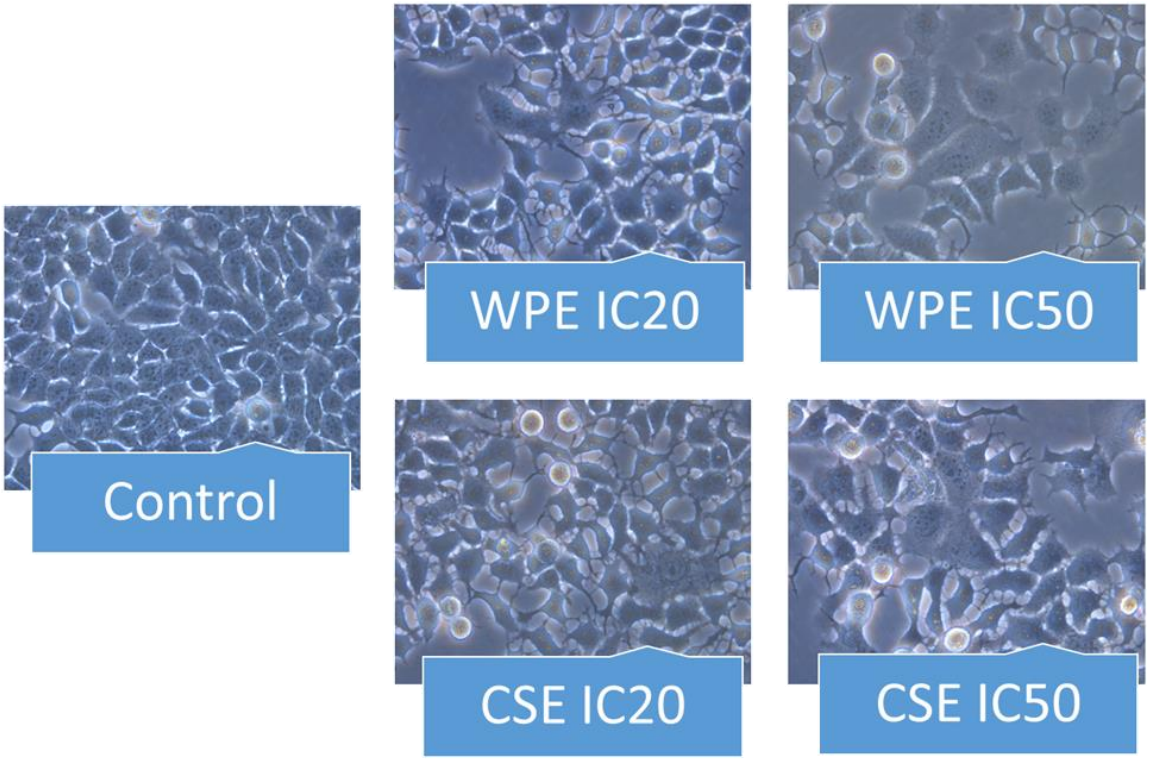


Figure 21 Microscopic images (40X) of the MCF-7 cells upon exposure to WPE and CSE IC20 and IC50 compared to control.

With respect to MDAMB-231, microscopic images showed that unexposed cells were already large in shape, and upon exposure to WPE and CSE IC20, they became elongated. With higher concentrations of exposure, the cells then started to shrink in size with evidence of apoptosis, as shown in **Figure 22**.

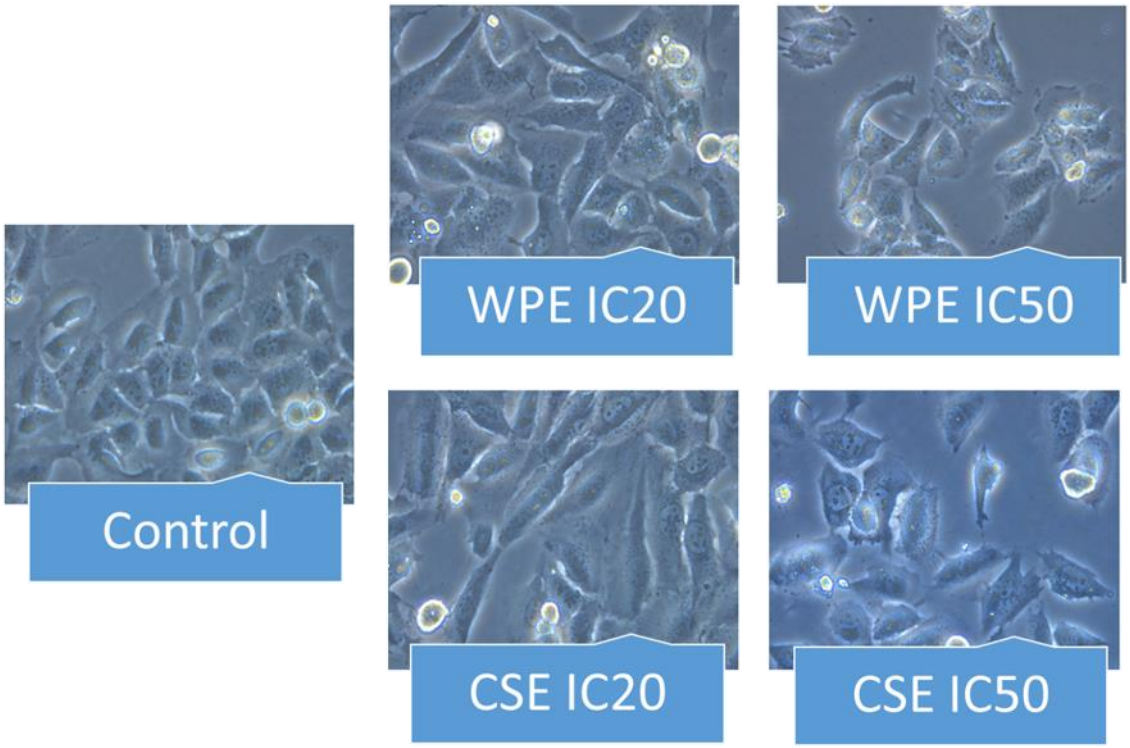


Figure 22 Microscopic images (40X) of the MDAMB-231 cells upon exposure to WPE and CSE IC20 and IC50 compared to control.

F. EMT markers

Concerning the MCF-7 cell line, the expression of the SNAIL mesenchymal marker increased upon WPE and CSE exposure with a significant increase with the WPE and CSE IC50 concentration (**Figure 23**). While, there was a significant decrease in the expression vimentin mesenchymal marker in both WPE and CSE of IC20 and IC50 concentrations, the expression of vimentin was already low in the MCF7 cell line (**Figure 24**), a finding that is known in the literature for MCF7. And for the epithelial

markers, the expression of E-cadherin did not show any change, while that of CDH-1 showed a trend of decrease upon both exposures to both concentrations (**Figure 23**).

With respect to the MDAMB-231, the epithelial markers (E-cadherin and CDH-1) were not expressed. As for the mesenchymal markers, the SNAIL showed an increase in expression in all exposures (WPE IC20, IC50 and CSE IC20, IC50) with a significant increase in the WPE IC20 and CSE IC50. In contrast, vimentin expression showed a significant but less marked decrease in all exposures.

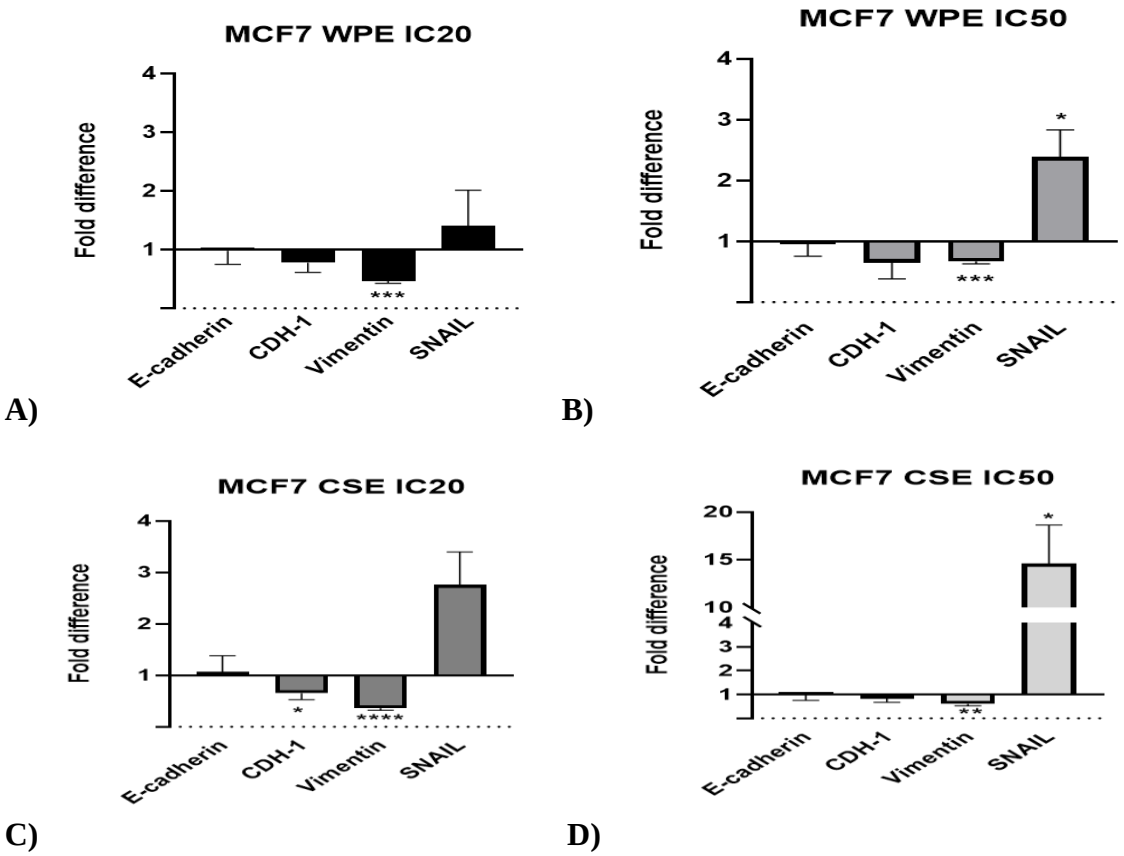


Figure 23 The levels of expression for both epithelial (E-cadherin and CDH-1) and mesenchymal markers (SNAIL and vimentin) in MCF-7 cell line upon exposure to WPE and CSE at IC20 **(A** and **C)** and IC50 **(B** and **D)**. Data were compared to untreated controls using Student t-test.

