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ASSOCIATION OF THE CYTOTOXIC EFFECTS OF WATERPIPE AND CIGARETTE SMOKE EXTRACT WITH RELATIVE TELOMERE LENGTH (RTL) IN BREAST CANCER CELL LINES

by
CAROLE SAMI ABDEL KARIM

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Pharmacology and Toxicology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
April 2021

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ACKNOWLEDGEMENTS

My recognition and sincere gratitude are addressed to my advisor and role model Dr. Nathalie Zgheib for her continuous support and guidance in my research work. I learned from Dr. Zgheib a lot during this period from reading literature reviews, performing experimental work, analyzing data, facilitating the topics in pharmacogenetics to helping me finalizing my thesis write-up. I am grateful that I was her student and proud that I have worked with a superwoman like her.

I would like to extend my gratitude to the Dean of the Maroun Semaan Faculty of Engineering and Architecture, Dr. Alan Shihadeh and his awesome lab team colleagues Mrs. Rola Salman and Mr. Mario El Hourani for their collaboration and support in explaining about the smoking machine and their contribution to this project by providing us with waterpipe and cigarette filters.

I would like to also thank Dr. Fouad Zouein who believed in my potential since the day of the interview. I still remember all his questions, and they truly guided me into knowing what to expect in research work. He was also supportive for this project and provided us with reference cigarettes that were essential for this experimental work.

I would like to thank Dr. Rihab Nasr for her collaboration with our lab on the same project, her guidance in all the stages of the experimental work and for answering my questions pertaining to cell lines and specific assays.

Special thanks are for Dr. Pamela Melki and Mrs. Reem Akika for helping me a lot in performing the experiments, learning the techniques, analyzing the data on specific softwares and for answering my several questions.

I want to thank my lab colleagues, Mrs. Ruwayda Kabbani, Dr. Farah Nassar, Mrs. Dania Kabbani, Ms. Karna Jabotian and Dr. Halim Saad for being supportive during the experiments and creating a friendly environment in the lab.

Last but not least, I want to thank my Intensive Care Unit Nurse manager Ziad El Ibrik and all the nursing colleagues who have been supportive during my Masters and facilitated my shift schedule in order to attend classes and later lab work.

Finally, I owe all this success to my family, my parents, my sister Maria, and my brother Karim who have been my greatest support all these years with all the challenges that I passed through. I think words are not enough to express my thankfulness for their indirect contribution to this work!

ABSTRACT
OF THE THESIS OF

Carole Sami Abdel Karim for Master of Science
Major: Pharmacology and Therapeutics

Title: Association of the Cytotoxic Effects of Waterpipe and Cigarette Smoke Extract with Relative Telomere Length (RTL) in Breast Cancer Cell Lines

Background: Breast cancer ranks the first leading cause of cancer worldwide in 2020 as declared by the International Agency for Research on Cancer (IARC). Breast cancer development and progression involve a wide range of genetic alterations including shortening or lengthening of telomere length. Telomeres maintain the genetic integrity hence cellular stability as they are important in protecting the end of chromosomes from fusion, and in avoiding the loss of coding nucleotides during each DNA replication. Any replication problem can result in shortening of telomere during each cell division reaching a critical length and causing the cell to become senescent. There is currently no comparative literature on telomerase expression and telomere length of waterpipe and cigarette smoking, and this is an important area of research given the strong carcinogenic effects of waterpipe smoke exposure and its increasing use worldwide.
Methods: The aim of this study is to identify the change in relative telomere length associated with the cytotoxic potential of waterpipe smoke in breast cancer cell lines in comparison to cigarette smoke. We exposed MCF-7 and MDAMB-231 cells to cigarette and waterpipe smoke extracts and tested cytotoxicity with MTT assay for choice of sub-toxic concentrations, trypan blue assay for cell counting, RNA and protein isolation for telomerase expression and activity respectively and DNA for relative telomere length.
Results: Exposure of breast cancer cells to acute subtoxic waterpipe and cigarette smoke extract showed a decrease in telomerase activity that was compensated by an increase in *h*TERT telomerase expression to potentially escape the proliferation barriers in cell senescence from G0/G1 cell cycle arrest resulting in enhanced carcinogenesis.
Conclusion: Our results went a step further in elucidating a telomerase-linked mechanism between telomerase activity and its rate-limiting determinant *h*TERT expression. Further studies on cell cycle, genotoxicity, invasion, and migration are required to explain our results, in addition to exposing cells to chronic subtoxic concentrations that would mimic the lifetime exposure of smokers *in-vivo*. The proposed approach may pave the way for the use of RTL as a biological marker for toxicity of cigarette and waterpipe in breast cancer.

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ABBREVIATIONS

AA	Aortic Aneurysm
ALT	Alternative lengthening of telomeres
ANOVA	one way analysis of variance
ATCC	American Type Culture Collection
BaP	Benzo[a]pyrene
BSA	Bovine serum albumin
CDC	Centers for Disease Control and Prevention
CHAPS	3[(cholamidopropyl)-dimethyl-ammonium]-1-propanesulfonate
CHD	Coronary Heart Disease
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
CSC	Cigarette Smoke Condensate
CSE	Cigarette Smoke Extract
CT	Cycle Threshold
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial Mesenchymal Transition
EPIC	European Prospective Investigation into Cancer and Nutrition
ER	Estrogen Receptor
ETS	Environmental Tobacco Smoke
FBS	Fetal Bovine Serum
HER2	Human Epidermal growth factor Receptor 2
HSD	Honestly significant difference
hTERT	human Telomere Reverse Transcriptase
IARC	International Agency for Research on Cancer
IC	Inhibitory concentration
IC20	Inhibitory concentration by 20%
IC50	Inhibitory concentration by 50%
ISO	International Standardization Organization
LPM	Liters per minute
MMPs	Matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	No amplification control
NHIS	National Health Interview Survey
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	*N*-nitrosonornicotine
NO	Nitric Oxide
NTC	No template control

PAF	Platelet-activating factor
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PR	Progesterone receptor
PVD	Peripheral Vascular Disease
qPCR	quantitative Polymerase Chain Reaction
RINS	Real-time in situ sampling
RPMI	Roswell Park Memorial Institute
RTL	Relative Telomere Length
RT-PCR	Reverse transcription-polymerase chain reaction
SCG	Single Copy Gene
SD	Standard deviation
SEER Program	Surveillance, Epidemiology, and End Results Program
SEM	standard error of mean
SIDS	Sudden Infant Death Syndrome
TER	Telomerase RNA
TERT	Telomerase Reverse Transcriptase
TMI	Tail Moment Index
TPM	Total Particulate Matter
TRAP	Telomeric Repeat Amplification Protocol
US	United States
WHO	World Health Organization
WPS	Waterpipe Smoke
WSE	Waterpipe Smoke Extract

To my lovely parents who always support me, and

to my grandparents who would have been proud.

CHAPTER I

INTRODUCTION

A. Smoking

1. Smoking Epidemiology

Tobacco smoking is a worldwide problem that is increasing tremendously while targeting different age groups without any deterrence. According to the Global Health Observatory data of the World Health Organization (WHO), the prevalence of tobacco smokers increased to over 1.1 billion in 2015 in many countries especially in the Eastern Mediterranean and the African Regions [1, 2]. In the U.S. alone, around 34 million people still smoke despite the increase in smoking cessation organizations that help people cope with the withdrawal symptoms after quitting smoke. As noted by the Centers for Disease Control and Prevention (CDC), the trend of smoking is high among specific groups classified according to gender, age, education, socio-economic status, physical and psychological health. For example, men aged between 25-64 years old, with lower education, below poverty level, disabled, and have serious psychological distress are more prone for smoking among the American population [3]. Of those, more than 16 million people are prone to live with diseases caused by smoking. Statistics also show that, by itself, secondhand smoke contributes to mortality of 41000 nonsmoking adults and 400 infants each year[4]. According to the WHO, it was estimated in 2008 that tobacco contributed to the deaths of 1 in 10 adults worldwide equivalent to 5 million people per year. It is predicted that tobacco might kill 1 in 6 adults that is equal to 10 million people annually in the next 20 to 30 years. In this

report, approximately one third of the world's population was reported as current smokers with estimates of 47% in men and 12% in women [5].

2. Smoking Related Diseases

All types of tobacco smoke can predispose the body to cardiovascular, respiratory, gastrointestinal, reproductive, growth and cancer problems (**Figure 1**). Though lung cancer accounts for 70% of the cases, smoke emitted from smoking tobacco can also lead to mouth, throat, breast, bladder, kidney, liver, and pancreatic cancer, among others. Smoking affects the heart by increasing the coronary vascular resistance and decreasing the coronary blood flow thereby increasing coronary vasoconstriction and decreasing blood supply to the myocardial tissue which in turn can increase the risks of stroke, cerebrovascular disease, peripheral vascular disease, and coronary artery disease. Smoking not only affects the systemic vasoconstriction but can also lead to pulmonary vasoconstriction by predisposing the lungs to asthma, respiratory tract infections, and pneumonia that can all lead to chronic obstructive pulmonary disease (COPD) among other bronchial and alveolar pathologies. In one study, Rammah *et al.* [6] proved that waterpipe contributes to COPD risk. Smoking effects are not only limited to the cardiovascular and respiratory system, but also can interfere with the cell barrier of the stomach and intestines leading to gastric and duodenal ulcers. Smoking may contribute to gastrointestinal problems like peptic ulcer disease, Crohn's disease and increase the risk of pancreatic and colorectal cancers. Besides, smoking can lead to infertility problems among both genders, and can lead to miscarriages in women [7]. For example, women who smoke are predisposed to low-birth-weight pregnancies because carbon monoxide, a chemical component present in cigarettes, binds to

hemoglobin leading to a decrease in oxygen supply to the fetus. Therefore, the environmental tobacco smoke (ETS) emitted from either cigarettes or waterpipes can increase the risk of miscarriages, premature births, sudden infant death syndrome (SIDS), decreased fertility in women and erectile dysfunction in men [2, 8]. In a recent Spanish study entitled The HELIX Project, the authors also showed that smoking during maternity can affect biological aging by accelerating telomere shortening in children from an early age which is an innovative effect corresponding to the genetic field [9]. Therefore, smoking not only contributes to well-known systemic diseases but also branches to cover genetic influences contributing to these diseases (**Figure 2**).

Figure 1: Effects of Tobacco Smoking [8]. (AA) Aortic Aneurysm; (CHD) Coronary Heart Disease; (PVD) Peripheral Vascular Disease; (COPD) Chronic Obstruction Pulmonary Disease.

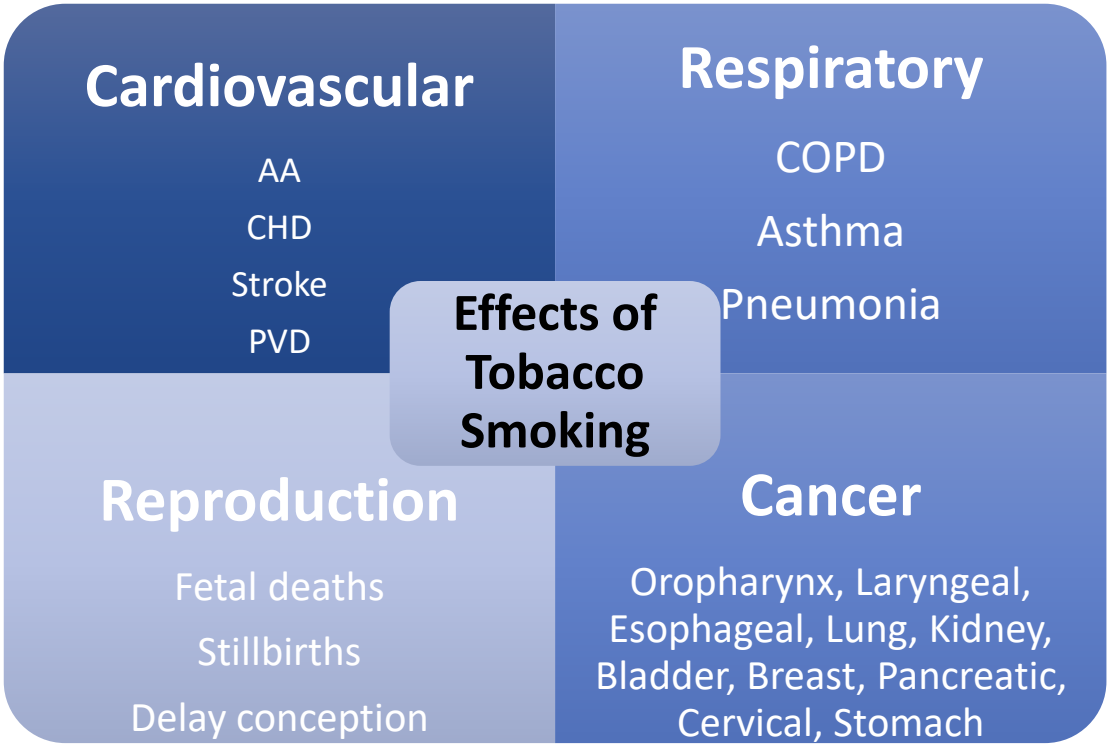
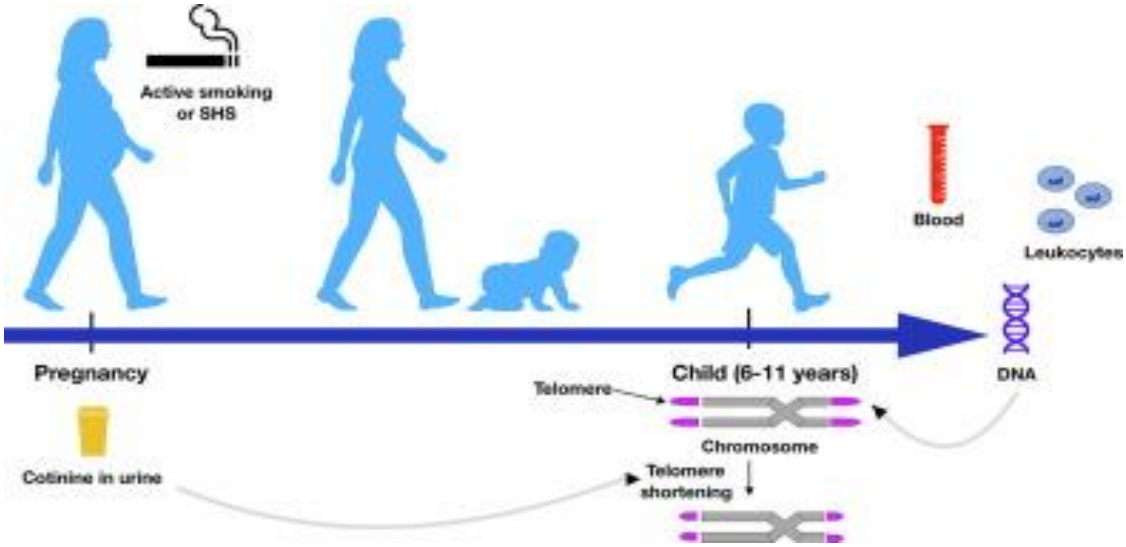