*P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001

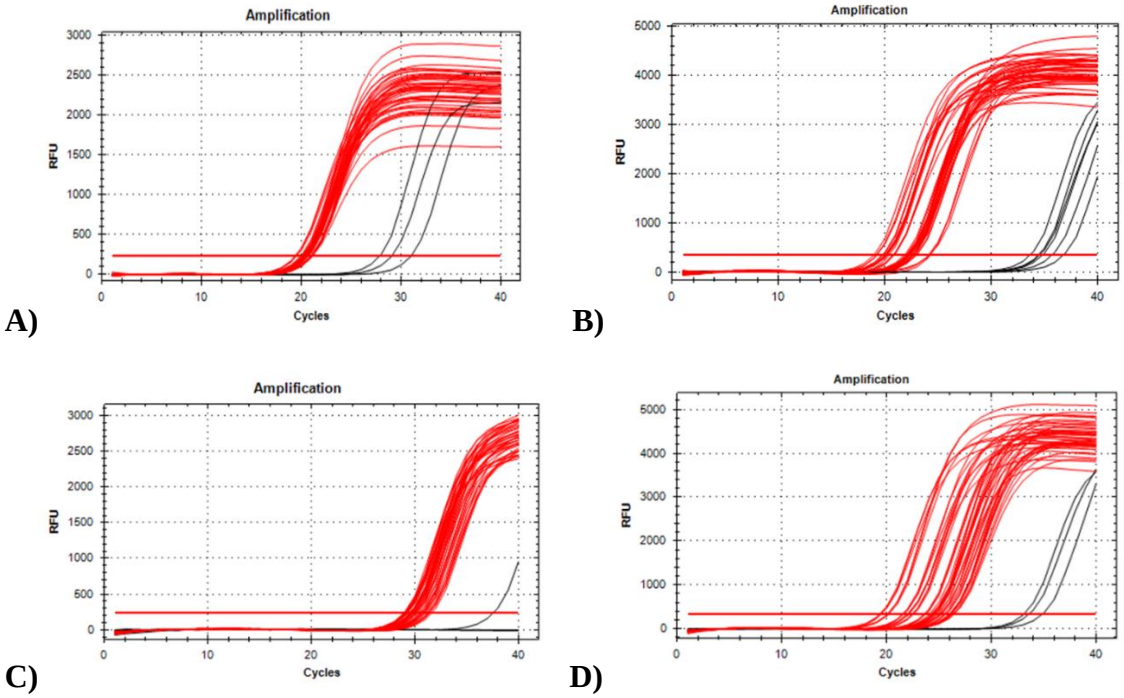
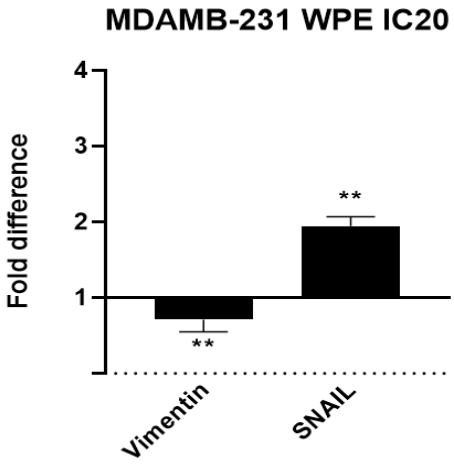
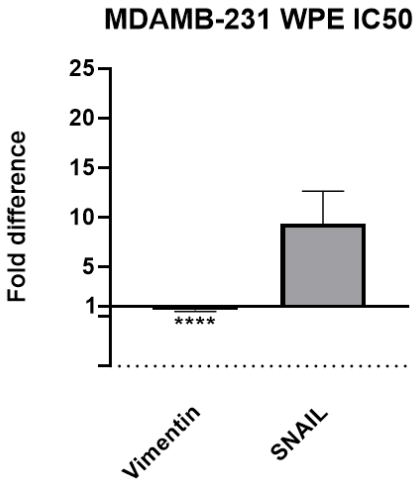


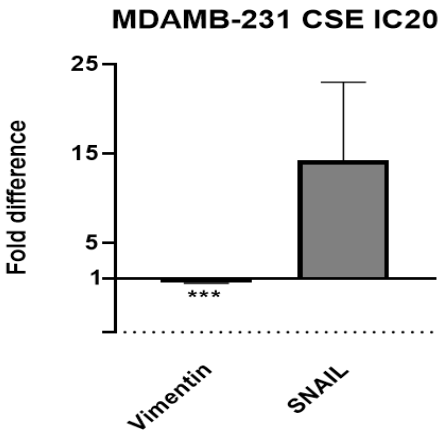
Figure 24 The Real-Time PCR amplification plots of the epithelial markers E-cadherin **(A)** and CDH-1 **(B)** and the mesenchymal markers vimentin **(C)** and SNAIL **(D)** in MCF-7 cell line upon exposure to WPE and CSE at IC20 and IC50.



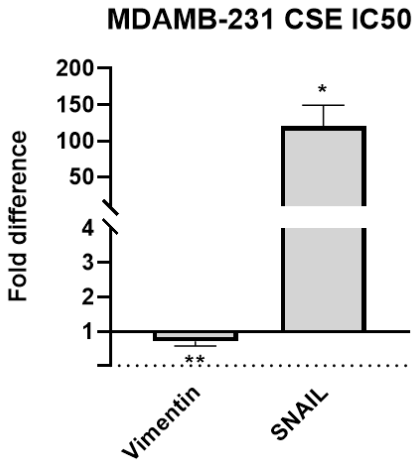
A)



B)



C)



D)

Figure 25 The levels of expression for both epithelial (E-cadherin and CDH-1) and mesenchymal markers (SNAIL and vimentin) in MDAMB-231 cell line upon exposure to WPE and CSE at IC20 **(A** and **C)** and IC50 **(B** and **D)**. Data were compared to untreated controls using Student t-test.

*P<0.05; ** P<0.01; *** P<0.001; **** P<0.0001

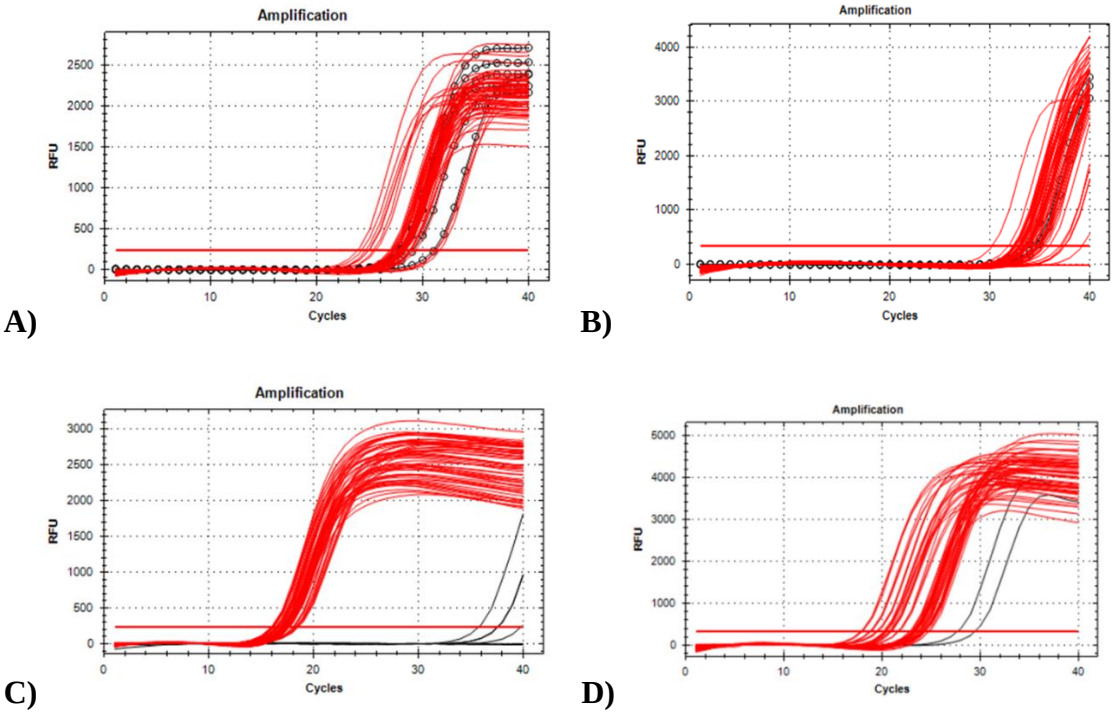
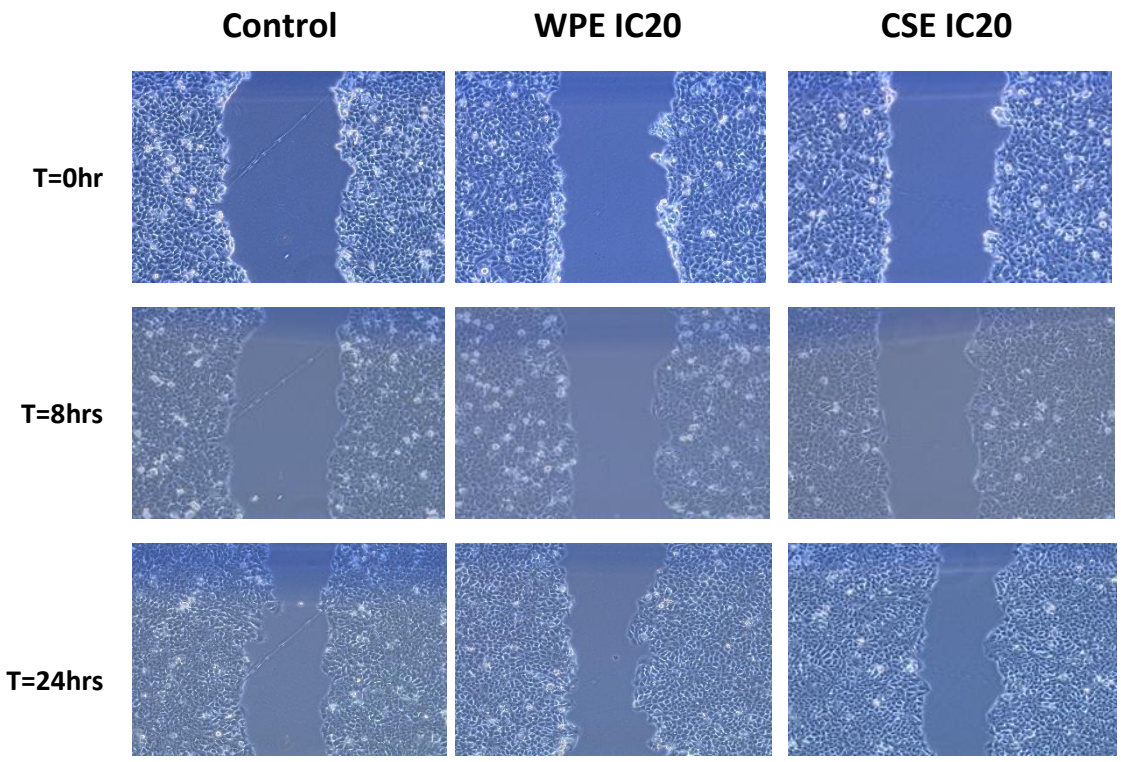


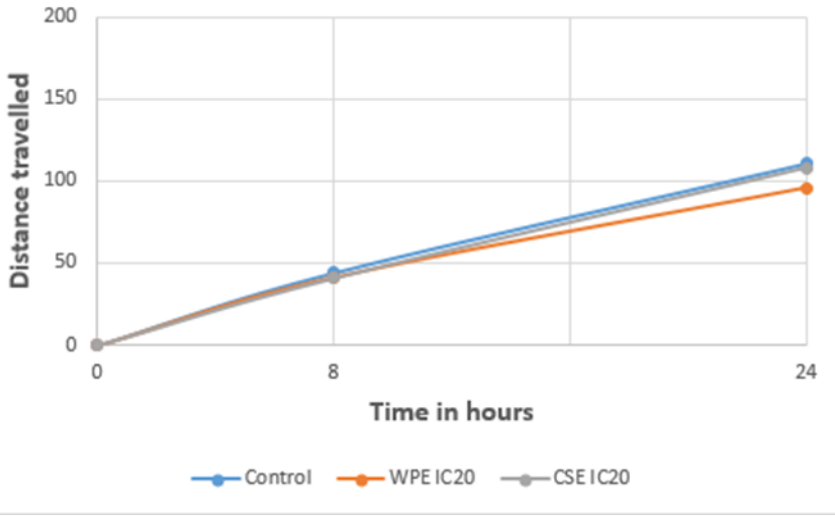
Figure 26 The Real-Time PCR amplification plots of the epithelial markers E-cadherin **(A)** and CDH-1 **(B)** and the mesenchymal markers vimentin **(C)** and SNAIL **(D)** in MDAMB-231 cell line upon exposure to WPE and CSE at IC20 and IC50.

G. Migration assay

The results of the scratch assay showed that at 24hrs, both exposures lead to a decrease in the distance travelled compared to the control and this was statistically significant for MDAMB-231 (**Figures 27 and 28**).



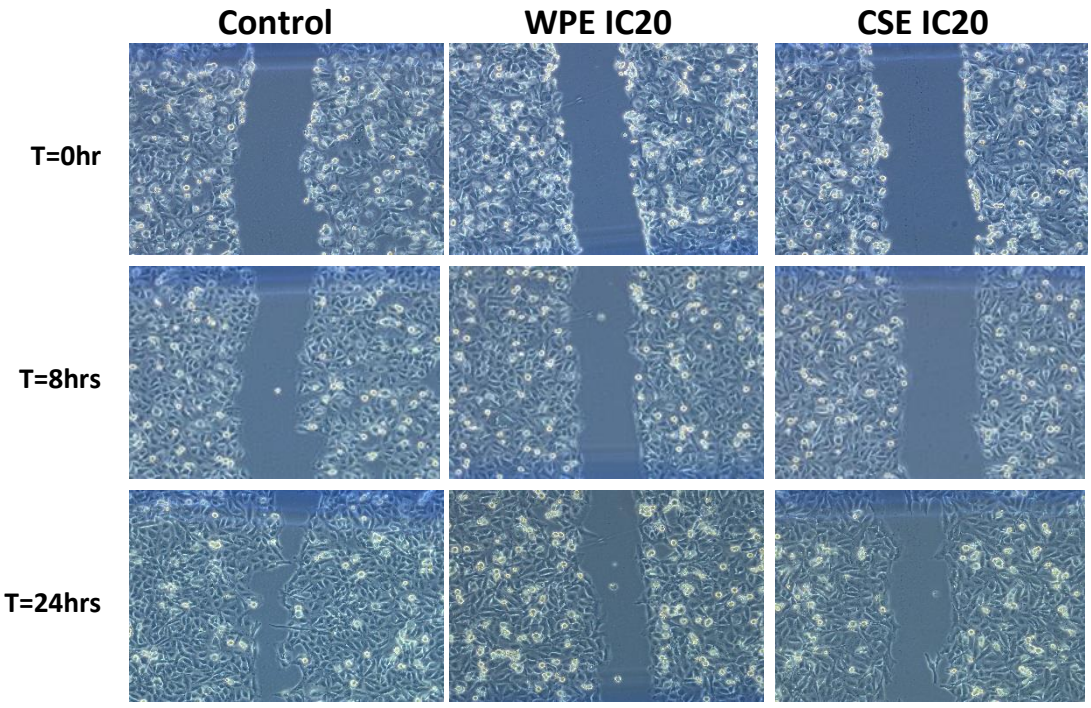
A)



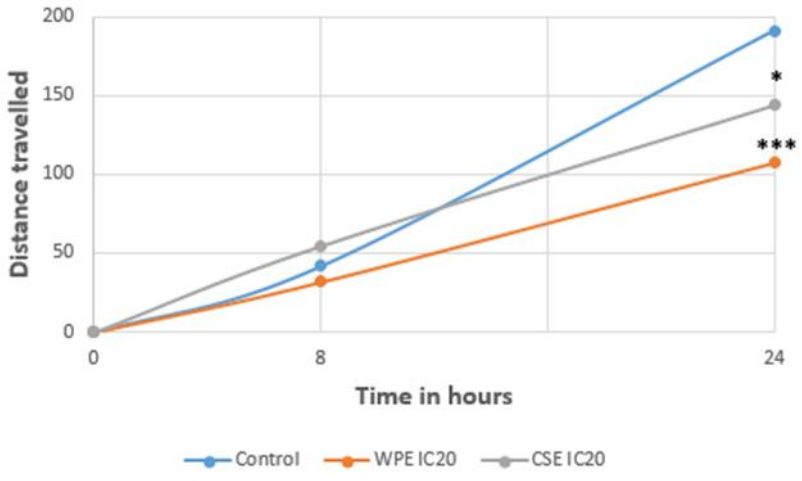
B)

Figure 27 Representative images of MCF-7 cell line migration upon exposure to WPE and CSE IC20 **(A)** with plots of the distance travelled measured for three trials **(B)**.

Data were compared using One-way ANOVA followed by Dunnett.



A)



B)

Figure 28 Representative images of MDAMB-231 cell line migration upon exposure to WPE and CSE IC20 **(A)** with plots of the distance travelled measured for three trials**(B)**.

Data were compared using One-way ANOVA followed by Dunnett.

*P<0.05; *** P<0.001 compared to control

H. DNA methylation

1. Validation of methylation experiments

In order to check for the validity of the DNA methylation data, we tried the primers on peripheral blood samples from a small number of subjects previously evaluated from the Greater Beirut Cohort as described above.

Concerning *AhRR* as a biomarker for cigarette smoke, **Figure 29** shows an example of methylation percentages as analyzed by ESME. Comparison between never and current cigarette smokers showed that the percentage of methylation at the region of interest decreased in current smoker with 58% of methylation compared with 90% of methylation in never smoker **(Figure 29)**. In **Figure 30** the comparison of peripheral blood methylation of current cigarette smokers (N=11) vs. never smokers (N=7) showed a significant hypomethylation of *AhRR* gene in current smokers. These results validate that the *AhRR* ROI is a biomarker for cigarette smokers, specifically hypomethylation at the CpG site cg05575921.

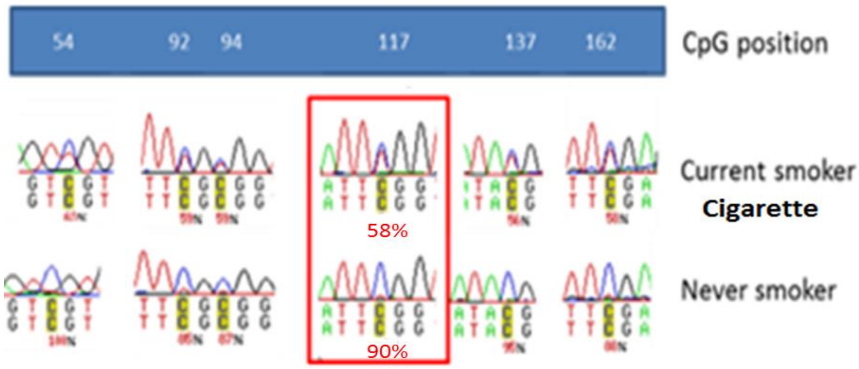


Figure 29 Representative example of methylation results of the *AhRR* region of interest, which is highlighted in red rectangle, for current cigarette smokers and never smokers.

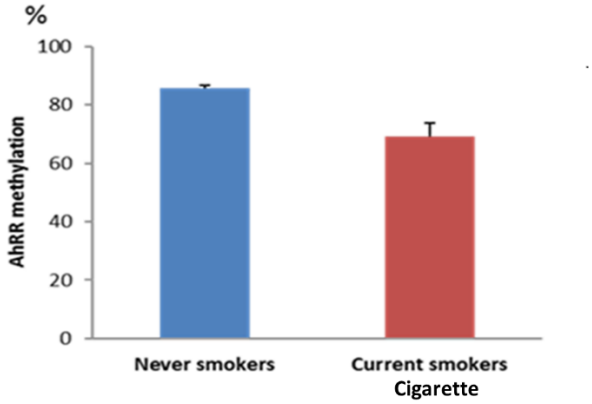


Figure 30 Comparison of *AhRR* ROI methylation percentages in peripheral blood of never vs. current cigarette smokers (*P<0.05 by Student t-test)

With respect to the *MYT1L* gene, representative individual sequence results at the ROI are highlighted in the red rectangle in **Figure 31**, and **Figure 32** represents hypermethylation results in waterpipe smokers compared to non-smokers at the site of ROI with 3 samples of each group. The data reflect 13.6% of difference, validating *MYT1L* gene as a biomarker for waterpipe smokers.