Figure 2: Effect of early life Tobacco Smoking and children's telomere length [9]



3. Human Exposure to Smoking

Human exposure to tobacco from cigarettes or waterpipe smoke can be either direct or indirect. Direct exposure is by inhaling the smoke emitted from smoking cigarettes or waterpipes referring to the primary smoker, and the indirect exposure is by inhaling from the exhaled environmental tobacco smoke (ETS) referring to the secondhand smoker. Over the years, studies showed that secondhand smokers, like primary smokers, can encounter similar risk factors and health-related diseases caused by carcinogens and harmful chemicals found in the heated tobacco. Secondhand smoking also called passive or involuntary smoking is as dangerous as primary smoking. It is referred to ETS, and it is divided into two forms released from the heated tobacco: the mainstream smoke and the side stream smoke. The mainstream smoke is simply the exhaled smoke released by the smoker's mouth or nose, while the side stream smoke is the smoke released from the lighted end of a cigarette, and this form is

considered more dangerous as it contains smaller particles with higher concentrations of cancer-causing carcinogens[10].

Exposure to cigarettes or waterpipe can be present anywhere around us at home, at work, in the car and in public places. Some laws ban smoking in indoor areas, but still anybody can be exposed to smoke in outdoor areas. Children are the most critical age group, and they are at high risk of being exposed to harmful carcinogens especially if their parents are smokers. These children can be physically and psychologically affected. For instance, some studies showed effects of smoking on mental health such as depression. Children exposed to such chemicals are also more prone to lung and ear infections and may develop shortness of breath, cough and wheezes more often than non-exposed children[10]. Therefore, all age groups from children to adults are exposed to cigarettes, and recently waterpipe everywhere, and the exposure can be direct or indirect and still be as harmful.

4. Smoking Delivery Methods

Smoking is a silent killer irrespective of the type of tobacco and the smoke inhaled technique. For instance, there are many types of smoking methods that differ in the way tobacco is inhaled when heated. First, cigarettes are among the oldest and most common types of smoking methods; they are made up of tobacco, filter and are wrapped by paper. Second, cigars and cigarillos resemble the cigarettes, but they are made up of tobacco wrapped by leaf tobacco. The large cigars contain 10 times more nicotine, 2 times more tar and 5 times more carbon monoxide when compared to the regular cigarette. Third, Electronic or E-cigarettes or vaping nowadays is common, not safer than other tobacco forms as some might think, and they are powered by a battery.

Fourth, waterpipe smoking also called “hookah”, “shisha”, “narghile” or “argileh” is heated by a charcoal, and the heated tobacco can have a special flavor like apple, lemon, cherry, orange, or mint. The emergence of flavored tobacco (maassel) and tobacco-free waterpipe products made it popular and attractive to youth groups. Waterpipe products were misconceived as safer, and people believed that they are less harmful than cigarette products [11]. Nevertheless, by now it is known that one session of waterpipe is more toxic than one regular cigarette at least with respect to the smoking duration whereby one hour-session of waterpipe can deliver up to 200 times more smoke when compared to one cigarette [12, 13]. Though there are several types and methods for smoking tobacco, what is covered here will be related to cigarette and waterpipe.

a. Cigarette Smoking

i. Common Use of Cigarettes

Cigarette smoking was among the oldest and most common choice of tobacco among adults according to the 2015 National Health Interview Survey (NHIS) in the U.S.[14]. Besides, adults are not the only age group who smoke, but youth and peer groups are as common. According to the Executive Summary of the U.S. Surgeon General Office in 2012, the report identified that everyday 3800 youths are at risk of smoking their first cigarette under the age of 18, and this was the case of 88% of adult smokers until now[15]. Recently, a decline in the trend of cigarette smoking was noticed, and such decline in the use of cigarettes was not attributed to its impact on the health status but rather to the fact of increased taxes and prices of tobacco products, in addition to implementing policies and regulations that govern their public use. Cigarette smoking also declined among youth and young adulthood as reported by the National

Survey on Drug Use and Health in 2012, but the craving for cigarette smoking shifted to an increase in electronic cigarettes and other shared forms of smoking like waterpipes, where today they are considered the highest and the most prevalent in U.S. people between 18-24 years of age [8, 16, 17]. In the BREATHE study by Khattab *et al.* [18], Lebanon was classified as the highest in cigarette smoking exposure compared to the other 10 MENA region countries. Lebanese men, especially, ranked higher in cigarette smoking compared to Lebanese women.

ii. Chemical Components of Cigarettes

Cigarettes are composed of a filter, cigarette paper, tobacco filler, tipping paper and additives containing harmful chemicals. Cigarettes are wrapped by papers holding the tobacco fillers that contain dangerous chemicals. These are usually added by manufacturers to control the burning rate of the cigarette [19]. There are two types of chemicals; those that are processed during the formation of the cigarette called particulate phase chemicals such as nicotine, tar, polycyclic aromatic hydrocarbons (PAHs), nitrosamines, metals like arsenic and lead. Others are created at the time the filler is burned called gaseous phase chemicals such as carbon monoxide and aldehydes like formaldehydes, acrolein, and acetaldehyde. Most of those chemicals are carcinogens **(Table 1)** and are inhaled through the filter that is found in the filtration zone of the tipping paper directly into the lungs through the ventilation holes. These holes help the smoker take deeper puffs which can block the largest tar particles, but at the same time enable the smallest particles to travel farther in the lungs. In addition, these holes usually are responsible for diluting the inhaled smoke with air, yet some smokers block them with their fingers or lips to get more of the nicotine rendering them

ineffective. The high levels of nicotine present in cigarettes increase the psychological dependence or craving by adult smokers to fulfill their need and desire. Besides, the additives added by the manufacturers are not less harmful, on the contrary: sugar, when burned, can cause cancer; ammonia, when added, can increase the absorption of nicotine rendering the cigarette more addictive, and bronchodilators increase the absorption of harmful substances in the lungs.

Table 1: Tobacco smoke carcinogens evaluated in the Monographs of the *International Agency for Research on Cancer* (IARC) [20]

Chemical Class	Number of Carcinogens	Representative Carcinogens
Polycyclic aromatic hydrocarbons (PAHs) and their heterocyclic analogues	15	Benzo[*a*]pyrene (BaP) Dibenz[*a*,*h*]anthracene
N-Nitrosamines	8	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) *N*′-Nitrosonornicotine (NNN)
Aromatic amines	12	4-Aminobiphenyl 2-Naphthylamine
Aldehydes	2	Formaldehyde Acetaldehyde
Phenols	2	Catechol Caffeic acid
Volatile hydrocarbons	3	Benzene 1,3-Butadiene Isoprene
Other organics	12	Ethylene oxide Acrylonitrile
Inorganic compounds	8	Cadmium Polonium-210

b. Waterpipe Smoking

i. Common Use of Waterpipes

Waterpipe smoking is one of the most common sources of inhaled tobacco. It initially began in India and China, then became prevalent in Asia, Africa and Middle East before the 1990s, and extended in the last few years to the West and globally [21]. In the Middle East, waterpipe was common among adult men. In the early 1990s, it started spreading among young people replacing the cigarettes, ranking top popularity among different types of tobacco and globalizing to other parts of the world [22]. Compared to cigarettes, data in 20 European countries showed the prevalence of waterpipe becoming the second most commonly smoked tobacco product after cigarettes. Lebanon is among the countries ranking the highest in waterpipe prevalence among young people with 36.9% compared to 32.7% in West Bank, and much higher in comparison to the Eastern Europe with 21.9% in Estonia, 22.1% in Czech Republic and 22.7% in Latvia [23]. In the BREATHE study, Saudi Arabian women ranked the highest in waterpipe consumption in comparison to women from the other 10 MENA region countries. Lebanese women, in particular, occupied the highest proportion in waterpipe smoking at least the last 5 years in Lebanon compared to men [18].

Waterpipe smoking is not only confined to the Eastern Mediterranean region, but it also spread to the West to countries like UK, Australia, Canada, and USA [24]. Recently, for example in U.S., waterpipe became common among college students even more than cigarette smoking, but it is threatening since it is being considered a vector for cigarette smoking, tobacco dependence and addiction in this age group[25].

ii. Chemical Components of Waterpipe

Waterpipes are composed of the tobacco bowl, the body, the water bowl, the hose, and the mouthpiece. Waterpipes work by placing a charcoal on top of the flavored tobacco, and smoke would pass from the head of the waterpipe into the water bowl through the hose to the smoker. In comparison to a single cigarette, the smoke of the waterpipe contains 36 times more tar, 15 times more carbon monoxide, heavy metals, more than 16 polycyclic aromatic hydrocarbons, and 70 percent more nicotine than one cigarette among other cancer-causing chemicals [26]. These harmful and toxic chemicals found in cigarettes too are being more absorbed in the body by waterpipe smokers than cigarette smokers over a longer period. For example, a one-hour session of waterpipe smoking results in 200 puffs compared to 20 puffs with an average cigarette. According to Eissenberg and Shihadeh [27], the evidence that cigarette and waterpipe have similar nicotine levels is disproved since the duration taken while smoking a cigarette can reach a maximum of 5-7 minutes compared to a single waterpipe smoking session that takes longer around 45 minutes to 1 hour; therefore nicotine exposure is definitely higher with waterpipe smoking. According to CDC [28], the inhaled smoke in a waterpipe session can reach 90 000 milliliters compared to 500-600 ml with cigarette smoking. A meta-analysis of 17 studies comparing a single waterpipe smoking session to a single cigarette identified the content of smoke (**74.1 vs 0.6 L**), nicotine (**4.1 vs 1.8 mg**), tar (**619 vs 24.5 mg**) and carbon monoxide (**192 vs 17.7 mg**) found in each respectively **(Table 2)**. In conclusion, these numbers among others highlight the fact that a session of waterpipe is more toxic than a single cigarette when comparing their common chemical constituents and the duration of smoking of each [29]. A single waterpipe session generates greater smoke exposure, nicotine, tar,

and CO than a single cigarette. For example, it is estimated that the exposure to nicotine from waterpipe daily use is like smoking ten cigarettes per day. This is likely associated with a higher risk for tobacco/nicotine dependence [30, 31].

Table 2: Smoke, Nicotine, Tar and Carbon Monoxide estimates in one waterpipe session vs one cigarette [29]

	Summary estimate (95% CI)	
Toxicant	*Waterpipe*	*Cigarette*
Smoke (L)	74.1 (38.2, 110.0)	0.6 (0.5, 0.7)
Nicotine (mg)	4.1 (2.7, 5.4)	1.8 (1.3, 2.3)
Tar (mg)	619.0 (244.0, 994.0)	24.5 (15.5, 33.6)
Carbon monoxide (mg)	192.0 (77.5, 307.0)	17.7 (15.6, 19.9)

B. Breast Cancer and Smoking

1. Breast Cancer Definition

a. Breast Cancer Types

Breast cancer is a disease that originates primarily in the breast from growing cells that become cancerous. The type of breast cancer is divided based on its location in the breast and the expression of specific receptors. Based on its location, breast cancer has two types: ductal and lobular. Ductal carcinoma, referring to the ducts that carry milk to the nipples, is invasive and can spread and metastasize to different body parts. Lobular carcinoma, referring to the lobules that produce initially milk, is also invasive and can spread to nearby breast tissues and to other body parts [32]. Breast cancer cells also express receptors that can indicate if the cancer is positive or negative. A tested positive breast cancer refers to the presence of any of the three receptors: estrogen, progesterone, and human epidermal growth factor receptor 2 (HER2). The presence of any of the receptors is also called Luminal breast cancer type divided into luminal A and luminal B. If the receptors for estrogen and progesterone are positive and

HER2 receptor is negative, then it is a luminal A breast cancer. If the receptors for estrogen and HER2 are positive and progesterone is negative, then it is a luminal B breast cancer. The absence of estrogen and progesterone receptors while HER2 receptor presence is called a HER2 positive breast cancer [33]. Additionally, the presence of all three receptors is a triple-positive breast cancer while a triple negative breast cancer refers to the receptors' absence and is also called basal-like breast cancer [34].