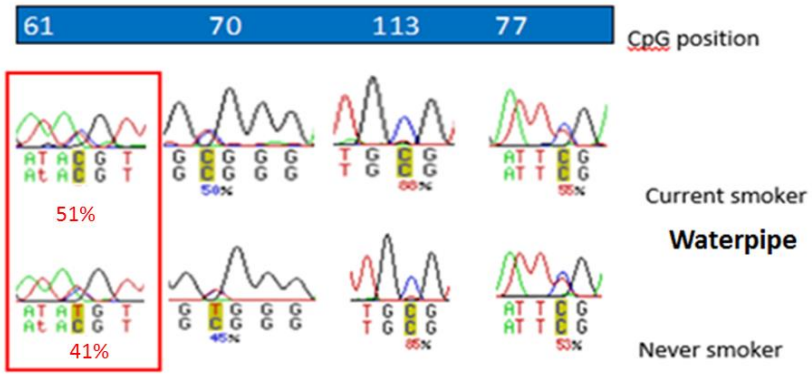


Figure 31 Representative example of methylation results of the *MYT1L* region of interest, which is highlighted in red rectangle, for current waterpipe smokers and never smokers.

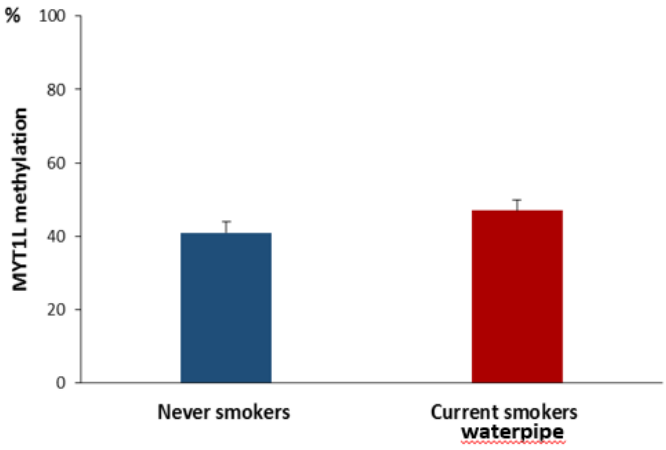
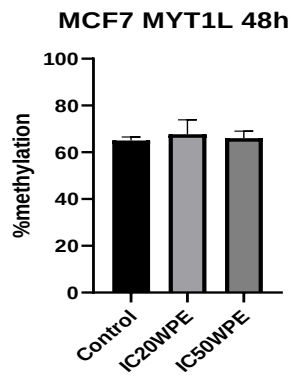


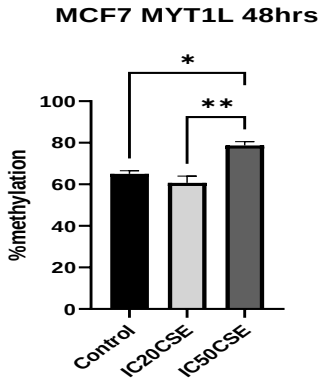
Figure 32 Comparison of *MYT1L* ROI methylation percentages in peripheral blood of never vs. current waterpipe smokers.

2. AhRR and MYT1L ROI methylation in MCF-7 and MDAMB-231 cell lines after exposure to cigarette and waterpipe smoke

For the *AhRR* DNA methylation, no statistically significant change was observed for MDAMB-231 with all exposure conditions (**Figure 34 A and B**). As for MCF-7, the percent *AhRR* methylation was zero for all exposures including control.

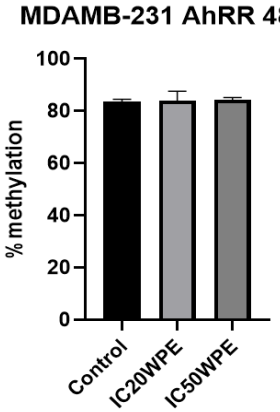
As shown in **Figure 33**, DNA methylation results for *MYT1L* in MCF-7 breast cancer cell line showed a statistically significant hypermethylation with CSE IC50 with respect to control, while no significant changes were seen with WPE and CSE IC20 exposure. As for the MDAMB-231 cell line, there was a trend of hypermethylation especially upon exposure to WPE, though there was some variability and the differences were not statistically significant (**Figure 34 C and D**).

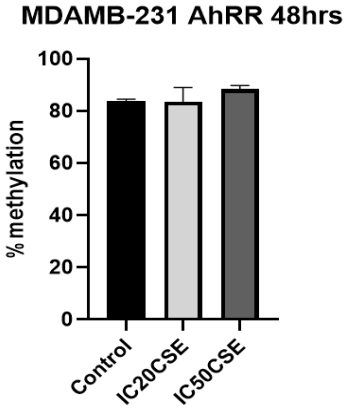




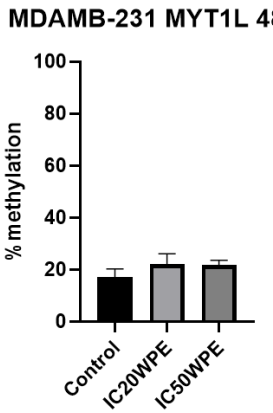
A) **B)**

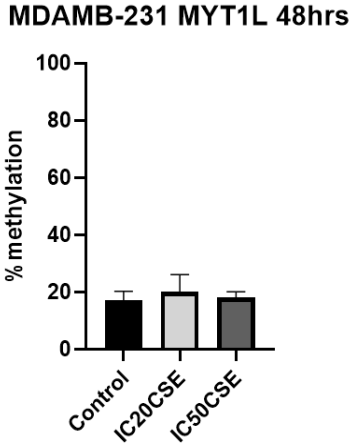
Figure 33 The methylation % of *MYT1L* ROI in MCF-7 cell line 48hrs after exposure to WPE **(A)** and CSE **(B)** at IC20 and IC50 concentrations (****P<000.1 with One-Way ANOVA followed by Tukey posthoc)





A) **B)**





C) **D)**

Figure 34 The methylation % for *AhRR* **(A, B)** and *MYT1L* **(C, D)** ROI in MDAMB-231 cell lines 48hrs after exposure with WPE and CSE at IC20 and IC50 concentrations.

CHAPTER V

DISCUSSION

According to the 2020 data of the WHO, breast cancer incidence ranked the first worldwide and in Lebanon [45, 46]. Also, tobacco smoke is one of the major risk factors for breast cancer development [63, 68]. Tobacco constituents are able to form DNA adducts, leading to DNA damage and mutations[30] [31]. DNA damage induces cell cycle arrest response in order to repair any DNA damage. Moreover, some studies showed that tobacco products have the potential to enhance the carcinogenic effects and induce the transition of epithelial to mesenchymal making cells more aggressive and invasive [89] [96]. Beside the genotoxic and carcinogenic effects, waterpipe and cigarette smoke showed to cause epigenetic changes related to tumor suppressor genes or genes responsible for expressing detoxifying enzymes in peripheral blood samples [132]. Our experiments showed that both WPE and CSE caused DNA damage in MCF-7 and MDAMB-231 breast cancer cell lines. Also, the DNA damage induced cell cycle arrest at S phase for both cell lines. Moreover, the exposure to WPE and CSE caused increase in the expression of *SNAIL*, a mesenchymal marker for the cell transition. These changes were not translated into any increase in cell migration ability. Instead, there was a decrease in cell migration in the exposed compared to the control at 24hrs time point for both cell lines. This may be related to the fact that the increased percentage of cell death hindered the mesenchymal property of live cells compared to the control that consists of a higher percentage of migrating cells. Also, we may see more significant changes with a longer duration of exposure. Moreover, we need to perform other EMT markers, since *SNAIL* expression might have increased in relation

to genotoxic stress resistance and anti-apoptotic response, and not necessarily mesenchymal transformation. With respect to methylation, results showed that the methylation process is highly specific and different between peripheral blood samples and cell lines, thus we did not observe any change in the specific waterpipe and cigarette smoke methylation markers that we reported in peripheral blood samples.

Studies showed that cigarette smoke causes a decrease in cell viability upon increase in concentration and duration of exposure on different cell lines. For example, a study was done on alveolar epithelial cells to determine the effect of cigarette smoke on these cells, and results of MTT showed that cigarette smoke causes a concentration dependent decrease in cell viability that is reversed by sulforaphane, a natural compound that exhibits a cytoprotective effect [159]. Another study was done on fibroblasts using CSE and showed time and dose dependent reduction in cell viability [160]. Others done on different cell lines such as MCF-10A, A549, and SV-HUC-1 also observed with the MTT assay a decrease in cell viability upon exposure to CSE that is dose and time dependent [86-88]. Our results showed a decrease in MCF-7 and MDAMB-231 cell viability with waterpipe and cigarette smoke that is dose and time dependent, similar to the results of other studies on different cell lines. These results were validated with the trypan blue assay that showed a decrease in the cell count with waterpipe and cigarette exposure in both MCF-7 and MDAMB-231 cell lines.

Beside the cytotoxic effect, cigarette and waterpipe smoke also exhibit genotoxic effect. The reason behind tobacco product being one of the risk factors for the development of cancer is because it consists of carcinogenic compounds that cause DNA damage. As a matter of fact with our results, ɣH2AX assay showed that both cigarette and waterpipe caused DNA damage in MCF-7 compared to control cells in a

dose dependent manner. While the MDAMB-231 cells exposed to the IC20 showed more DNA damage compared to the control, cells exposed to IC50 concentrations of both waterpipe and cigarette smoke extracts showed more genotoxic resistance compared to the IC20 exposure illustrated by a lesser percentage of cells with DNA damage. This could be related to the higher expression of SNAIL in the IC50 concentrations which may causes alteration in genotoxic stress response [161]. Of note that the MDAMB-231 showed in the unexposed control cells more ɣH2AX foci than the unexposed MCF-7 cell line (p53 wild type); this was expected since MDAMB-231 is a p53 negative cell line that has a longer persistence of ɣH2AX compared to wild type p53 cell line whereby the loss of ɣH2AX is faster [162]. Also, in MDAMB-231, the number of cells in the field were low and their shape changed upon exposure to higher concentrations of (IC50) of WPE and CSE; this may be because the cells were stressed with a congruent decrease in cell viability as seen in the MTT and trypan blue results. Our results showed that the genotoxic effect caused cell cycle arrest at S phase. This S phase cell cycle arrest was also observed by Narayan et al [86] in MCF10A cell line treated with cigarette smoke condensate. The authors also showed that the level of PCNA (proliferating cell nuclear antigen) which is an accessory factor of DNA polymerase that is involved in DNA replication and DNA repair mechanism increased, thus reflecting activation of S phase cell cycle arrest and DNA repair mechanism after exposure to cigarette carcinogenic constituents [86]. Moreover, a review article reported that exposure of cells to B[a]P (benzo[a]pyrene), which is a major constituent of tobacco product, caused DNA strand breakage, inhibition in DNA synthesis, and induction of S phase cell cycle arrest [163].

With respect to the potential carcinogenic effect of WPE and CSE on MCF-7 and MDAMB-231 cell lines, the EMT markers showed an increase in *SNAIL* expression, a marker for mesenchymal cells, in a dose dependent manner. The expression of *SNAIL* might cause changes in apoptotic response as SNAIL decreases the expression of genes that have a role in apoptosis such as apoptotic nuclease DFF40 and Mst4 [161]. Also, SNAIL binds to the *CDH-1* promoter thus decreasing its expression [161], as observed in our results whereby there is a trend of decrease in *CDH-1* expression. Moreover, *SNAIL* expression decreases the enzymatic activity involved in Krebs cycle and the metabolic activity, shown in MDCK cells, thereby decreasing cell viability [164]. Our results showed a decrease in the levels of *vimentin* and this may be related to the fact that some cells were stressed upon exposure and hence may have initiated apoptosis [88, 165]. Caspases are activated during apoptosis, and vimentin is cleaved by several caspases including caspase-3, 7, 6 and 8 [165]. So the level of *SNAIL* may have increased in the live cells that may be resistant to apoptosis compared to the control, while the level of *vimentin* decreased since cell viability and cell count decreased in the treatment which might reflect activation of apoptotic program and increase in cell death compared to control [88, 165]. This thought requires further investigation through performing apoptosis assay. For the wound healing assay, a study was done on MCF-7 cell line using WPE as an exposure, and results showed enhancement of migration ability of cells compared to the unexposed ones, but the starting concentration was low and the duration was 48hrs [96]. Also, another study was done to determine the migration ability of MCF10A, MCF12A, and MCF-7 cell line upon exposure to low concentration of cigarette smoke for weeks, and results showed an enhancement in the migration ability for all tested cell lines [89]. This shows that the

cytotoxic concentrations that we exposed our cells to, may have potentially delayed the observation of the mesenchymal phenotype. It is thus better if we repeat the experiments with lower concentrations of exposure and for a longer duration, or to take the alive cells and seed them again with the same number of control cells to specifically observe the mesenchymal changes in exposed live cells. Also, performing other EMT markers might help us to ascertain whether there is an EMT or not for the concentrations and duration that we followed.

GSK-3β phosphorylates SNAIL for ubiquitination [166], and cigarette smoke was shown to increase the phosphorylation of GSK-3β in vitro such as in human alveolar epithelial cell line, thus decreasing the level of GSK-3β protein and increasing the level of SNAIL [167]. In this same study [167], microscopic images showed that CSE caused an increase in interspaces between cells and, upon higher concentrations of exposure, cells were shrunken with formation of granules and vacuoles, as shown in our results regarding the MCF-7 and MDAMB-231 cell line respectively. The increase in space between exposed cells may be due to either development of mesenchymal property or a decrease in the number of cells. Also, inhibition of GSK-3β was shown to prolong the ɣH2AX elevation and to cause cell cycle arrest in chondrocytes at the S phase [168]. Therefore, the carcinogenic constituents of WPE and CSE in the breast cancer cell line might affect the level of GSK-3beta resulting in an increase in the expression of SNAIL and prolonging the expression of the DNA damage marker (ɣH2AX) that led to cell cycle arrest at S phase [168]. Moving forward, we plan to perform western blots to determine the levels of GSK-3β and its phosphorylated form to determine whether our results are related to the GSK-3β inactivation pathway.

Concerning the DNA methylation changes, our recently conducted pilot study with peripheral blood of never smokers vs. current cigarette or waterpipe smokers showed that *AhRR* (cg05575921) hypomethylation and *MYT1L* (cg06201514) hypermethylation are potential markers of cigarette and waterpipe smoking respectively. Based on these results, we performed PCR and Sanger sequencing for both ROIs on the bisulphite converted DNA of breast cancer cell lines (MCF-7 and MDAMB-231) after acute exposure to cytotoxic concentrations of waterpipe and cigarette smoke. With respect to the *AhRR*, results showed that MCF-7 cell line has zero percentage of methylation in the control and treated samples, while MDAMB-231 had hypermethylation in all samples. In comparison to the literature, a study showed that *AhRR* gene is actually overexpressed in the MCF-7 cell line, hence potentially explaining the baseline no methylation (zero %) of the ROI. Therefore, DNA methylation changes cannot be observed since the gene is already hypomethylated in MCF-7 cell line. No such data are available for the MDAMB-231 cell line. Nevertheless, the differences that we saw in the levels of *AhRR* DNA methylation between MCF-7, MDAMB-231, and peripheral blood samples reiterate the point that DNA methylation is tissue and biologic sample specific. The same applies to *MYT1L* that, although its peripheral blood hypermethylation was a marker of waterpipe smoking, in breast cancer cell lines it was hypermethylated with exposure to both waterpipe and cigarette smoking with significant results with cigarette and MCF7, though with a lot of variability. These discrepancies are similar to those observed in lung cancer cell lines where the baseline levels of synuclein-ɣ mRNA were highly detected in H292, in contrast to A549 cell line whereby cigarette smoke exposure for 3 days lead to hypomethylation of *synuclein-ɣ* gene with secondary increase in its mRNA

level [149]. Also, another study was done to compare cells (LL29, Hs.888, and NL-20) DNA methylation of several genes (DAPK, ECAD, MGMT, and RASSFIA) upon exposure to low and high concentrations of cigarette smoke [153]. Results showed that fibroblast cell lines (LL29 and Hs888) methylation status did not significantly change, whereas in NL-20 lung cells, ECAD was significantly hypermethylated at both concentrations and MGMT was hypomethylated only at high concentrations of exposure. In the same study, NL-20 lung cells were exposed chronically and showed a change of methylation status for RASSF1A from hypomethylated at short term exposure (72hr) to hypermethylated after 28 days of exposure. These results reflect that DNA methylation is highly specific to the cell line and concentration and duration of exposure.

Our results and those of the literature on DNA methylation in cell lines upon exposure to smoke extract lead us to conclude that several factors affect DNA methylation changes. These include the type of cell line or biological samples, concentrations of exposure, and duration of exposure. Accordingly, it is best to perform whole methylome analysis after cell line exposure and at different time points being acute and chronic.

CHAPTER VI

LIMITATIONS

The limitations of the work relays on not being able to perform the treatment exposure on normal breast cell lines since it requires cholera toxin that we are not allowed to ship through the Lebanese border. Also, the cells were exposed to high concentrations with acute exposure, therefore cell toxicity may have hindered the carcinogenic effect of chronic exposure to cigarette and waterpipe smoke. Moreover, we need to determine the differentially methylated regions for the breast cancer cell lines MCF-7 and MDAMB-231 exposed to CSE and WPE.

CHAPTER VII

CONCLUSION

Waterpipe and cigarette smoke are commonly used tobacco products and are major risk factors for the development of several types of cancer including breast cancer. We tested the potentially genotoxic and carcinogenic effect of WPE and CSE at acute cytotoxic concentrations on MCF-7 and MDAMB-231 cell lines. We found that cytotoxic exposure caused more DNA damage with cell cycle arrest at the S-phase, and triggered the expression of the SNAIL mesenchymal marker in both cell lines. These findings were likely coupled with a change in the shape and behavior of the cells into more aggressive ones on microscopy, though the observation might also be related to a decrease in the number of cells, hence the lesser migration ability at 24 hours. With respect to the epigenetic analyses, no differential effect was seen between exposure and control, and the results were different from those previously shown in peripheral blood.

CHAPTER VII

FUTURE PERSPECTIVES

- Perform whole DNA genome for the acute CSE and WPE exposure to figure out the epigenetic biomarker for MCF-7 and MDAMB-231 breast cancer cell lines.
- Launch chronic exposure experiments to investigate the DNA methylation changes of breast cancer cells on long term.
- Expose cells to both types of tobacco products in order to see the effect of the carcinogenic constituents when combined.
- Perform experiments, such as western blot, that help us determine the level of expression of proteins involved in cell cycle arrest and EMT, such as GSK-3β and PCNA.
- Perform the apoptosis experiment to elicit the big picture about cell viability, cell cycle arrest, and cell death.
- Perform the migration assay in a different way either by prolonging the duration of exposure, or expose the cells, count the live ones and re-seed them (same number of exposed live cells and the control), in addition to the invasion assay.
- Attempt to test our hypotheses on normal like breast cells such as the MCF-10A.
- Perform a quantitative analysis for the genotoxicity assay in order to determine the exact percentage difference of DNA damage between the exposed and unexposed conditions.
- Measure the RNA expression of additional EMT markers, such as *TWIST1*, *ZEB1*, *CK7*, and *EpCAM*, to determine the specific marker responsible for any EMT changes in MCF-7 and MDAMB-231 exposed to CSE and WPE.