b. Breast Cancer Epidemiology

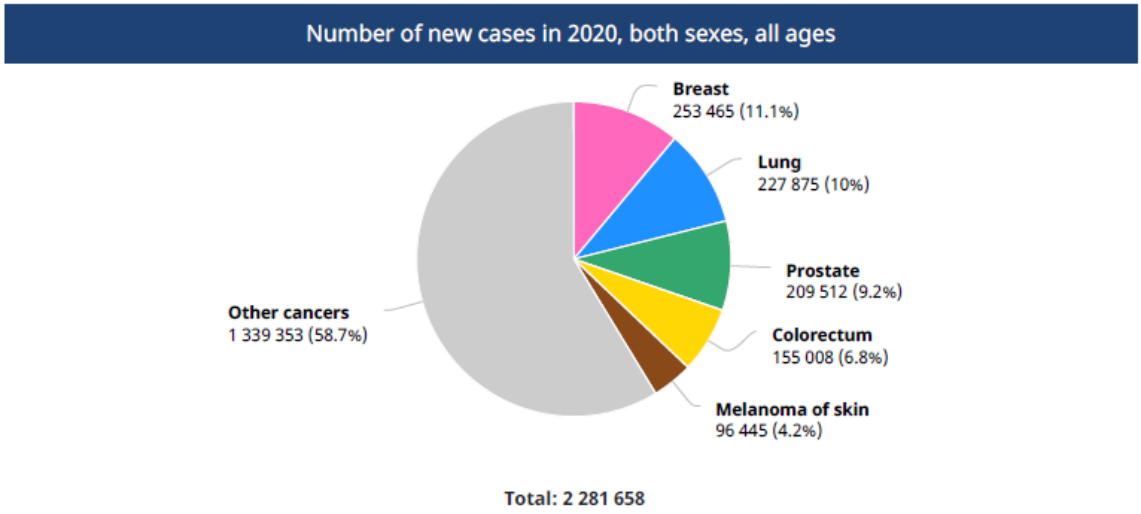
According to recent Globocan statistics in 2020, breast cancer ranked as the third leading cause of mortality after lung and pancreatic cancer in the U.S. and the fourth worldwide, but it overpassed lung cancer by ranking the first leading cause of cancer not only in U.S. but also in the world **(Figure 3)** [35, 36]. In a review by Waks *et al.* in 2019 [37], breast cancer was classified as the most common type of cancer among females in addition to skin cancer reaching more than 250, 000 new cases in 2017. Today, breast cancer occupies the highest incidence rate worldwide. Cases in females were increasing with a 12% risk of developing cancer during the lifetime course of a woman in the U.S. In males, it is a rare disease that represents only 1% of all breast cancer cases around the world [38]. Today, breast cancer accounts for 11.7% of all newly diagnosed cases in both genders [36]. Breast cancer is also common in other Western countries and the four countries affiliated to the Middle East Cancer Consortium (MECC).

In the new 2020 Globocan statistics, Lebanon ranked also the first in breast cancer incidence with breast cancer being the second leading cause of death after lung cancer **(Figure 4)** [39]. Breast cancer is very common compared to other cancers in

females, and it occurs in younger age groups compared to Western countries. Based on previous data from the Lebanese National Cancer reports, breast cancer constitutes a ratio of one-third, and more cases are diagnosed compared to other types of cancers in women [40]. According to the **National Cancer Institute** (Surveillance, Epidemiology, and End Results Program), the estimated number of female breast cancer in 2020 was 276, 480 cases that counts for a rate of 128.5 per 100, 000 women per year. On the other side, the estimated number of deaths from breast cancer in 2020 was 42, 170 cases that counts for a death rate of 20.3 per 100, 000 women per year [41].

Figure 3: The Globocan Statistics of 2020 representing the number and percentage of new cancer cases in **a.** The United States of America and **b.** The World [35, 42]

a.



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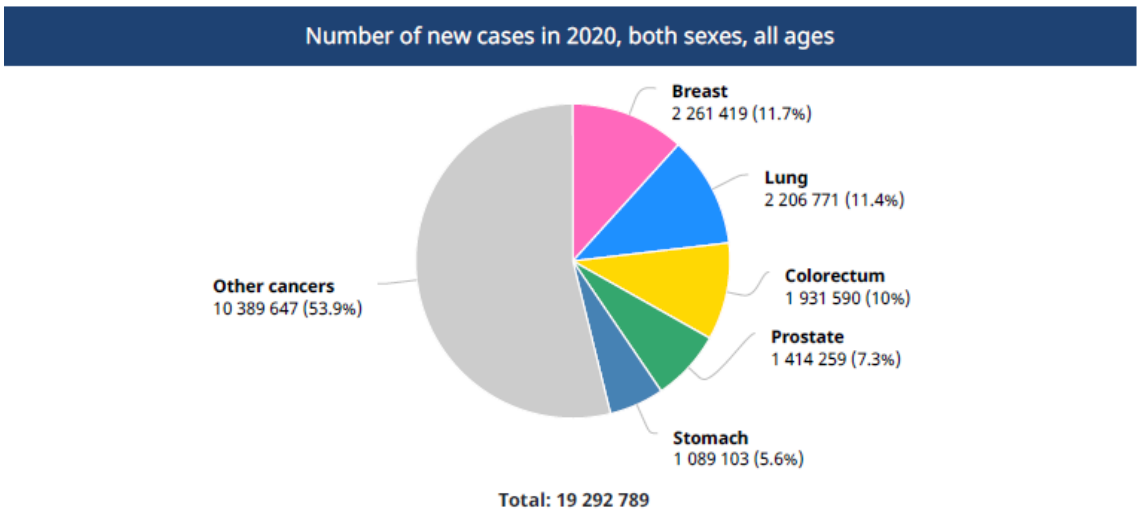
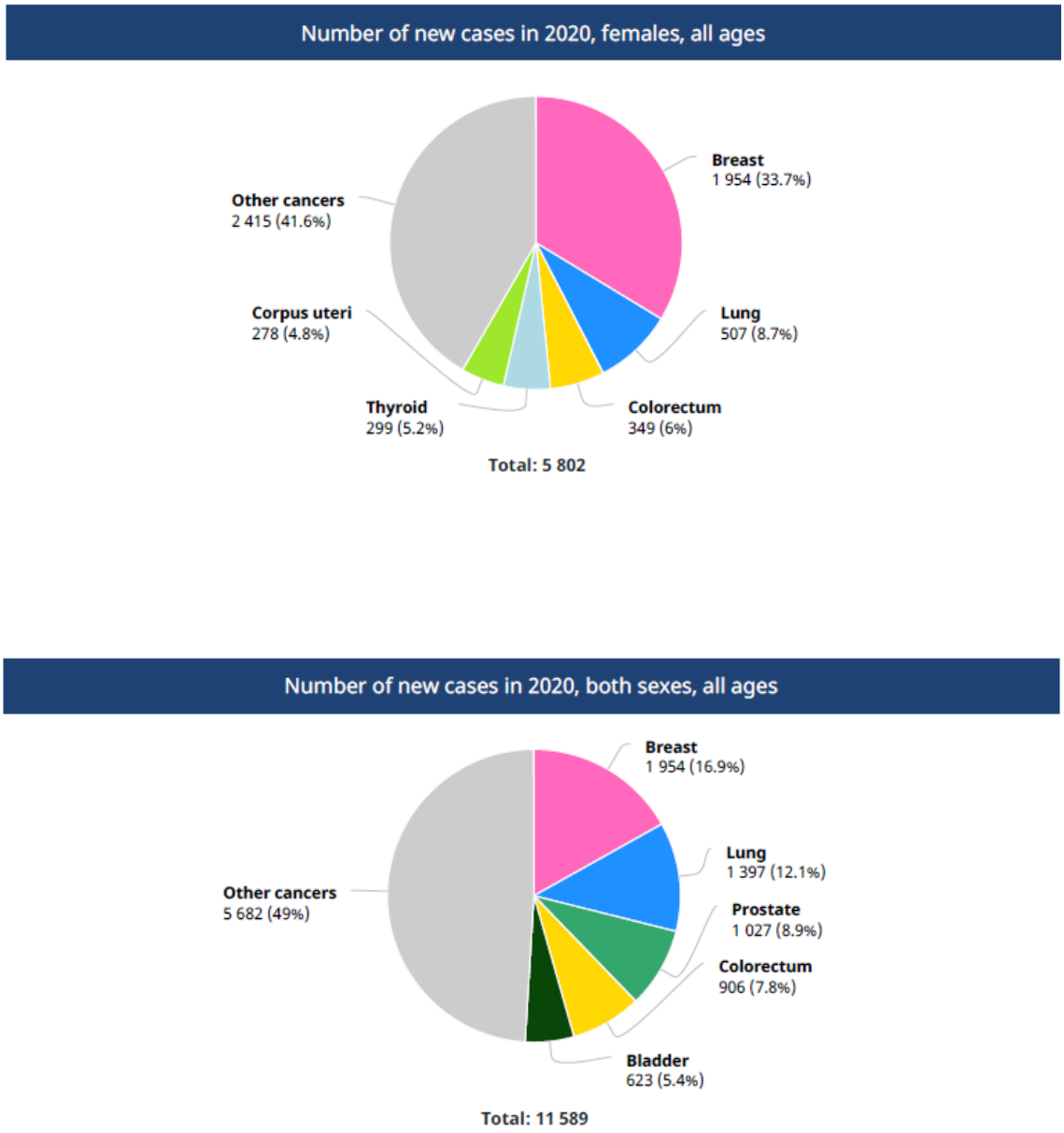


Figure 4: The Globocan Statistics of 2020 representing the number and percentage of new cancer cases in Lebanon [39]



c. Breast Cancer Risk Factors

There are many risk factors that predispose women to breast cancer. According to the CDC, some of these risks include family history, alcohol intake, obesity, the use of oral contraceptives, hormonal therapy, genetic mutations, age group and gender predisposition. Recently, it is hypothesized that exposure to tobacco from cigarettes, waterpipes and other methods also affect the carcinogenicity in breast cancer as reviewed and assessed by the International Agency for Research on Cancer (IARC) [43].

2. Smoking-Associated Breast Cancer

a. Effects of Cigarette and Waterpipe Smoke on Breast Cancer Development and Progression

Smoking is a modifiable risk factor that increases breast cancer development and progression. Most of the studies focused on participants who mainly smoke cigarette and disregarded all other types such as waterpipe. For example, Jones *et al.* [44] showed in the Generations Study cohort of over 113,700 women aged 16 years or older that those who started cigarette smoking at an early age such as adolescence or peri-menarche were at a greater risk of developing breast cancer in adulthood. Moreover, women who smoked more than five cigarettes daily, used more than 10 packs yearly, or had ceased smoking for less than 20 years were likely at a significant risk for invasive breast cancer as reported in this cohort. Also, this study showed that women with a family history of the disease or women smoking before their first childbirth are at an increased risk of developing breast cancer.

In addition to that, it is important to highlight that, during puberty, the breast forms the terminal ductal and lobular structures from undifferentiated cells that are very sensitive to carcinogenesis. Therefore, women who started smoking before the age of puberty or their first childbirth are more prone for breast cancer. As stated earlier, cigarette and waterpipe smoke are composed of different chemical yields including polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, aromatic amines among other constituents [45]. These tobacco chemicals can be taken up by mammary tissues through blood circulation causing DNA damage and adduct formation in mammary glandular epithelial cells [46, 47]. Hence, the binding of a chemical to DNA could lead to a mutation, and without adequate DNA repair, may result in cancer [48]. In a recent study by Takada *et al.* [47], smoking was shown to influence biological changes in the breast through receptor expression. Among the estrogen receptor (ER), progesterone receptor (PgR) and HER2, the study showed a significant correlation between smoking and HER2 expression patterns especially after recurrent disease. Therefore, smoking not only contributes to breast cancer development at initial phases, but also to progression at recurrent stages.

b. Epidemiological Studies

Cohort studies on the association of smoking with breast cancer began since the 1990s, but little evidence was found at the time and the association remained unclear [49]. Many agencies, including the IARC, the U.S. Surgeon General, California's Environmental Protection Agency and the Canadian Panel, reported findings concerning the contribution of smoking to breast cancer **(Table 3)** [50]. It was not until the release of the Canadian Expert Panel on Tobacco Smoke and Breast Cancer in 2009 that

smoking was identified as a causal factor for breast cancer development [51]. In a cohort study by Luo *et al.* [52], the risk of breast cancer among former smokers compared to non-smokers increased by 9% and by 16% among current smokers. That study displayed the risk for developing breast cancer, yet there are other studies showing recurrence of breast cancer especially among smokers. For example, Pierce *et al.* [53] showed that breast cancer had a 37% increased recurrence risk especially among former smokers with history of smoking more than a 30 pack-year. Moreover, the overall mortality risk from breast cancer among smokers showed an increase by 54% compared to nonsmokers. In one of the largest cohort analysis conducted by the European Prospective Investigation into Cancer and Nutrition (EPIC), breast cancer risk was the highest among former and current smokers, women exposed to passive smoking compared to nonsmokers, and those unexposed to passive smoking respectively [54]. According to the IARC, there are several confounding factors that need control such as unreported alcohol consumption and less screening rates among current active smokers [55]. For instance, alcohol is an additional potential risk factor for breast cancer; therefore, patients who do not report their consumption of alcohol may lead to unreliable results confounding the main risk factor for breast cancer development [43, 55].

c. Animal Studies

The studies conducted on animals exposed to cigarette smoke and breast cancer are so far scarce especially that they necessitate long-term exposure. One study by Di Cello *et al.* [56] demonstrated the effect of cigarette smoke on the phenotype of mammary epithelial cells that changes from normal to fibroblastoid, a phenomenon so called mesenchymal transition. Also, introducing cigarette smoke extract (CSE) to

normal mammary epithelial cells of mice resulted in the colonization of mammary ducts, a process leading to metastasis. Moreover, injecting a mammary pad of mice with MCF-7 breast tumor cells exposed to CSE contributed to lung metastasis compared to untreated MCF-7 cells injected to mice that did not result in metastasis, hence stressing the effect of cigarette smoke on cell invasion. In another study, different constituents present in cigarette smoke such as isoprene, benzene, ethylene oxide and benzo[*a*]pyrene (BaP) were identified as mammary carcinogens in a group of rodents [57]. These identified carcinogens were also reported earlier by IARC in 2004 and proven to be present in secondhand smoke [20]. In summary, studies with animals so far showed a tumorigenic effect of cigarette smoke on mammary cells from development to progression and metastasis, but future research should be directed on experimenting more with *in vivo* studies [43].