REFERENCES

1. Bacha, Z.A., et al., *Factors associated with smoking cessation success in Lebanon.* Pharm Pract (Granada), 2018. **16**(1): p. 1111.
2. Hollands, G.J., et al., *Interventions to increase adherence to medications for tobacco dependence.* Cochrane Database Syst Rev, 2015(2): p. CD009164.
3. Aires, C.C., et al., *Studies on the extra-mitochondrial CoA -ester formation of valproic and Delta4 -valproic acids.* Biochim Biophys Acta, 2007. **1771**(4): p. 533-43.
4. Khattab, A., et al., *Smoking habits in the Middle East and North Africa: results of the BREATHE study.* Respir Med, 2012. **106 Suppl 2**: p. S16-24.
5. DiFranza, J.R., et al., *Tobacco promotion and the initiation of tobacco use: assessing the evidence for causality.* Pediatrics, 2006. **117**(6): p. e1237-48.
6. Badran, M. and I. Laher, *Waterpipe (shisha, hookah) smoking, oxidative stress and hidden disease potential.* Redox Biol, 2020. **34**: p. 101455.
7. Al-Kaabba, A.F., et al., *Prevalence and associated factors of cigarette smoking among medical students at King Fahad Medical City in Riyadh of Saudi Arabia.* J Family Community Med, 2011. **18**(1): p. 8-12.
8. Messner, B. and D. Bernhard, *Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis.* Arterioscler Thromb Vasc Biol, 2014. **34**(3): p. 509-15.
9. Rezk-Hanna, M. and N.L. Benowitz, *Cardiovascular Effects of Hookah Smoking: Potential Implications for Cardiovascular Risk.* Nicotine Tob Res, 2019. **21**(9): p. 1151-1161.
10. Benowitz, N.L., et al., *Interindividual variability in the metabolism and cardiovascular effects of nicotine in man.* J Pharmacol Exp Ther, 1982. **221**(2): p. 368-72.
11. Adams, K.F., et al., *Acute elevation of blood carboxyhemoglobin to 6% impairs exercise performance and aggravates symptoms in patients with ischemic heart disease.* J Am Coll Cardiol, 1988. **12**(4): p. 900-9.
12. 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.
13. Garbin, U., et al., *Cigarette smoking blocks the protective expression of Nrf2/ARE pathway in peripheral mononuclear cells of young heavy smokers favouring inflammation.* PLoS One, 2009. **4**(12): p. e8225.
14. Salonen, J.T., et al., *Autoantibody against oxidised LDL and progression of carotid atherosclerosis.* Lancet, 1992. **339**(8798): p. 883-7.
15. Morrow, J.D., et al., *Increase in circulating products of lipid peroxidation (F2-isoprostanes) in smokers. Smoking as a cause of oxidative damage.* N Engl J Med, 1995. **332**(18): p. 1198-203.
16. Pilz, H., et al., *Quitting cigarette smoking results in a fast improvement of in vivo oxidation injury (determined via plasma, serum and urinary isoprostane).* Thromb Res, 2000. **99**(3): p. 209-21.
17. Reilly, M., et al., *Modulation of oxidant stress in vivo in chronic cigarette smokers.* Circulation, 1996. **94**(1): p. 19-25.

18. Lavi, S., et al., *Smoking is associated with epicardial coronary endothelial dysfunction and elevated white blood cell count in patients with chest pain and early coronary artery disease.* Circulation, 2007. **115**(20): p. 2621-7.
19. Barbieri, S.S., et al., *Cytokines present in smokers' serum interact with smoke components to enhance endothelial dysfunction.* Cardiovasc Res, 2011. **90**(3): p. 475-83.
20. Kangavari, S., et al., *Smoking increases inflammation and metalloproteinase expression in human carotid atherosclerotic plaques.* J Cardiovasc Pharmacol Ther, 2004. **9**(4): p. 291-8.
21. Cavusoglu, Y., et al., *Cigarette smoking increases plasma concentrations of vascular cell adhesion molecule-1 in patients with coronary artery disease.* Angiology, 2004. **55**(4): p. 397-402.
22. Zuo, L., et al., *Interrelated role of cigarette smoking, oxidative stress, and immune response in COPD and corresponding treatments.* Am J Physiol Lung Cell Mol Physiol, 2014. **307**(3): p. L205-18.
23. Viegi, G., et al., *Definition, epidemiology and natural history of COPD.* Eur Respir J, 2007. **30**(5): p. 993-1013.
24. Yang, S.R., et al., *Cigarette smoke induces proinflammatory cytokine release by activation of NF-kappaB and posttranslational modifications of histone deacetylase in macrophages.* Am J Physiol Lung Cell Mol Physiol, 2006. **291**(1): p. L46-57.
25. Taylor, J.D., *COPD and the response of the lung to tobacco smoke exposure.* Pulm Pharmacol Ther, 2010. **23**(5): p. 376-83.
26. van Wetering, S., et al., *Defensins: key players or bystanders in infection, injury, and repair in the lung?* J Allergy Clin Immunol, 1999. **104**(6): p. 1131-8.
27. Gaschler, G.J., et al., *Cigarette smoke exposure attenuates cytokine production by mouse alveolar macrophages.* Am J Respir Cell Mol Biol, 2008. **38**(2): p. 218-26.
28. Chung, K.F. and I.M. Adcock, *Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction.* Eur Respir J, 2008. **31**(6): p. 1334-56.
29. Samet, J.M., *Tobacco smoking: the leading cause of preventable disease worldwide.* Thorac Surg Clin, 2013. **23**(2): p. 103-12.
30. Perera, F.P., et al., *Carcinogen-DNA adducts in human breast tissue.* Cancer Epidemiol Biomarkers Prev, 1995. **4**(3): p. 233-8.
31. in *The Health Consequences of Smoking-50 Years of Progress: A Report of the Surgeon General*. 2014: Atlanta (GA).
32. Phillips, D.H. and S. Venitt, *DNA and protein adducts in human tissues resulting from exposure to tobacco smoke.* Int J Cancer, 2012. **131**(12): p. 2733-53.
33. Chen, R.J., et al., *Epigenetic effects and molecular mechanisms of tumorigenesis induced by cigarette smoke: an overview.* J Oncol, 2011. **2011**: p. 654931.
34. U.S. Food and Drug Administration. *Tobacco Products*. Available from: https://www.fda.gov/tobacco-products.
35. Maziak, W., et al., *The global epidemiology of waterpipe smoking.* Tob Control, 2015. **24 Suppl 1**: p. i3-i12.
36. Primack, B.A., et al., *US health policy related to hookah tobacco smoking.* Am J Public Health, 2012. **102**(9): p. e47-51.

37. Schivo, M., M.V. Avdalovic, and S. Murin, *Non-cigarette tobacco and the lung.* Clin Rev Allergy Immunol, 2014. **46**(1): p. 34-53.
38. Walters, M.S., et al., *Waterpipe smoking induces epigenetic changes in the small airway epithelium.* PLoS One, 2017. **12**(3): p. e0171112.
39. Shihadeh, A., et al., *Toxicant content, physical properties and biological activity of waterpipe tobacco smoke and its tobacco-free alternatives.* Tob Control, 2015. **24 Suppl 1**: p. i22-i30.
40. Shihadeh, A., *Investigation of mainstream smoke aerosol of the argileh water pipe.* Food Chem Toxicol, 2003. **41**(1): p. 143-52.
41. Shihadeh, A. and R. Saleh, *Polycyclic aromatic hydrocarbons, carbon monoxide, "tar", and nicotine in the mainstream smoke aerosol of the narghile water pipe.* Food Chem Toxicol, 2005. **43**(5): p. 655-61.
42. Schubert, J., et al., *Waterpipe smoke: source of toxic and carcinogenic VOCs, phenols and heavy metals?* Arch Toxicol, 2015. **89**(11): p. 2129-39.
43. Monzer, B., et al., *Charcoal emissions as a source of CO and carcinogenic PAH in mainstream narghile waterpipe smoke.* Food Chem Toxicol, 2008. **46**(9): p. 2991-5.
44. Rammah, M., et al., *In vitro cytotoxicity and mutagenicity of mainstream waterpipe smoke and its functional consequences on alveolar type II derived cells.* Toxicol Lett, 2012. **211**(3): p. 220-31.
45. International Agency for Research on Cancer. *Lebanon Source: Globocan 2020*. March 2021; Available from: https://gco.iarc.fr/today/data/factsheets/populations/422-lebanon-fact-sheets.pdf.
46. International Agency for Research on Cancer, *GLOBOCAN 2020: New Global Cancer Data.* 15 February 2021.
47. Voduc, K.D., et al., *Breast cancer subtypes and the risk of local and regional relapse.* J Clin Oncol, 2010. **28**(10): p. 1684-91.
48. Fares, J., et al., *Diagnostic Clinical Trials in Breast Cancer Brain Metastases: Barriers and Innovations.* Clin Breast Cancer, 2019. **19**(6): p. 383-391.
49. Fares, Y., et al., *Trauma-related infections due to cluster munitions.* J Infect Public Health, 2013. **6**(6): p. 482-6.
50. Ban, K.A. and C.V. Godellas, *Epidemiology of breast cancer.* Surg Oncol Clin N Am, 2014. **23**(3): p. 409-22.
51. van den Brandt, P.A., et al., *Pooled analysis of prospective cohort studies on height, weight, and breast cancer risk.* Am J Epidemiol, 2000. **152**(6): p. 514-27.
52. Suzuki, R., et al., *Body weight and incidence of breast cancer defined by estrogen and progesterone receptor status--a meta-analysis.* Int J Cancer, 2009. **124**(3): p. 698-712.
53. Toniolo, P.G., et al., *A prospective study of endogenous estrogens and breast cancer in postmenopausal women.* J Natl Cancer Inst, 1995. **87**(3): p. 190-7.
54. Evans, J.P., et al., *Genetics and the young woman with breast cancer.* Breast Dis, 2005. **23**: p. 17-29.
55. Mavaddat, N., et al., *Cancer risks for BRCA1 and BRCA2 mutation carriers: results from prospective analysis of EMBRACE.* J Natl Cancer Inst, 2013. **105**(11): p. 812-22.
56. Hwang, S.J., et al., *Germline p53 mutations in a cohort with childhood sarcoma: sex differences in cancer risk.* Am J Hum Genet, 2003. **72**(4): p. 975-83.