Table 3: Expert panel agency reviews of active smoking and breast cancer [50]

Agency	Year Published	Literature Covered	Findings
IARC Monograph 38	1986	(18 studies)	"suggestion of a decreased risk was noted"
California EPA	1997	Through 1996 (21 studies)	"the results are inconclusive"
U.S. Surgeon General	2001	Through 1999 (24 studies)	"active smoking does not appear to appreciably affect breast cancer risk"
IARC Monograph 83	2004	Through mid-2002 (44 studies)	"the evidence is indicative of no association between smoking and breast cancer"
U.S. Surgeon General	2004	Through 2002 (3 additional large studies since Palmer review)	"the evidence is suggestive of no causal relationship between smoking and breast cancer"
California EPA	2005	Through 2005 (52 studies)	"the data provide support for a causal association between active smoking and elevated breast cancer risk"
Canadian Panel	2009	Through 2008 (Terry meta-analysis, plus 40 more studies)	"the relationship between active smoking and both pre- and post-menopausal breast cancer is consistent with causality"
IARC Monograph 100E	2012	Through 2008 plus one 2009 study in preparation (>130 studies)	"a positive association has been observed between tobacco smoking and cancer of the female breast"

C. Cell Culture Studies with Cell Lines Exposed to Smoke

There are many studies on cigarette smoke exposure in cancer cells [58]; however, in this section the focus will be on cigarette and waterpipe exposure in breast cancer cell lines and on studies evaluating waterpipe exposure in other cancer cell lines.

1. Cigarette and Waterpipe Experiments in Breast Cancer Cell Lines

One of the hallmarks in cancer progression is the ability of the cells to metastasize and cause invasion. In a study by Sadek *et al.* [59], this was manifested by morphological change such as the change of MCF-7 breast cancer cell line from epithelial-like into fibroblast-like (mesenchymal). Under the microscope, cells exposed to waterpipe smoke (WPS) of 100-200 μg/ml using the Aleppo Method became elongated in shape and decreased their cell to cell contact when compared to unexposed cells treated with the same volume of Phosphate-buffered saline (PBS) or Roswell Park Memorial Institute (RPMI) medium. This event is known as epithelial-mesenchymal transition, and it is proven that WPS can play a role in initiating such transition. Similarly, a study by Di Cello [56] demonstrated a transformed phenotypical change in both non-tumorigenic (MCF-10A and MCF-12A) and tumorigenic (MCF-7) breast cancer cell lines when exposed to cigarette smoke extract or cigarette smoke condensate (0.5% CSE or 25 μg/ml CSC) chronically for a long-term. Like waterpipe, this study concludes the association of smoking with epithelial to mesenchymal transition leading to an increase in the metastatic ability of breast cancer cells. In addition, in the study by Sadek *et al.* [59], WPS plays a role in deregulating some key controller proteins that are considered important in preventing cancer invasion. It was shown that the expression of E-cadherin, a tumor invasion suppressor, becomes downregulated while FAK gene, a

key controller gene in cell metastasis, becomes upregulated when exposed to WPS. This leads to the activation of Erk1/Erk2 pathway and the initiation of EMT through the deregulation of specific proteins that are targeted by waterpipe. As a conclusion, WPS can increase the risk of metastasis in breast cancer cells such as MCF-7 by promoting EMT via Erk1/Erk2 signaling pathways. Similarly, two articles by Kispert *et al.* [60, 61] showed an increase in the platelet-activating factor (PAF) and its receptor after breast cancer cells' exposure to cigarette smoke. One of the studies concludes that CSE (20µg/mL) can contribute to tumor progression and metastasis by increasing the expression of COX-2 expression resulting in an increase in PGE_2 release in both MCF-7 and MDAMB-231 cell lines [60]. The other study concludes that CSE (20µg/mL) can induce cell motility via PAF accumulation mainly in triple negative MDAMB-231 breast cancer cells showing its mechanistic potential for metastasis through the inflammatory mediator PAF [61].

2. Waterpipe Experiments in Other Cancer Cell Lines

A large number of studies evaluated the exposure of cancer cell lines with cigarette smoke and only few ones with waterpipe. In a study by Shihadeh *et al.* [62], treatment of A549 (human alveolar epithelial) cells with 4 mg/ml waterpipe derived from smoking machine protocol resulted in reduced cell proliferation in both tobacco-based and tobacco-free waterpipe after 72 hours of treatment. In addition, the smoke extract from both tobacco preparations impaired the cellular growth as indicated by a decrease in the percentage of alveolar cells in the S phase consistent with the fact that waterpipe total particulate matter (TPM) induced cell cycle arrest at G0/G1. The doubling time of cells also increased in both tobacco preparations with no significant

difference in outcome. Finally, comparing both tobacco and nontobacco products with respect to topography parameters (puff drawn, puff duration, interpuff interval, total smoke volume) and toxicant yields (TPM, CO, NO) did not show significant difference across any product. Therefore, the similarities in the outcomes suggest that tobacco free product impose the same health hazards as the conventional one contributing to pulmonary disease risk factor that can lead to abnormal cell repair in case of injury. The only noticed difference among both products is the nicotine addiction resulting more with the conventional waterpipe tobacco products. Additionally, this study highlighted the pro-inflammatory effect of waterpipe smoke that could be derived from burning charcoal rather than from the type of tobacco itself whereby 90% of the toxicants such as polyaromatic hydrocarbons and carbon monoxide are derived from the charcoal.

In a more recent study, Khalil *et al.* [63] used different flavored tobacco brands generating smoke through a special filter setup without cartridges thus smoke remained in the form of gas. Among the used flavors, the Double Apple proved to be the most potent smoke and the most toxic among the other flavors when applied to A549 cells for 24 hours. For instance, the percentage of viability of A549 cells tested by MTS and NR cytotoxicity assays decreased to 25 % at 120 minutes when exposed to Double Apple (Nakhla brand) unlike the Lemon and mint (Mazaya brand) that decreased by 30% at 120 minutes remaining the least toxic among all other brands. By comparing the immediate exposure to the 24-hour post exposure to tobacco flavors, the Double Apple also significantly reduced cell viability to 80% immediately post exposure yet with a more severe drop to 40% after 24 hours. This was not noticed with other types of flavors. For instance, the Watermelon and Mint (Mazaya brand) had a toxic response that was only significant after a 50-minute time point of the 24-hour post exposure with

a 70% drop in viability. It was argued that nicotine could be the reason behind the higher cytotoxicity reported when using the Double Apple flavor. However, even though 0.5% nicotine is present similarly in the Lemon and Mint (El Fakher brand) and the Double Apple (Nakhla brand), the first did not have the same cytotoxic effect as the second. Thus, nicotine is not the only reason behind the potent cytotoxicity.

When exposing A549 cells to Double Apple tobacco brand using the comet assay, the tail moment index (TMI) showed also significant cellular DNA damage earlier at 30 minutes of exposure and increased to significant alarming levels at 50- and 120-minutes post exposure. Under the microscope, A549 cells displayed significant changes in the cell's structural membrane when exposed to Double Apple tobacco brand with 50% displaying cell rounding at 20 minutes after 24-hour exposure confirming the loss of membrane integrity. In addition, those cells were stained positive for both Annexin V/FITC and PI apoptosis assay indicating cell death by either late apoptosis or necrosis after 5 hours post exposure to Double Apple [63]. Altogether, this concludes that the loss of cell membrane integrity after smoke exposure increases cell detachment as displayed by the cell rounding shape that will likely result in cell necrosis. Therefore, the consumption of a single session of nargileh smoke is cytotoxic by decreasing cell viability, genotoxic by causing cellular DNA damage, and necrotic to the cells by changing their morphological shape resulting in their cellular death.

Since nicotine is the primary toxic substance of waterpipe smoke (WPS), Bodas *et al.* [65] showed that both nicotine and WPS resulted in similar effects when inducing COPD-emphysema progression as an endpoint. In this study, human bronchial epithelial cells (Beas2b) were treated either with WPS (5%) or Nicotine (5mM) using the procedure listed in the Beirut method protocol [64, 65]. In comparison to CS and e-cig

vapor (e-CV), treatment of Beas2b cells with WPS and Nicotine for 6-24 hours decreased cell viability in a dose-dependent manner. Nicotine induced cell apoptosis and senescence through impairment of autophagy mechanism. The autophagy impairment in the lung stimulated by nicotine exposure is due to an increase in ROS levels that led to exacerbated COPD- emphysema like symptoms. Not only nicotine causes exacerbation of COPD and cystic fibrosis cases, it also inhibits bacterial clearance capacity by impairing the xenophagy machinery contributing to increased infections and pulmonary pathogenesis. Therefore, nicotine, a toxic constituent present in all tobacco forms, plays a role in COPD-emphysema by impairing autophagy and inducing cell apoptosis/senescence thus leading to increased bacterial infections and further exacerbations. Altogether, the study refutes the perception that waterpipe smoke (WPS) is less toxic than cigarette smoke (CS) [45] and disagrees with the general belief that waterpipe and e-cigarettes are safer than regular cigarettes [11, 66]. Therefore, this concludes the fact that WPS is harmful as the CS on the lung health [11, 27, 66]

In humans, lungs are initially affected by smoking tobacco and in particular, alveolar cells become inflamed when exposed to smoke. Rammah *et al.* [6] exposed alveolar type II derived cells (A549) to WPS concentrations of 0.5, 1, 3, 4, 6, 8 mg/ml using the Beirut smoking protocol for up to 3 days, waterpipe decreased cell proliferation and increased doubling time in a dose-dependent manner. It induced cell cycle arrest but not apoptosis through the p53-p21 pathway contributing to cellular senescence. The resulting senescent cells increased Toll-like receptor 4 and matrix metalloproteinase (MMPs) expression. Nitric Oxide (NO), a pro-inflammatory and damaging marker, indicates the presence of inflammation. Therefore, measuring its level showed an increase in NO production after WPS exposure (4mg/ml) suggesting an

underlying inflammatory response. As a conclusion, the increased transcriptional expression of inflammatory mediators and the increased NO production after waterpipe exposure resulted in an inflammatory process that eventually plays a role in the pathogenesis of COPD by impairing cellular growth and inducing inflammation that can lead to abnormal cell repair. When the epithelium fails to repair itself by alveolar cell proliferation and the MMPs expression leads to further alveolar destruction, this results in progressive pulmonary diseases that impair the normal lung function. Additionally, in this study by Rammah *et al.* [6], cigarettes were found to be more mutagenic than waterpipe, and this was explained based on the difference in heating temperature and toxicants dilution. According to White *et al.* [67], relatively high burning temperatures as high as 400 to 475 degrees Celsius resulted in mutagenic effect with cigarettes. However, Rammah *et al.* [6] showed that heat from the burning coal did not reach higher than 450 degrees Celsius and went down to as low as 50 degrees Celsius leading to a less mutagenic effect with waterpipe.

In the study by Mortaz *et al.* [68], A549 cells were treated with waterpipe smoke (WPS) 4 mg/ml at different time points (24, 48, 72 and 96 hours) compared to only PBS in the control group. The smoke extracts were prepared as indicated by Rammah *et al.* [6]. Like previous studies, WPS in comparison to PBS- treated cells decreased cell viability with the highest effect seen after 96 hours. It also decreased the relative proliferation rate in a time-dependent manner. What was exclusive for this study is the effect of WPS in increasing the A549 cell infection rate by the uptake of FITC-BCG after 72 and 96 hours. Here, WPS enhanced the uptake of BCG by A549 cells thus increasing the internalization of Mycobacterium Bovis [68]. This concludes that

waterpipe consumption contributes to higher infection rates through bacterial internalization by micropinocytosis.

D. Telomerase, RTL, Smoking and Breast Cancer

1. Telomerase

Telomerase is a ribonucleoprotein enzyme that maintains the length of telomeres by adding a guanine repetitive sequence thus preserving genome stability [69]. The enzyme was first discovered and named telomerase after the Russian scientist A. M. Olovnikov in the 1980s, but its existence in preventing telomere shortening was proposed since the 1970s. The telomerase enzyme consists of two main components: telomerase RNA (TER) and the telomerase reverse transcriptase (TERT). Both form the core of the enzyme by providing its functional activity *in vitro.* The cycle of the telomerase reactions is composed of different stages *in vitro* that include primer binding, elongation, translocation, and dissociation [69, 70].

a. Telomerase RNA (TER)

The structure of the telomerase RNA (TER) has two functions. The first is telomere synthesis since it contains the template region that affects its formation. The second is enzymatic activity since it consists of elements that maintain the assembly, localization, and stability of the RNA. However, it is hypothesized that TER alone without TERT cannot form the right structure [70].

b. Telomerase Reverse Transcriptase (TERT)

The structure of the telomerase reverse transcriptase (TERT) is more conserved than TER. TERT contains enzyme catalytic domains and telomerase-specific domains. The enzyme catalytic domain consists of a large number of motifs resembling other reverse transcriptases. For instance, the telomerase-specific domain is important for TER, the substrate for DNA binding and the functional activity of the telomerase [69, 70].

c. Telomerase Activity and Expression in Breast Cancer

Telomerase activity differs with different cell types. For example, telomerase activity of stem cells is lower than embryonic cells allowing only partial compensation for telomere shortening. Unlike stem cells, embryonic cells activate telomerase at a greater unlimited potential, while somatic cells lack any telomerase activity resulting in early senescence due to telomere length reduction [70]. The dysregulation in telomere elongation contributes to cell immortality that characterizes cancer cells. For example, the telomerase activity is upregulated in several cancer types such as breast cancer where telomerase is expressed in 90% of these cancerous cells unlike most normal cells [71, 72]. Therefore, the enzyme, telomerase, was identified as a **diagnostic marker** for cancer in several studies since telomerase activity was detected in most cancer cases [73, 74]. On the other side, telomerase expression differs based on the stage of cancer. During early carcinogenesis, the expression is low like normal cells that lack any expression, while in tumor invasion it increases to prevent the senescence or apoptosis of cancer cells [71, 75]. Since telomerase expression increases with the invasiveness of the cancer, then telomerase was considered a **prognostic marker** in determining the disease stage of breast cancer [72]. Additionally, low expression of telomerase in

normal cells unlike cancer cells suggests treating cancer with telomerase inhibitors [76]. These inhibitors serve as a viable target for the treatment of telomerase-positive cancer cells, but the challenge remains in sparing the normal telomerase-carrying cells [77].