57. Mouchawar, J., et al., *Population-based estimate of the contribution of TP53 mutations to subgroups of early-onset breast cancer: Australian Breast Cancer Family Study.* Cancer Res, 2010. **70**(12): p. 4795-800.
58. Walsh, T. and M.C. King, *Ten genes for inherited breast cancer.* Cancer Cell, 2007. **11**(2): p. 103-5.
59. Tan, M.H., et al., *Lifetime cancer risks in individuals with germline PTEN mutations.* Clin Cancer Res, 2012. **18**(2): p. 400-7.
60. Fares, J., et al., *Current state of clinical trials in breast cancer brain metastases.* Neurooncol Pract, 2019. **6**(5): p. 392-401.
61. Smith-Warner, S.A., et al., *Alcohol and breast cancer in women: a pooled analysis of cohort studies.* JAMA, 1998. **279**(7): p. 535-40.
62. Chen, W.Y., et al., *Moderate alcohol consumption during adult life, drinking patterns, and breast cancer risk.* JAMA, 2011. **306**(17): p. 1884-90.
63. Cui, Y., A.B. Miller, and T.E. Rohan, *Cigarette smoking and breast cancer risk: update of a prospective cohort study.* Breast Cancer Res Treat, 2006. **100**(3): p. 293-9.
64. Friedenreich, C.M., *Physical activity and cancer prevention: from observational to intervention research.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(4): p. 287-301.
65. Michels, K.B., et al., *Diet and breast cancer: a review of the prospective observational studies.* Cancer, 2007. **109**(12 Suppl): p. 2712-49.
66. John, E.M. and J.L. Kelsey, *Radiation and other environmental exposures and breast cancer.* Epidemiol Rev, 1993. **15**(1): p. 157-62.
67. Preston, D.L., et al., *Radiation effects on breast cancer risk: a pooled analysis of eight cohorts.* Radiat Res, 2002. **158**(2): p. 220-35.
68. MacNicoll, A.D., et al., *Metabolism and activation of carcinogenic polycyclic hydrocarbons by human mammary cells.* Biochem Biophys Res Commun, 1980. **95**(4): p. 1599-606.
69. Conway, K., et al., *Prevalence and spectrum of p53 mutations associated with smoking in breast cancer.* Cancer Res, 2002. **62**(7): p. 1987-95.
70. Sibai, A.M., et al., *Prevalence and covariates of obesity in Lebanon: findings from the first epidemiological study.* Obes Res, 2003. **11**(11): p. 1353-61.
71. Fares, M.Y., et al., *Breast Cancer Epidemiology among Lebanese Women: An 11-Year Analysis.* Medicina (Kaunas), 2019. **55**(8).
72. Terry, P.D. and T.E. Rohan, *Cigarette smoking and the risk of breast cancer in women: a review of the literature.* Cancer Epidemiol Biomarkers Prev, 2002. **11**(10 Pt 1): p. 953-71.
73. Palmer, J.R., et al., *Breast cancer and cigarette smoking: a hypothesis.* Am J Epidemiol, 1991. **134**(1): p. 1-13.
74. Lash, T.L. and A. Aschengrau, *Active and passive cigarette smoking and the occurrence of breast cancer.* Am J Epidemiol, 1999. **149**(1): p. 5-12.
75. Marcus, P.M., et al., *The associations of adolescent cigarette smoking, alcoholic beverage consumption, environmental tobacco smoke, and ionizing radiation with subsequent breast cancer risk (United States).* Cancer Causes Control, 2000. **11**(3): p. 271-8.
76. Millikan, R.C., et al., *Cigarette smoking, N-acetyltransferases 1 and 2, and breast cancer risk.* Cancer Epidemiol Biomarkers Prev, 1998. **7**(5): p. 371-8.

77. Calle, E.E., et al., *Cigarette smoking and risk of fatal breast cancer.* Am J Epidemiol, 1994. **139**(10): p. 1001-7.
78. Terry, P.D., A.B. Miller, and T.E. Rohan, *Cigarette smoking and breast cancer risk: a long latency period?* Int J Cancer, 2002. **100**(6): p. 723-8.
79. Baron, J.A., C. La Vecchia, and F. Levi, *The antiestrogenic effect of cigarette smoking in women.* Am J Obstet Gynecol, 1990. **162**(2): p. 502-14.
80. Li, D., et al., *DNA adducts in normal tissue adjacent to breast cancer: a review.* Cancer Detect Prev, 1999. **23**(6): p. 454-62.
81. Li, D., et al., *Aromatic DNA adducts in adjacent tissues of breast cancer patients: clues to breast cancer etiology.* Cancer Res, 1996. **56**(2): p. 287-93.
82. Firozi, P.F., et al., *Aromatic DNA adducts and polymorphisms of CYP1A1, NAT2, and GSTM1 in breast cancer.* Carcinogenesis, 2002. **23**(2): p. 301-6.
83. Godschalk, R.W., et al., *Modulation of DNA and protein adducts in smokers by genetic polymorphisms in GSTM1,GSTT1, NAT1 and NAT2.* Pharmacogenetics, 2001. **11**(5): p. 389-98.
84. Jaiswal, A.S., et al., *Adenomatous polyposis coli-mediated accumulation of abasic DNA lesions lead to cigarette smoke condensate-induced neoplastic transformation of normal breast epithelial cells.* Neoplasia, 2013. **15**(4): p. 454-60.
85. Jorgensen, E.D., et al., *DNA damage response induced by exposure of human lung adenocarcinoma cells to smoke from tobacco- and nicotine-free cigarettes.* Cell Cycle, 2010. **9**(11): p. 2170-6.
86. Narayan, S., et al., *Cigarette smoke condensate-induced transformation of normal human breast epithelial cells in vitro.* Oncogene, 2004. **23**(35): p. 5880-9.
87. Deng, Q.F., et al., *Cigarette smoke extract induces the proliferation of normal human urothelial cells through the NF-kappaB pathway.* Oncol Rep, 2016. **35**(5): p. 2665-72.
88. Agraval, H. and U.C.S. Yadav, *MMP-2 and MMP-9 mediate cigarette smoke extract-induced epithelial-mesenchymal transition in airway epithelial cells via EGFR/Akt/GSK3beta/beta-catenin pathway: Amelioration by fisetin.* Chem Biol Interact, 2019. **314**: p. 108846.
89. Di Cello, F., et al., *Cigarette smoke induces epithelial to mesenchymal transition and increases the metastatic ability of breast cancer cells.* Mol Cancer, 2013. **12**: p. 90.
90. Kispert, S., J. Marentette, and J. McHowat, *Cigarette smoke induces cell motility via platelet-activating factor accumulation in breast cancer cells: a potential mechanism for metastatic disease.* Physiol Rep, 2015. **3**(3).
91. Malik, D.E., R.M. David, and N.J. Gooderham, *Mechanistic evidence that benzo[a]pyrene promotes an inflammatory microenvironment that drives the metastatic potential of human mammary cells.* Arch Toxicol, 2018. **92**(10): p. 3223-3239.
92. Kundu, C.N., et al., *Cigarette smoke condensate-induced level of adenomatous polyposis coli blocks long-patch base excision repair in breast epithelial cells.* Oncogene, 2007. **26**(10): p. 1428-38.
93. Botlagunta, M., et al., *Oncogenic role of DDX3 in breast cancer biogenesis.* Oncogene, 2008. **27**(28): p. 3912-22.

94. Zaarour, R.F., et al., *Waterpipe smoke condensate influences epithelial to mesenchymal transition and interferes with the cytotoxic immune response in non-small cell lung cancer cell lines.* Oncol Rep, 2021.
95. Shihadeh, A., et al., *Comparison of tobacco-containing and tobacco-free waterpipe products: effects on human alveolar cells.* Nicotine Tob Res, 2014. **16**(4): p. 496-9.
96. Sadek, K.W., et al., *Water-pipe smoking promotes epithelial-mesenchymal transition and invasion of human breast cancer cells via ERK1/ERK2 pathways.* Cancer Cell Int, 2018. **18**: p. 180.
97. Weinhold, B., *Epigenetics: the science of change.* Environ Health Perspect, 2006. **114**(3): p. A160-7.
98. Moore, L.D., T. Le, and G. Fan, *DNA methylation and its basic function.* Neuropsychopharmacology, 2013. **38**(1): p. 23-38.
99. Jin, B., Y. Li, and K.D. Robertson, *DNA methylation: superior or subordinate in the epigenetic hierarchy?* Genes Cancer, 2011. **2**(6): p. 607-17.
100. Robertson, K.D., *DNA methylation and human disease.* Nat Rev Genet, 2005. **6**(8): p. 597-610.
101. Probst, A.V., E. Dunleavy, and G. Almouzni, *Epigenetic inheritance during the cell cycle.* Nat Rev Mol Cell Biol, 2009. **10**(3): p. 192-206.
102. Li, E., *Chromatin modification and epigenetic reprogramming in mammalian development.* Nat Rev Genet, 2002. **3**(9): p. 662-73.
103. Kareta, M.S., et al., *Reconstitution and mechanism of the stimulation of de novo methylation by human DNMT3L.* J Biol Chem, 2006. **281**(36): p. 25893-902.
104. Suzuki, M.M. and A. Bird, *DNA methylation landscapes: provocative insights from epigenomics.* Nat Rev Genet, 2008. **9**(6): p. 465-76.
105. Kuchiba, A., et al., *Global methylation levels in peripheral blood leukocyte DNA by LUMA and breast cancer: a case-control study in Japanese women.* Br J Cancer, 2014. **110**(11): p. 2765-71.
106. Xu, X., et al., *DNA methylation in peripheral blood measured by LUMA is associated with breast cancer in a population-based study.* FASEB J, 2012. **26**(6): p. 2657-66.
107. Delgado-Cruzata, L., et al., *Global DNA methylation levels in white blood cell DNA from sisters discordant for breast cancer from the New York site of the Breast Cancer Family Registry.* Epigenetics, 2012. **7**(8): p. 868-74.
108. Choi, J.Y., et al., *Association between global DNA hypomethylation in leukocytes and risk of breast cancer.* Carcinogenesis, 2009. **30**(11): p. 1889-97.
109. Brennan, K., et al., *Intragenic ATM methylation in peripheral blood DNA as a biomarker of breast cancer risk.* Cancer Res, 2012. **72**(9): p. 2304-13.
110. Cho, Y.H., et al., *Aberrant promoter hypermethylation and genomic hypomethylation in tumor, adjacent normal tissues and blood from breast cancer patients.* Anticancer Res, 2010. **30**(7): p. 2489-96.
111. Wu, H.C., et al., *Repetitive element DNA methylation levels in white blood cell DNA from sisters discordant for breast cancer from the New York site of the Breast Cancer Family Registry.* Carcinogenesis, 2012. **33**(10): p. 1946-52.
112. Kitkumthorn, N., et al., *LINE-1 methylation in the peripheral blood mononuclear cells of cancer patients.* Clin Chim Acta, 2012. **413**(9-10): p. 869-74.

113. Deroo, L.A., et al., *Global DNA methylation and one-carbon metabolism gene polymorphisms and the risk of breast cancer in the Sister Study.* Carcinogenesis, 2014. **35**(2): p. 333-8.
114. Brooks, J., P. Cairns, and A. Zeleniuch-Jacquotte, *Promoter methylation and the detection of breast cancer.* Cancer Causes Control, 2009. **20**(9): p. 1539-50.
115. Hu, X.C., I.H. Wong, and L.W. Chow, *Tumor-derived aberrant methylation in plasma of invasive ductal breast cancer patients: clinical implications.* Oncol Rep, 2003. **10**(6): p. 1811-5.
116. Niwa, Y., T. Oyama, and T. Nakajima, *BRCA1 expression status in relation to DNA methylation of the BRCA1 promoter region in sporadic breast cancers.* Jpn J Cancer Res, 2000. **91**(5): p. 519-26.
117. Murata, H., et al., *Genetic and epigenetic modification of mismatch repair genes hMSH2 and hMLH1 in sporadic breast cancer with microsatellite instability.* Oncogene, 2002. **21**(37): p. 5696-703.
118. Widschwendter, M., et al., *Methylation and silencing of the retinoic acid receptor-beta2 gene in breast cancer.* J Natl Cancer Inst, 2000. **92**(10): p. 826-32.
119. Graff, J.R., et al., *Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression.* J Biol Chem, 2000. **275**(4): p. 2727-32.
120. Bachman, K.E., et al., *Methylation-associated silencing of the tissue inhibitor of metalloproteinase-3 gene suggest a suppressor role in kidney, brain, and other human cancers.* Cancer Res, 1999. **59**(4): p. 798-802.
121. Hoque, M.O., et al., *Detection of aberrant methylation of four genes in plasma DNA for the detection of breast cancer.* J Clin Oncol, 2006. **24**(26): p. 4262-9.
122. Dulaimi, E., et al., *Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients.* Clin Cancer Res, 2004. **10**(18 Pt 1): p. 6189-93.
123. Guo, Y., et al., *Regulation of DNA methylation in human breast cancer. Effect on the urokinase-type plasminogen activator gene production and tumor invasion.* J Biol Chem, 2002. **277**(44): p. 41571-9.
124. Xing, R.H. and S.A. Rabbani, *Transcriptional regulation of urokinase (uPA) gene expression in breast cancer cells: role of DNA methylation.* Int J Cancer, 1999. **81**(3): p. 443-50.
125. Grigorian, M., et al., *Effect of mts1 (S100A4) expression on the progression of human breast cancer cells.* Int J Cancer, 1996. **67**(6): p. 831-41.
126. Johansson, A. and J.M. Flanagan, *Epigenome-wide association studies for breast cancer risk and risk factors.* Trends Cancer Res, 2017. **12**: p. 19-28.
127. Martin, E.M. and R.C. Fry, *Environmental Influences on the Epigenome: Exposure- Associated DNA Methylation in Human Populations.* Annu Rev Public Health, 2018. **39**: p. 309-333.
128. Philibert, R.A., et al., *Changes in DNA methylation at the aryl hydrocarbon receptor repressor may be a new biomarker for smoking.* Clin Epigenetics, 2013. **5**(1): p. 19.
129. 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.
130. Ambatipudi, S., et al., *Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study.* Epigenomics, 2016. **8**(5): p. 599-618.

131. Mouneimne, Y., et al., *Bisphenol A urinary level, its correlates, and association with cardiometabolic risks in Lebanese urban adults.* Environ Monit Assess, 2017. **189**(10): p. 517.
132. Shenker, N.S., et al., *Epigenome-wide association study in the European Prospective Investigation into Cancer and Nutrition (EPIC-Turin) identifies novel genetic loci associated with smoking.* Hum Mol Genet, 2013. **22**(5): p. 843-51.
133. 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.
134. Denison, M.S., et al., *Ligand binding and activation of the Ah receptor.* Chem Biol Interact, 2002. **141**(1-2): p. 3-24.
135. Larigot, L., et al., *AhR signaling pathways and regulatory functions.* Biochim Open, 2018. **7**: p. 1-9.
136. Elizondo, G., et al., *Altered cell cycle control at the G(2)/M phases in aryl hydrocarbon receptor-null embryo fibroblast.* Mol Pharmacol, 2000. **57**(5): p. 1056-63.
137. Abdelrahim, M., R. Smith, 3rd, and S. Safe, *Aryl hydrocarbon receptor gene silencing with small inhibitory RNA differentially modulates Ah-responsiveness in MCF-7 and HepG2 cancer cells.* Mol Pharmacol, 2003. **63**(6): p. 1373-81.
138. Tian, Y., et al., *Ah receptor and NF-kappaB interactions, a potential mechanism for dioxin toxicity.* J Biol Chem, 1999. **274**(1): p. 510-5.
139. Kolluri, S.K., et al., *p27(Kip1) induction and inhibition of proliferation by the intracellular Ah receptor in developing thymus and hepatoma cells.* Genes Dev, 1999. **13**(13): p. 1742-53.
140. Puga, A., A. Maier, and M. Medvedovic, *The transcriptional signature of dioxin in human hepatoma HepG2 cells.* Biochem Pharmacol, 2000. **60**(8): p. 1129-42.
141. Huang, G. and C.J. Elferink, *Multiple mechanisms are involved in Ah receptor-mediated cell cycle arrest.* Mol Pharmacol, 2005. **67**(1): p. 88-96.
142. Jiang, Y., et al., *A novel family of Cys-Cys, His-Cys zinc finger transcription factors expressed in developing nervous system and pituitary gland.* J Biol Chem, 1996. **271**(18): p. 10723-30.
143. Jandrig, B., et al., *ST18 is a breast cancer tumor suppressor gene at human chromosome 8q11.2.* Oncogene, 2004. **23**(57): p. 9295-302.
144. De Rocker, N., et al., *Refinement of the critical 2p25.3 deletion region: the role of MYT1L in intellectual disability and obesity.* Genet Med, 2015. **17**(6): p. 460-6.
145. Masserdotti, G., S. Gascon, and M. Gotz, *Direct neuronal reprogramming: learning from and for development.* Development, 2016. **143**(14): p. 2494-510.
146. Manukyan, A., et al., *Analysis of transcriptional activity by the Myt1 and Myt1l transcription factors.* J Cell Biochem, 2018. **119**(6): p. 4644-4655.
147. Hu, J., et al., *From the Cover: Neutralization of terminal differentiation in gliomagenesis.* Proc Natl Acad Sci U S A, 2013. **110**(36): p. 14520-7.
148. Wang, B., et al., *The crucial role of DNA-dependent protein kinase and myelin transcription factor 1-like protein in the miR-141 tumor suppressor network.* Cell Cycle, 2019. **18**(21): p. 2876-2892.

149. Liu, H., et al., *Cigarette smoke induces demethylation of prometastatic oncogene synuclein-gamma in lung cancer cells by downregulation of DNMT3B.* Oncogene, 2007. **26**(40): p. 5900-10.
150. Ostrow, K.L., et al., *Cigarette smoke induces methylation of the tumor suppressor gene NISCH.* Epigenetics, 2013. **8**(4): p. 383-8.
151. Yang, W., et al., *Cigarette smoking extract causes hypermethylation and inactivation of WWOX gene in T-24 human bladder cancer cells.* Neoplasma, 2012. **59**(2): p. 216-23.
152. Chen, L.M., et al., *Long-term exposure to cigarette smoke extract induces hypomethylation at the RUNX3 and IGF2-H19 loci in immortalized human urothelial cells.* PLoS One, 2013. **8**(5): p. e65513.
153. Word, B., et al., *Cigarette smoke condensate induces differential expression and promoter methylation profiles of critical genes involved in lung cancer in NL-20 lung cells in vitro: short-term and chronic exposure.* Int J Toxicol, 2013. **32**(1): p. 23-31.
154. Vaz, M., et al., *Chronic Cigarette Smoke-Induced Epigenomic Changes Precede Sensitization of Bronchial Epithelial Cells to Single-Step Transformation by KRAS Mutations.* Cancer Cell, 2017. **32**(3): p. 360-376 e6.
155. Kopp, B., L. Khoury, and M. Audebert, *Validation of the gammaH2AX biomarker for genotoxicity assessment: a review.* Arch Toxicol, 2019. **93**(8): p. 2103-2114.
156. Niida, H. and M. Nakanishi, *DNA damage checkpoints in mammals.* Mutagenesis, 2006. **21**(1): p. 3-9.
157. Lamarche, B.J., N.I. Orazio, and M.D. Weitzman, *The MRN complex in double-strand break repair and telomere maintenance.* FEBS Lett, 2010. **584**(17): p. 3682-95.
158. 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.
159. Jiao, Z., et al., *Sulforaphane increases Nrf2 expression and protects alveolar epithelial cells against injury caused by cigarette smoke extract.* Mol Med Rep, 2017. **16**(2): p. 1241-1247.
160. Yang, G.Y., et al., *Effects of cigarette smoke extracts on the growth and senescence of skin fibroblasts in vitro.* Int J Biol Sci, 2013. **9**(6): p. 613-23.
161. Kajita, M., K.N. McClinic, and P.A. Wade, *Aberrant expression of the transcription factors snail and slug alters the response to genotoxic stress.* Mol Cell Biol, 2004. **24**(17): p. 7559-66.
162. Kuo, L.J. and L.X. Yang, *Gamma-H2AX - a novel biomarker for DNA double-strand breaks.* In Vivo, 2008. **22**(3): p. 305-9.
163. Shackelford, R.E., W.K. Kaufmann, and R.S. Paules, *Cell cycle control, checkpoint mechanisms, and genotoxic stress.* Environ Health Perspect, 1999. **107 Suppl 1**: p. 5-24.
164. Osorio, L.A., et al., *SNAIL transcription factor increases the motility and invasive capacity of prostate cancer cells.* Mol Med Rep, 2016. **13**(1): p. 778-86.
165. Byun, Y., et al., *Caspase cleavage of vimentin disrupts intermediate filaments and promotes apoptosis.* Cell Death Differ, 2001. **8**(5): p. 443-50.

166. Lundgren, K., B. Nordenskjold, and G. Landberg, *Hypoxia, Snail and incomplete epithelial-mesenchymal transition in breast cancer.* Br J Cancer, 2009. **101**(10): p. 1769-81.
167. Tian, D., et al., *Cigarette smoke extract induces activation of beta-catenin/TCF signaling through inhibiting GSK3beta in human alveolar epithelial cell line.* Toxicol Lett, 2009. **187**(1): p. 58-62.
168. Yang, K., et al., *The Key Roles of GSK-3beta in Regulating Mitochondrial Activity*. Cell Physiol Biochem, 2017. **44**(4): p. 1445-1459.