Collado [78], in her study on telomerase in three breast cell lines, identified the relation between telomerase activity and expression. MCF-10A (a normal-like immortalized breast cell line), MCF-7 (a noninvasive breast cancer cell line) and HTB26 (an invasive breast cancer cell line) were assessed for their telomerase activity and were found to express telomerase reverse transcriptase *h*TERT, but the levels were much higher in the cancerous breast cell lines compared to the normal immortalized cell line. These findings asserted the role of telomerase activity in the diagnosis of cancer, while telomerase expression in the prognosis of cancer.

d. Telomerase and its Regulation in Cancer Cells

Two factors play a role in the regulation of telomerase activity: cell cycle and *h*TERT expression. Telomerase activity appears *in-vitro* in the G1-phase, but it works by adding telomere repeats during the S-phase by acting on the shortest telomere [79]. Both immortal and cancer cells that are characterized by an infinite number of cell divisions are regulated by this mechanism to compensate for shortening of chromosomes. However, cells that exit the cell cycle (G0 phase) and enter a state of quiescence or senescence where there is no cell division have a down-regulated telomerase activity **(Figure 5)** [80, 81].

Additionally, cellular proliferation is another barrier for telomerase regulation and maintenance of telomere length. As telomeres shorten and reach the Hayflick limit, telomerase enzyme is unable to escape the two barriers of proliferation; M1, the

permanent growth arrest also called replicative senescence and M2, the crisis or mortality [82]. To escape the proliferation barriers especially in cancer cells, the expression of *h*TERT, a rate-limiting determinant of telomerase activity, is required for long-term proliferation supporting the role of telomerase in cell immortalization and oncogenesis **(Figure 6)**. Therefore, an increased expression of TERT in breast epithelial cells suggests mammary carcinogenesis, while in breast cancer cells it confirms cell immortalization due to bypassing the crisis stage [83]. This concludes that telomerase is active in cells undergoing division and regulated (up and down) by the expression of *h*TERT, its catalytic subunit.

Figure 5: A model of telomerase activity being down-regulated in cells that exit the cell cycle [80]

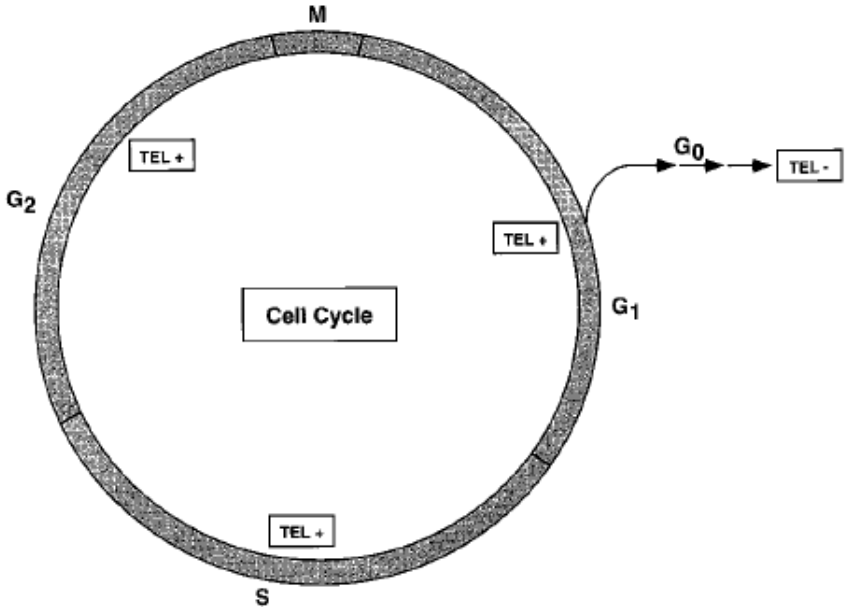
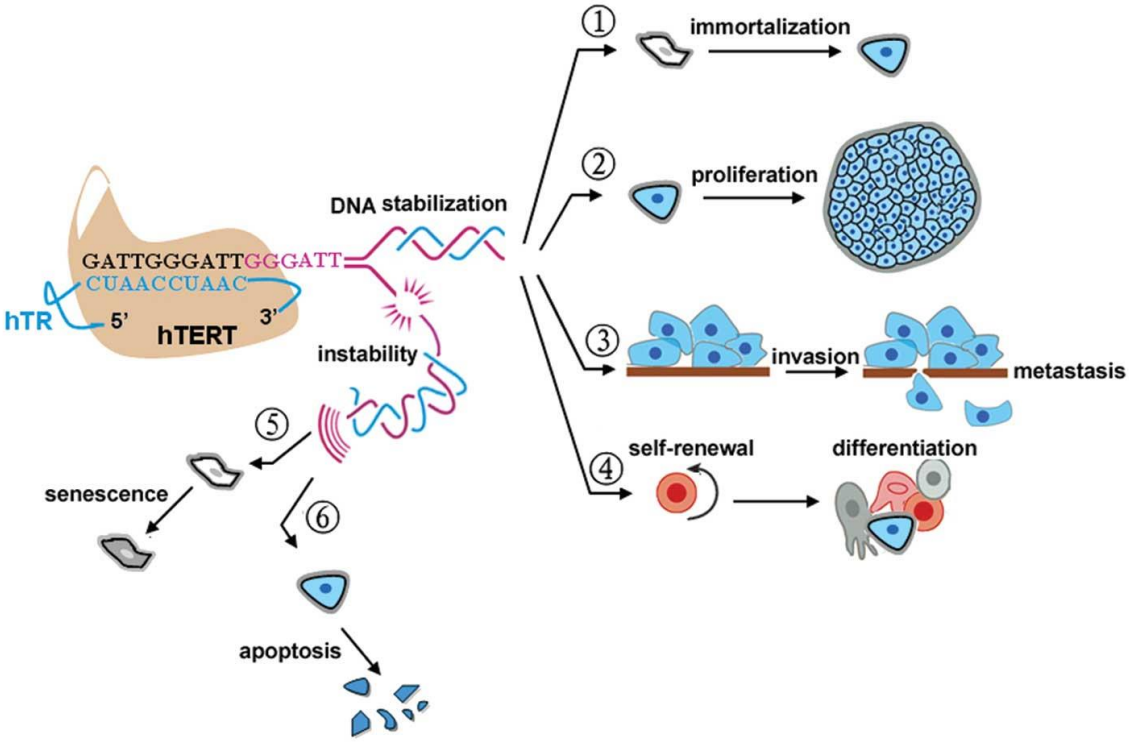


Figure 6: The resulting end-points of overexpression (1-4) and repression (5-6) of *h*TERT gene [84]



2. Relative Telomere Length (RTL)

a. Telomere Definition

Telomeres are ribonucleoprotein DNA structures found at the end of human chromosomes, made up of 6 base pairs of repeated TTAGGG sequences that usually maintain genomic integrity by saving the chromosomal ends from shortening during replication [85, 86]. Telomeres shorten with every cellular division, and they may reach a critical shortening phase whereby cells will stop to proliferate resulting in their senescence or even death. Each cellular division will cause the loss of 50 to 100 base pairs of telomeric DNA [87]. Telomeres help the replication machinery by protecting its chromosomal ends from being recognized as DNA double strand breaks thus preventing

fusion in chromosomes. Therefore, measuring the length of the human's telomere is a marker for real biological age and cellular senescence [85, 86].

b. Factors Influencing Relative Telomere Length (RTL)

Telomere length shortens with age but is also influenced by several lifestyle, behavioral, dietary, and environmental factors. Some of the factors are disease promoting while others are disease protective **(Figure 7)**. In a cross-sectional study of 497 Lebanese subjects, short telomere length was associated with aging, central obesity, poor sleep, and hypertension [88]. In another study, cigarette smoking and vigorous physical activity had different impacts on telomere length highlighting the importance of lifestyle in determining telomere dynamics. Of interest, smoking was reported to be associated with shorter RTL, while physical activity with longer RTL [87]. Shammas [89] indicated that following an appropriate diet rich in high fibers, antioxidants and low protein can reduce the shortening rate of telomeres, but being exposed to environmental pollution can increase that rate.

c. RTL and Tobacco Smoking

Smoking is identified as a factor influencing telomere length. Most studies indicated shortening of telomere length with cigarette smoking. It has been postulated that this could be due to the generation of free radicals, induction of oxidative stress and inflammation leading to cellular senescence and possibly apoptosis [87, 90]. In a systematic review of 84 studies, smokers were divided into ever and never smokers into one category and current and former smokers into another category. Significantly shorter telomere length was identified among ever smokers and current smokers

compared to those who never smoked and former smokers respectively [85]. McGrath *et al.* [90] identified a correlation between shorter telomere length and bladder cancer risk with cigarette smokers. Others identified an enhanced effect of telomere loss with tobacco smoking in circulating lymphocytes in a dose-response relationship whereby smoking was associated with low-grade systemic inflammation contributing to high turnover in lymphocytes. According to Morla *et al.* [86], this increased turnover of lymphocytes shifted from naive to memory T-cells that are known for their shorter telomeres. In the Valdes *et al.* [91] study, white blood cells of women showed an average loss rate of 25.7 to 27.7 base pairs of telomeric DNA with daily smoking. Moreover, an additional 5 base pairs were lost with each pack-year smoked equivalent to 18% of the mean annual loss in telomere length from ageing. Also, smoking one pack of cigarettes per day for a duration of 40 years corresponded to 7.4 years of age-related shortening of telomere length. To the best of our knowledge, previous studies only investigated cigarette smoking in relation to RTL, and no epidemiological study identified the association between waterpipe and RTL.

d. RTL and Telomerase in Breast Cancer

Most of the studies identified a positive association of longer telomeres with breast cancer. In a systematic review for breast cancer patients in the last 20 years, telomere length in peripheral blood of 13 studies and telomere length in breast tumor tissue of 20 studies were analyzed [92]. The results showed longer telomeres with breast cancer patients which was an indicative of better prognosis especially if they were more physically active according to Ennour-Idrissi *et al.* [93]. However, there was one study that showed shorter telomeres from peripheral blood that was associated with needing

chemotherapy [94]. Other studies analyzing breast tumor tissues showed short telomeres either in high grade tumors or in normal breast tissues that were associated with tumor recurrence [95, 96]. Since there are several confounding factors in these studies (physical activity, chemotherapy, tumor stage etc.), further longitudinal studies with accurate telomere length measurement and proper control of variables must be conducted to determine the correlation of the telomere length with breast cancer.

Of note that few studies identified the association between telomerase expression, activity, and telomere length in breast cancer. A study by Thriveni *et al.* [97] and as noted above, patients who had advanced stage, high grade and lymph-node positive breast cancer had higher telomerase expression than patients with early stage, low grade and lymph-node negative breast cancer. In this study, 39% of the patients who had high *h*TERT expression had elongated telomeres while 23% of those patients with low expression had shorter telomere length suggesting the effective role of telomerase enzyme in maintaining telomere stability in cancer cells. However, in the same study also, 25% of the patients showed less telomere length with higher *h*TERT expression, and 13% showed elongated telomeres with a lower expression. This lengthening in telomeres despite the lower *h*TERT expression was attributed to an alternative lengthening mechanism (ALT) that is independent from telomerase, but dependent on homologous recombination. Although maintenance or lengthening of telomeres occurs mainly through telomerase enzyme, it is estimated that 10-15% of cancer cases can achieve lengthening through the alternate mechanism [98].

3. Smoking and Telomerase and RTL

In addition to cancer, smoking is a factor that shortens telomere length [85]. Likewise, smoking affects telomerase activity whereby patients with smoking history have increased levels of telomerase activity. Thus, a strong correlation is found between telomerase activity and ever-smokers compared to never-smokers. For instance, in a study by Yim *et al.* [99], exposure to tobacco from smoke carcinogens increased telomerase activity in normal bronchial epithelium. Similarly, telomerase activity could be increased in breast cancer cell lines in the presence of cigarette and waterpipe smoke extracts. Like relative telomere length, the number of pack-years of smoking affects the activity of telomerase. This concludes the fact that being exposed to smoke increases telomerase activity to compensate for shortened telomere length (**Figure 8**).

Figure 7: Disease promoting and disease protective factors affecting telomere length [100]

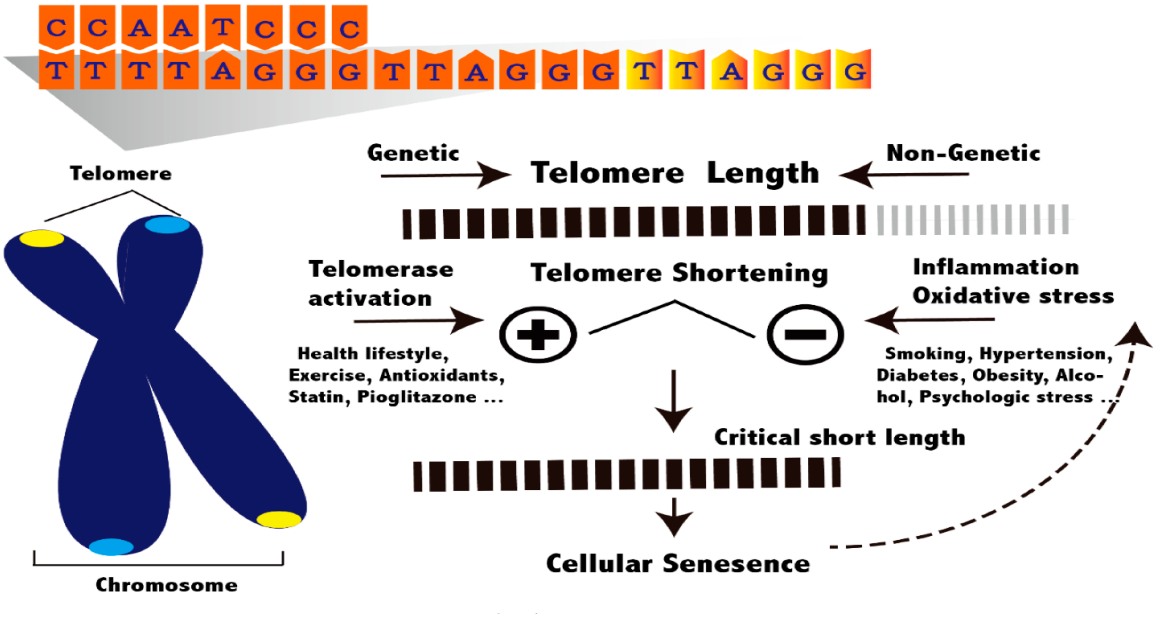
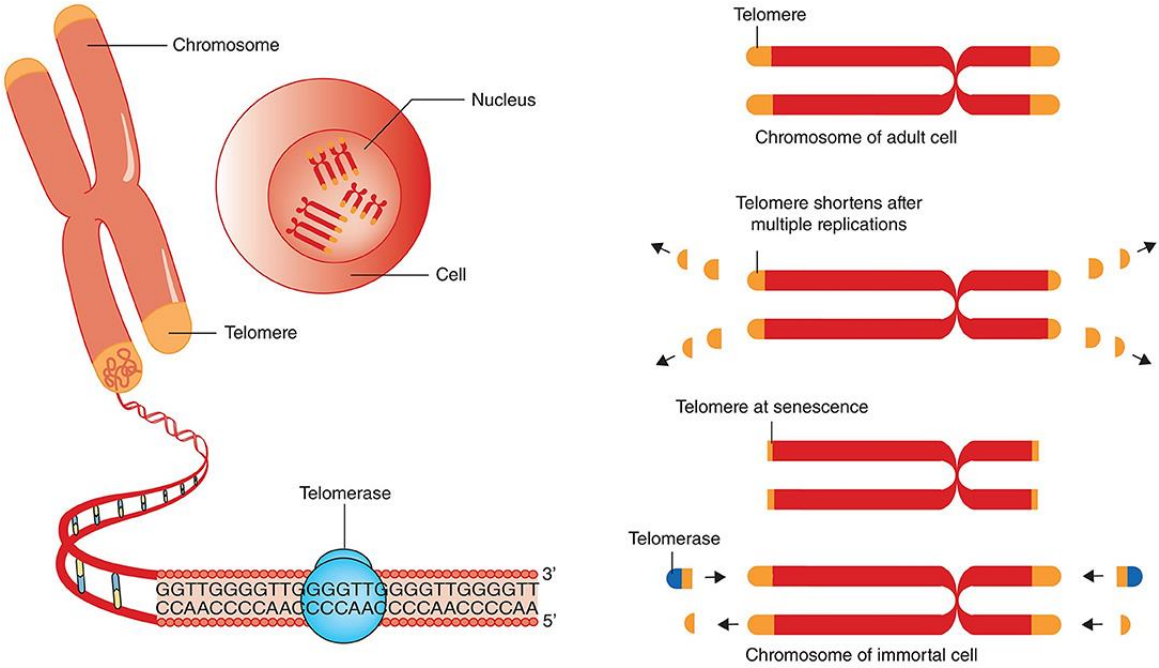


Figure 8: The telomerase enzyme extends telomere ends in response to telomere shortening [101, 102]



CHAPTER II

AIMS

Smoking is the number one leading cause of cancer in the world, and breast cancer is the most common and fatal cancer type among females globally including Lebanon. As a matter of fact, cigarette and waterpipe smoking have been linked to the development and metastasis of cancer in several *in-vitro* and *in-vivo* studies [6, 56, 59-63, 65, 68]. Nevertheless, there is limited knowledge regarding waterpipe exposure risk [59] compared to cigarette in mammary epithelial cells unlike alveolar and bronchial cells [43, 56, 60, 61, 103]. Besides, no studies compared the effect of smoking exposure on relative telomere length (RTL) and telomerase activity and expression in breast cells. As such, there are only studies on peripheral blood of cigarette smokers showing shortening of telomere length [86, 90]. Hence, the study aim in this thesis is the following:

Aim: To evaluate the effect of short-term acute subtoxic concentrations of waterpipe and cigarette smoke extract on RTL, telomerase activity and expression in two breast cancer cells: MCF-7 (ER-positive) and MDAMB-231 (ER-negative).

Of note, we initially planned to compare the effect of smoking with chronic subtoxic exposure of 2-3 months duration. Nevertheless, and due to the challenges faced from recurrent lockdowns and COVID-19 sicknesses, we started with acute subtoxic exposure. In the future, we plan to perform chronic subtoxic exposure on breast cancer cells and other cell lines such as alveolar, bladder and colon cancer cells to measure any changes in telomere length and telomerase expression and activity. Further work is currently ongoing in the lab to include cell cycle, genotoxicity, and apoptosis

experiments at these acute subtoxic concentrations. We also plan to perform invasion and migration assays on these cells *in-vitro.*

CHAPTER III

MATERIALS AND METHODS

A. Breast Cancer Cell Lines

MCF-7 and MDAMB-231 are breast cancer cell lines originating from human mammary gland breast epithelial adenocarcinomas isolated from pleural effusions. These cells are adherent in their growth properties as they attach to the flasks when sub-cultured [104, 105]. MCF-7 cells are smaller in morphological shape compared to MDAMB-231 cells.

1. MCF-7 Cell Line

MCF-7 is an ER- and PR-positive breast cancer cell line. It is hormone dependent and both estrogen and progesterone receptor positive belonging to the luminal A breast cancer type. MCF-7 cells are metabolically active and rely on ATP production from oxidative phosphorylation under normoxic conditions and increase glycolysis under hypoxic conditions [106]. MCF-7 cells are characterized with a low metastatic potential [107].

2. MDAMB-231 Cell Line

On the contrary, MDAMB-231 is an ER-, PR- and HER2-negative breast cancer cell line. It is hormone independent and triple receptor negative classified as basal-like breast cancer type. MDAMB-231 cells rely on glycolysis for ATP production under both normoxic and hypoxic conditions. They are insensitive to antiestrogen treatments

and have multidrug resistance properties [106]. MDAMB-231 cells are characterized by their invasive phenotype and metastatic potential [108].

B. Cell Culture and Media

MCF-7 and MDAMB-231 cell lines were obtained from the American Type Culture Collection (ATCC). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% sodium pyruvate for a complete medium at a temperature of 37 degrees Celsius in a humidified atmosphere with 5% CO2. Prior to each assay, counting for the number of cells was done after detaching them. MCF7 cells were detached using 2.5% Trypsin (10x) and 0.53 mM Ethylenediaminetetraacetic acid (EDTA) solution, while MDAMB-231 cells were detached using 1% Trypsin (1x) only.

C. Beirut Method Smoking Machine Protocol

This study is based on a standard smoking machine that is designed for high-flow smoking devices like waterpipe that can imitate the puff topography of smokers in their natural settings by replicating the smoker's puffing behavior. The topography measurements can be "played back" on the smoking machine and considered similar to a real smoker's natural smoking behavior [109]. It was based on a study done in a Beirut café whereby smoking topography measurements were recorded for 52 narghile smokers assessing their smoking sessions in terms of puff volume, duration and frequency [110]. This study showed an average smoker puff volume of 0.53 liters, a mean puff duration of 2.47 sec, a mean interpuff interval of 16.28 sec and a duration stay of 1 hour. The yielded puff topography data were validated by a real-time in situ

sampling technique (RINS) with a portable device that is automatically designed to deliver a fixed percentage of the smoke flow [111].

D. Generation Method of Cigarette and Waterpipe Particulate Matter

The generation method of cigarette and waterpipe particulate matter is shown in **Figures 9 and 10**. For waterpipe smoke extract (WSE), the primary components of waterpipe are head, body, water bowl, and hose. The waterpipe head was prepared by weighing 10 g of flavored tobacco (Nakhle brand, Double Apple) covering it with perforated aluminum foil according to the predefined 18-hole pattern [64]. Waterpipe smoke was generated using a digital controlled automatic smoking machine operating under the validated Beirut smoking protocol following a standard smoking regimen: 171 puffs, 530 ml volume, 17 s interpuff interval, and 2.6 s puff duration [110]. For each smoking session, the water bowl was filled with fresh 850ml of water, and the infiltration test for the hose was performed to ensure the allowable porous wall infiltration at waterpipe mouthpiece flow rate of 12.2 liters per minute (LPM) as determined by the method described in Saleh and Shihadeh [112]. Of note, the number of puffs was reduced to 105 puffs instead of 171 puffs. This reduction was based on the observation that one piece of charcoal (Three Kings, Holland) will serve for 105 puffs and for the remaining puffs, another ½ piece of charcoal is needed to reach 171 puffs [113]. For CSE, certified 3R4F research cigarettes were purchased from the University of Kentucky. Cigarette smoke was generated using a digital controlled smoking machine operating under the International Standardization Organization (ISO) smoking regimen: a puff volume of 35 ml over a puff duration of 2 sec with an interpuff duration of 60 sec without vent blocking [114]. The smoke generated from WSE and CSE was

collected on quartz fiber filter, and the total particle matter collected on the filters were weighed gravimetrically and stored in airtight containers at -20°C until extraction.

Figure 9: Waterpipe Setup, designed in Dr. Alan Shihadeh Laboratory, connected to four branches of which four glass filters are attached to a programmed smoking machine linked to a software controlling the puffing parameters

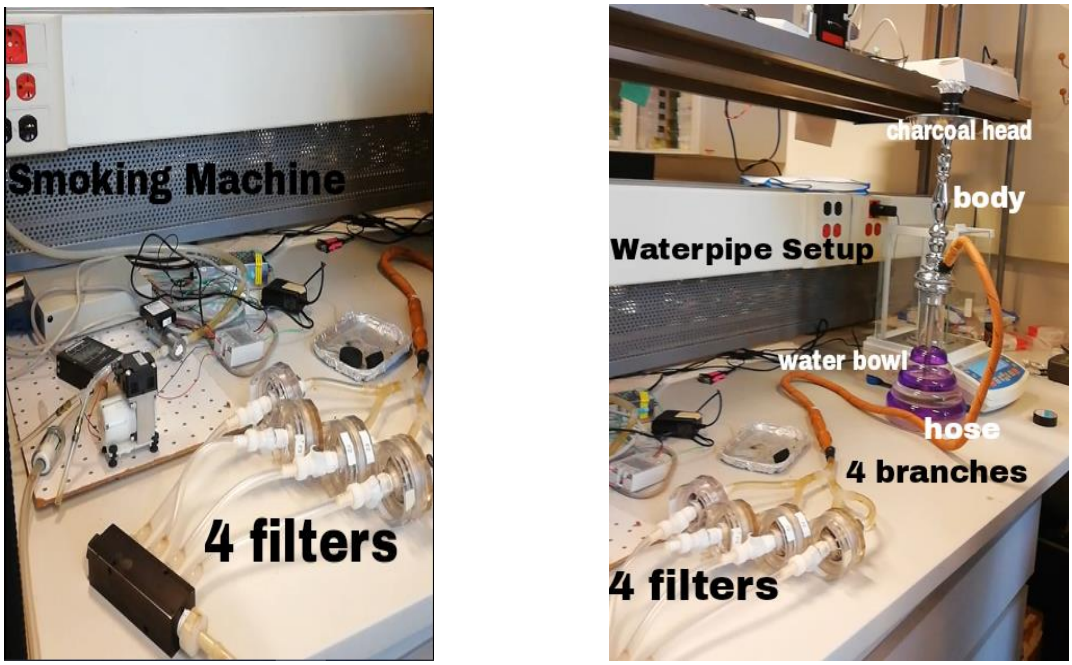
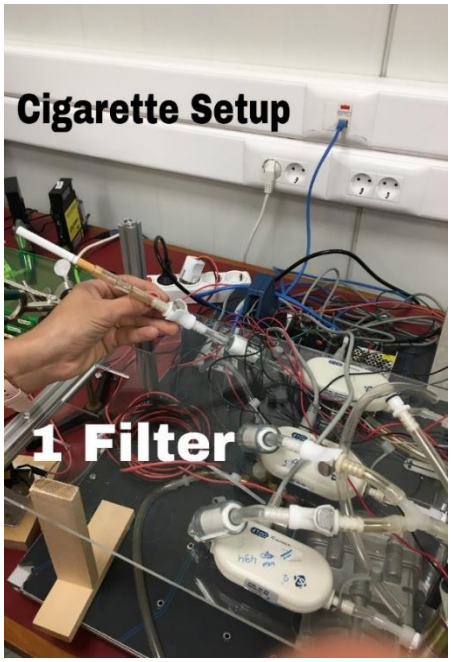
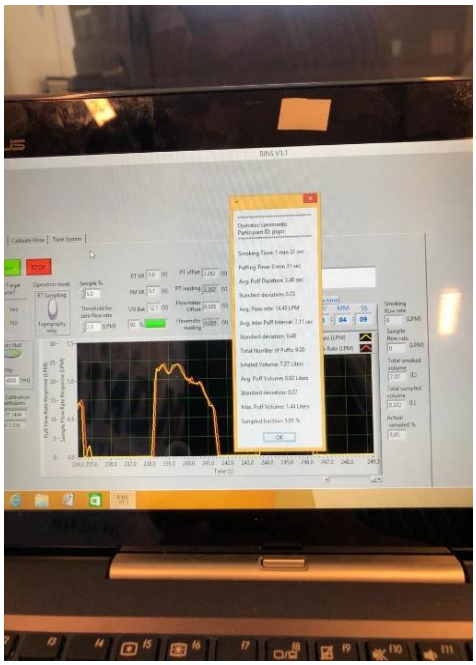


Figure 10: The setup of one single cigarette, designed in Dr. Alan Shihadeh Laboratory, connected to one filter attached to a programmed smoking machine linked to a software controlling the puffing parameters





E. Measuring and Weighing of Total Particulate Matter (TPM)

In one session of waterpipe, there are around 1200-2000 mg of particulate matter that are trapped by the filters. The maximum capacity of one filter is around 150 mg, so replacement of filters with clean filters is required throughout the session and 8-16 filters are needed for one session of 105 puffs. After the session, the filters are compiled into one extract so that one filter out of four would represent 25% of the session. The chosen number of filters is done in such a way that the compiled filters represent 100% of the session in every trial**.** Unlike waterpipe session, every cigarette represents one session on one filter, and it produces approximately 8-10 mg of particulate matter. To be noted that the filters are weighed before and after the session to know the amount of total particulate matter such that the delta weight of filters is equal to the weight of total particulate matter.

F. Cigarette and Waterpipe Smoke Extract Preparation

Cell culture incomplete media DMEM (Dulbecco's Modified Eagle Medium) without FBS (Fetal bovine serum) were added to each filter to yield a WSE stock concentration of 40 mg/ml and a CSE stock concentration of 2 mg/ml. The filter was then pressed in a syringe to ensure recovery of media added. All recovered media were then mixed together, sterilized using 0.22 µm filters and stored at -20°C until the day of treatment [6, 62]. On the exposure day, different concentrations were prepared from each stock extract, and a specific volume of each was diluted with media to get the appropriate concentration (mg of particulate matter/ml of extraction volume).

G. Choice of Concentrations

MCF-7 and MDAMB-231 cells were treated with cigarette and waterpipe extracts ranging from low concentrations (0.5 mg/ml for cigarettes and 1 mg/ml for waterpipe) to high concentrations (2 mg/ml for cigarettes and 30 mg/ml for waterpipe). For selection of the concentrations, cells were tested using the MTT assay for 3 consecutive days out of which the inhibitory concentrations (IC20 and IC50) were determined representing 80% and 50% of those cells that were still metabolically active thus alive. The resulting inhibitory concentrations (IC20 and IC50) of the 24-hour exposure to cigarette and waterpipe extracts were used for all the following experiments.

H. Cell Metabolic Activity Using MTT Assay

MTT assay was performed at 24, 48 and 72 hours for each treatment concentration initially including all cigarette and waterpipe concentrations. Briefly, MCF-7 and MDAMB-231 cells were seeded in a 96- well plate at a seeding density of 8000 and 4000 cells, respectively. After overnight incubation, cells were treated in triplicates with different doses of cigarette (0.5, 0.7, 1, 1.5, 2 mg/ml) and waterpipe (1, 3, 5, 7, 10, 12, 15, 20, 30 mg/ml) for 24, 48 and 72 hours. Control cells and blank wells in triplicates were treated with complete media only. After 24-hour exposure, cells were incubated with 25 µL of MTT reagent for 4 hours after which a 100 µL solubilizing agent was applied, and cells incubated overnight. On the next day, absorbance was detected using the ELISA plate reader at a wavelength of 595 nm. After subtracting the absorbance of the wells without cells (negative control), the obtained results were

calculated as percentage of metabolic activity relative to control for three independent trials.

I. Cell Viability Using Trypan Blue Assay

MCF-7 and MDAMB-231 cells were seeded in a 6-well plate at a seeding density of 300,000 and 150,000 cells, respectively. After overnight incubation, cells were treated with the IC20 (6.5 mg/ml for waterpipe, 0.5 mg/ml for cigarette of MCF-7 and 8.5 mg/ml for waterpipe and 0.7 mg/ml for cigarette of MDAMB-231) and the IC50 (9.5 mg/ml for waterpipe and 0.7 mg/ml for cigarette of MCF-7 and 13 mg/ml for waterpipe and 1 mg/ml for cigarette of MDAMB-231) for 24 and 48 hours. Control cells were treated with complete media composed of DMEM with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% FBS. After 24 and 48-hour treatment respectively, the medium on top of the wells was preserved, cells were detached with the corresponding Trypsin and added to the preserved medium, centrifuged, washed with PBS then alive and dead cells were counted by a hemocytometer using trypan blue dye. Results are presented in terms of percentage of viable cell count relative to control for three independent trials.

J. Cell Morphology Using Leica Microscope

Cell morphologic changes were identified under the Leica microscope at different magnifications 10x, 20x and 40x for MCF-7 and MDAMB-231 breast cancer cell lines. The images were taken after 24- and 48-hours post-exposure to waterpipe and cigarette sub-toxic concentrations and are compared to images of unexposed control cells.

K. Molecular Assays

Molecular effects of smoke extracts on both cell lines at the IC20 and IC50 of the 24-hour exposure were assessed for further interpretation.

MCF-7 and MDAMB-231 cells were seeded in 6-well plates at a seeding density of 3 x 10^5 and 1.5 x 10^5 respectively. After overnight incubation, MCF-7 cells were treated with WPE (6.5, 9.5 mg/ml) and CSE (0.5, 0.7 mg/ml) and MDAMB-231 cells were treated with WPE (8.5, 13 mg/ml) and CSE (0.7, 1 mg/ml) for 24 and 48 hours. Control cells were treated with complete media composed of DMEM with 1% penicillin/streptomycin, 1% sodium pyruvate and 10% FBS. After 24 and 48-hour treatment respectively, the medium on top of the wells was discarded, cells were detached with the corresponding Trypsin, centrifuged, washed with PBS then pellets were snap frozen in Liquid Nitrogen immediately and stored at -80°C until further assays.

1. RNA Isolation and Quantification

RNA was isolated using Trizol-based protocol (Sigma-Aldrich, Germany). Isolated RNA samples were treated with DNase using the DNase treatment and removal kit (Invitrogen, USA) as per manufacturer's protocol. The RNA concentrations were measured by Nanodrop spectrophotometer using Denovix DS-11 with both 260/230 and 260/280 ratios detected for assessment of the purity of samples. RNA samples for all trials were then run on an agarose gel to view RNA bands, and 25 µl of each sample was immediately reverse transcribed to cDNA using the high-capacity reverse transcription kit (Applied Biosystems, USA) as per manufacturer's protocol. No

amplification control (NAC) was run with the samples. The resulting cDNA samples by BIO-RAD (T100, Thermal Cycler) were stored at -20°C until further assays.

2. Telomerase Expression Using RT-PCR

RNA expression of *h*TERT was measured using RT-PCR. In brief, PCR was performed in a 384-well plate using cDNA template equivalent to 25ng RNA, 1 x SYBR Green master mix (Bio-Line sensifast, USA) and primers shown in **Table 4** (each at a final concentration of 200 nM). No template control (NTC) was run with the samples. PCR thermal cycling conditions were two cycling steps: 95°C for 2 min, and 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Each sample was measured in triplicate, and RNA expression of *h*TERT was calculated relative to GAPDH reference gene using $2^{-\Delta\Delta ct}$ method. To compare the efficiencies of the PCR amplification reactions of *h*TERT and GAPDH, a standard curve was generated using serial dilutions (0.2, 1, 5, 25, 125 ng) of an RNA sample, and delta threshold cycle (ct) (ct telomerase gene – ct GAPDH reference gene) was calculated and plotted *versus* log (RNA input amount). The standard curve was ideal with an r^2 reaching 1 for the tested two genes.

3. Protein Isolation and Quantification

Proteins were isolated using 3[(cholamidopropyl)-dimethyl-ammonium]-1-propanesulfonate (0.5% CHAPS) lysis buffer (Abcam, USA), 10 mM Tris-HCl (pH 7.5) (Bio-Rad, USA), 1 mM MgCl2 (Sigma-Aldrich, Germany), 1mM EGTA (Sigma-Aldrich, Germany), 5 mM β-mercaptoethanol (Bio-Rad, USA), 0.1 mM [4(2-aminoethyl)-benzenesulfonyl fluoride] hydrochloride (Sigma-Aldrich, Germany) and 10% glycerol (Sigma-Aldrich, Germany). For telomerase activity assay only, an RNase

inhibitor (Qiagen, Germany) was freshly added to the buffer prior to the assay. Proteins were incubated with the lysis buffer for 30 min on ice and vigorous vortexing was performed every 10 min interval. The lysate was then centrifuged at 14000 rpm for 30 min at 4°C, and the supernatant was collected. Protein quantification was performed using Lowry quantification method. In brief, a serial dilution of bovine serum albumin (BSA) (Amresco, Ireland) was prepared with concentrations ranging between 0.3 µg/ml and 1.5 µg/ml. Then, samples and standards were treated with Lowry reagents (Bio-Rad, USA), shaked for 1 min and incubated for 15 min. The absorbance was read at 750 nm with an ELISA plate reader. A standard curve was drawn using the absorbance and concentrations of BSA, and sample protein concentrations were calculated from the standard curve.

4. Measurement of Telomerase Activity

Telomerase activity was measured using Telomeric Repeat Amplification Protocol (TRAP) assay. Fresh protein isolates (0.25 µg) were added to a reaction mixture containing 1x SYBR Green buffer (Bio-Line sensifast, USA) and 10 µM of forward TS and reverse ACX primers (**Table 4**). The reaction mixture was first incubated at 37°C for 30 min during which telomerase in the protein samples was allowed to elongate the TS primer by inserting TTAGGG repeat sequences. Then, the PCR was initiated at 95°C for 10 min, which inactivates the telomerase enzyme. This was followed by 40 cycles at 95°C for 10 sec and 60°C for 60 sec. Telomerase activity was calculated based on the ct value at which SYBR green, by binding to the double stranded telomerase product, emits the threshold fluorescence. All samples were run in triplicate. Heat inactivated samples or negative controls were prepared from protein

samples incubated at 95°C for 10 min prior to activity measurement and included in the assay. Serial dilutions of protein extracts (1/625, 1/125, 1/25, 1/5, 1 µg) extracted from fresh HELA cells (ATCC, USA) that are known to have high telomerase activity were run with each plate to generate a standard curve. Telomerase activity of samples was calculated from this standard curve and reported as ratio of activity relative to HELA.

5. DNA Isolation and Quantification

DNA was isolated using Flexigene DNA isolation kit (Qiagen, Germany) as per manufacturer's protocol. The DNA concentrations were measured by Nanodrop spectrophotometer using Denovix DS-11 with both 260/230 and 260/280 ratios detected for assessment of the purity of samples. Some DNA samples were selected randomly in the first phase of isolation and run on an agarose gel to view the quality of the DNA bands. The non-converted DNA samples were stored at -20°C until further assays.

6. RTL Measurement

RTL was measured on the isolated DNA using quantitative PCR (qPCR) as previously described by Cawthon *et al.* [115]. Telomere and single copy gene (SCG) PCR were performed in separate 384-well plates using the telomere and human β-globin (SCG) primer pairs shown in **Table 4**. In both PCR experiments, telomere and SCG of a 3 µl DNA sample (10ng/µl) were amplified using 7 µl of the corresponding primer pairs and SYBR green master mix (Bio-Rad, USA) for a total of 10 µl/well. The thermal cycling protocol for telomere was: 50°C for 2 min, 95°C for 2 min, then two cycles of 95°C for 15 sec, 49°C for 15 sec, then 35 cycles of 95°C for 15 sec, 62°C for 10 sec, and 74°C for 15 sec. For SCG: 50°C for 2 min, 95°C for 2 min, then 35 cycles of 95°C

for 15 sec and 58°C for 1 min. A melt curve was generated to detect any primer dimer formation. In addition to the samples, standards (S1, S2, S3, S4, S5, S6) of concentrations ranging from 0.016 ng/µl to 50 ng/µl with a serial dilution of 1/5 were prepared from a pool of DNA samples and run in every plate along with a no template control (NTC). Standard curves for each of telomere and SCG ct with log (standard concentration) were ideal with an r^2 >0.9. Samples and standards were run in triplicates.

L. Statistical Analysis

For each experimental assay, results were calculated relative to control and shown as mean + standard error of mean (SEM) of at least three independent trials and displayed graphically using GraphPad Prism version 8.3.1. Comparisons between the treatments of categorical independent variables with at least one continuous dependent variable were performed using one way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD), as applicable. Differences of p value less than 0.05 were considered statistically significant.

Table 4: Primers used in the different molecular assays.

Primers	Reference
Relative Telomere Length	
Telomere	Lu *et al*. (2011)[71]
Forward primer 5’ ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT 3’	
Reverse primer 5’ TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA 3’	
Human β-globin	Cawthon *et al*. (2002)[115]
Forward primer 5’ GCTTCTGACACAACTGTGTTCACTAGC 3’	
Reverse primer 5’ CACCAACTTCATCCACGTTCACC 3’	
Gene Expression	
Telomerase	Zhu *et al.* (2006)[116] Lu *et al*. (2011)[71]
Forward primer 5’ CGTCGAGCTGCTCAGGTCTT 3’	
Reverse primer 5’ AGTGCTGTCTGATTCCAATGCTT 3’	
GAPDH	Lu *et al*. (2006) [117]
Forward primer 5’ GAAGGTGAAGGTCGGAGTC 3’	
Reverse primer 5’ GAAGATGGTGATGGGATTTC 3’	
Telomerase Activity	Hou *et al.* (2001)[118] Yaku *et al.* (2017)[119]
Forward TS 5’ AATCCGTCGAGCAGAGTT 3’	
Reverse ACX 5’ GCGCGG(CTT ACC)$_3$CTAACC 3’	

CHAPTER IV

RESULTS

A. PART I. CELL CULTURE EXPOSURE

1. Consistency of Representative Smoking Sessions of Waterpipe and Cigarette

Table 5a shows that different sessions of waterpipe result in an approximate average of 141 mg TPM per one filter for filters between F1 and F12 and an approximate average of 65 mg for filters between F13 and F16. Besides, the average TPM calculated per session of 16 filters is around 1965 mg which is consistent with previously published data using the Beirut protocol [120]. Table 5b identifies different cigarette sessions with an average of 9.4 mg per cigarette per filter following the ISO protocol [114] which is similar to previous published data that resulted in a TPM of ~11 mg per one 2R4F or 3R4F Kentucky reference cigarette [121-123].

Table 5: a. Summary of different sessions of waterpipe, the average TPM (mg) and SD of waterpipe filters and **b**. Summary of different sessions of cigarette, the average TPM (mg) and SD of cigarette filters.

a.

Waterpipe TPMs_mg/session

Filter Number	Session 1	Session 2	Session 3	Session 4		
F1	141.3	129.5	138.7	145.7		
F2	127.1	118.1	117.3	138.3		
F3	134.5	159.4	148.3	143.3		
F4	132.8	153.7	154.5	136.7		
F5	140	130.7	146.8	146.7		
F6	148.9	134.7	147.9	147.6		
F7	142.7	124.6	136.3	137		
F8	149.2	124.8	147.3	145.3		
F9	123.5	146	112.9	149.4		
F10	155	128	126.1	154.3		
F11	163.1	166.1	149.5	154.3	**Average**	**SD**
F12	150.6	166.1	150.3	144.2	141.85625	12.620364
F13	57.2	67.5	88.8	51.3		
F14	57.8	69.8	85.8	53		
F15	56	66.7	85	45.1		
F16	57.6	71.3	86.7	51	65.6625	14.392029
Sum of TPM/ session	1937.3	1957	2022.2	1943.2	1964.925	39.065533

b.

Cigarette TPMs_mg

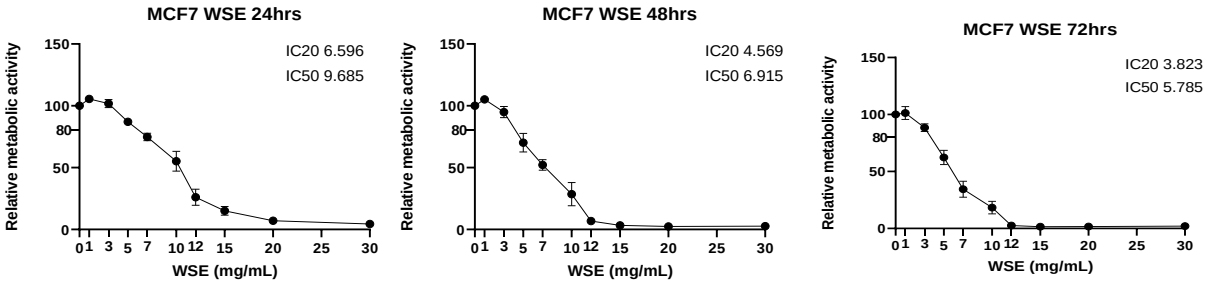
3R4F Ref Cigarette Number	TPM/Cigarette
1	10.2
2	6.2
3	8
4	9
5	9.6
6	10.8
7	9
8	9.5
9	10.7
10	10.5
11	9
12	9.2
13	10.8
14	7
15	11.3
16	8.6
17	9.7
18	11
19	10.2
20	10.1
21	7.8
Average	9.438095238
SD	1.351841777

2. Choice of Concentrations by MTT Assay

For this study, the inhibitory concentrations IC20 and IC50 were based on at least three trials of MTT for 3 consecutive days (24, 48 and 72 hours). Breast cancer cell lines (MCF-7 and MDAMB-231) were treated with all the previously listed concentrations in the **Materials and Methods section** of waterpipe and cigarette, respectively as shown in **Figures 11 and 12**. The graphs analyzed on GraphPad Prism represent the relative metabolic activity of MCF-7 and MDAMB-231 breast cancer cell lines respectively as a function of the exposed treatment. Graphs of both cell lines shift to the left with time after 48 and 72 hours highlighting the increasing cytotoxic effect of waterpipe and cigarette on the metabolic activity of the cells with time. The chosen inhibitory concentrations for the rest of the experiments were the IC20 and IC50 identified initially after 24-hour exposure to WSE and CSE. The concentrations were chosen as subtoxic concentrations based on cytotoxicity estimation in toxicological *in-vitro* studies [124].

Figure 11: a. Set of at least three trials metabolic activity (MTT assay) with MCF-7 exposed to different concentrations of waterpipe smoke extract (WSE) after 24, 48 and 72 hours and **b.** Set of at least three trials metabolic activity (MTT assay) with MCF-7 exposed to different concentrations of cigarette smoke extract (CSE) after 24, 48 and 72 hours.

a.



b.

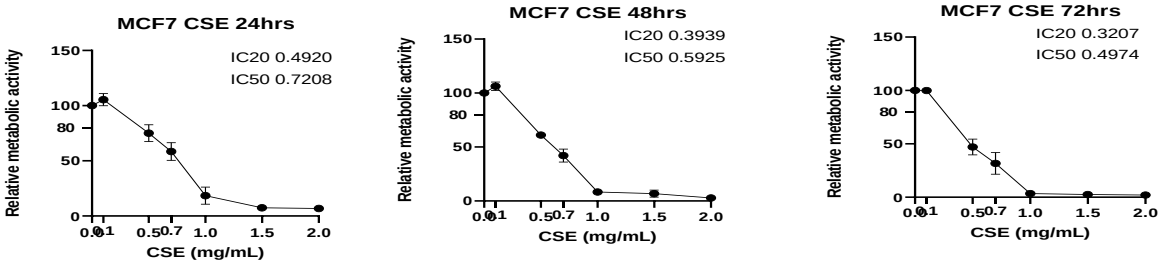
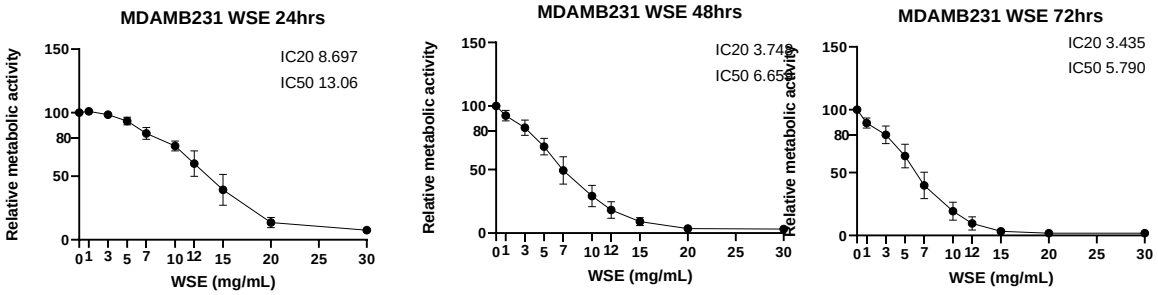
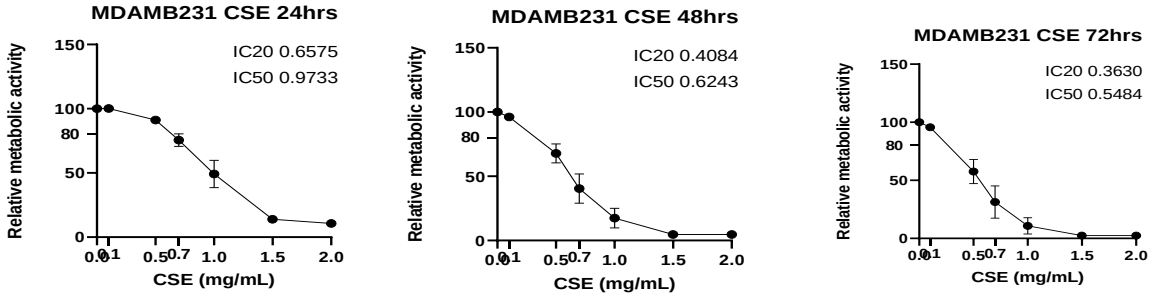


Figure 12: a. Set of at least three trials metabolic activity (MTT assay) with MDAMB-231 exposed to different concentrations of waterpipe smoke extract (WSE) after 24, 48 and 72 hours and **b.** Set of at least three trials metabolic activity (MTT assay) with MDAMB-231 exposed to different concentrations of cigarette smoke extract (CSE) after 24, 48 and 72 hours

a.



b.



Of note, these findings of IC20 and IC50 for waterpipe and cigarette are among the first data to indicate subtoxic concentrations of smoke extracts in breast cancer cell lines. No previous studies indicate the use of IC20 and IC50 clearly as presented here. Some studies used all the cigarette concentrations in their experiments; some relied on results from earlier studies without justification and others used concentrations that most accurately reflect a representation in human serum that does not affect cell viability [60, 61, 103]. There is only one study by Rammah *et al.* [6] on A549 cells using the Beirut smoking protocol indicating the choice of 4 mg/ml out of all the waterpipe concentrations (0.5, 1, 3, 4, 6, 8 mg/ml) based on a cytotoxic assay. This dose was selected by the author since it rapidly inhibited cell growth after 24 h of exposure, and the cells sustained their viability. While in the presence of up to 1 mg/ml of WSE, the A549 proliferative capacity was still maintained and at a dose of 8 mg/ml, the WSE induced a cytotoxic effect. Therefore, the effective inhibitory concentration sustaining cell viability was the only one used by Rammah *et al.* indicating a concentration almost producing 20% inhibition of the endpoint measured.

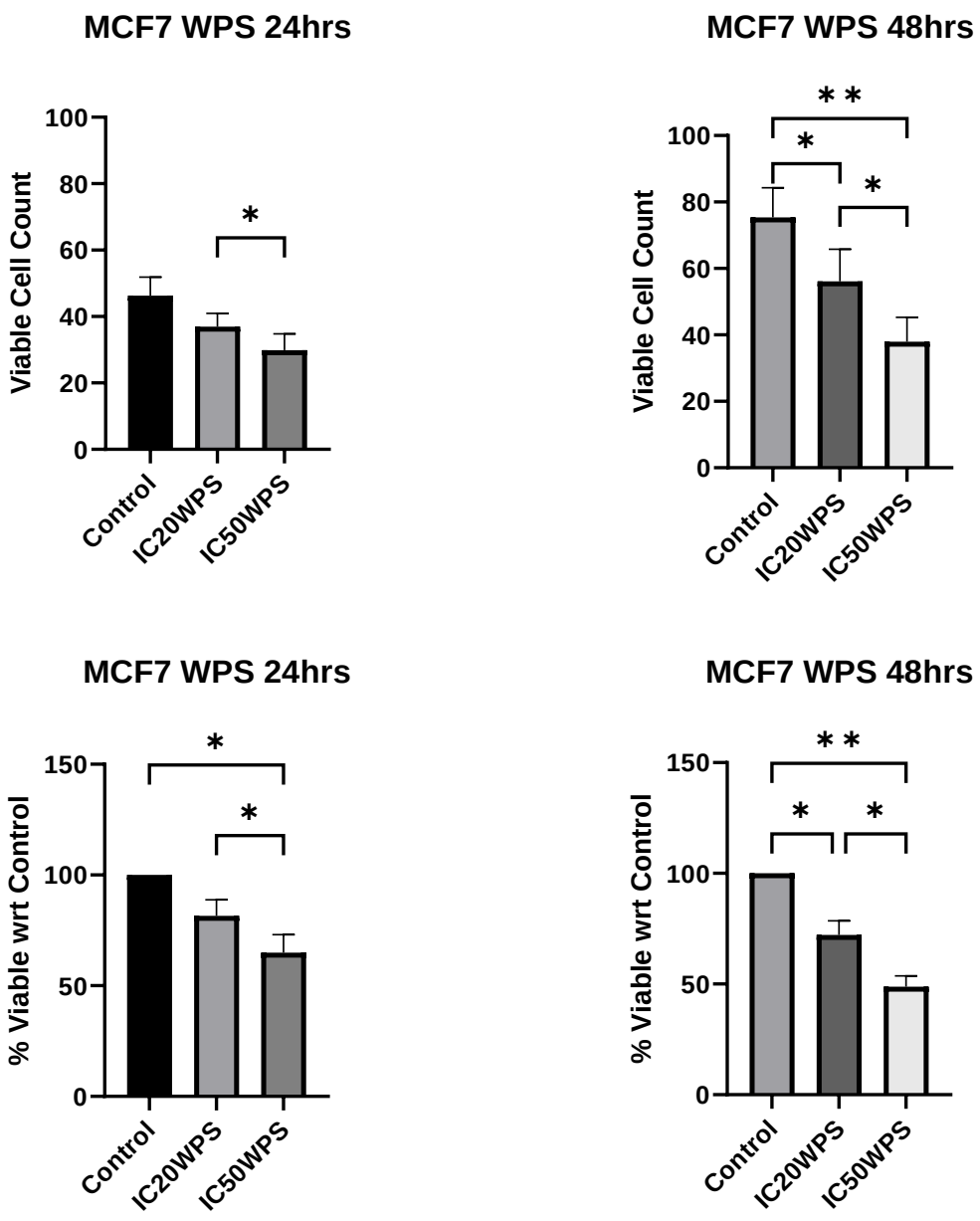
3. Cell Counting by Trypan Blue Assay for MCF-7 Breast Cancer Cells

The data in the bar graphs of **Figure 13** show that the number of MCF-7 cells exposed to subtoxic concentrations of waterpipe and cigarette extracts decreased gradually with respect to the control. Both alive and dead cells were counted after 24 and 48 hours. There is no previous data on counting MCF-7 cells exposed to waterpipe and cigarette smoke by trypan blue assay as presented in **Figure 13**. The studies that included MCF-7 breast cancer cell line exposed to cigarette smoke extract [56, 60, 61] and to waterpipe smoke extract [59] did not include data about cell count and percentage viability using the Trypan blue assay. There are only two studies with A549 cell line that clearly show a decrease in cell count like the one with MCF-7 when exposed to waterpipe extract [6, 63]. Since they are different cell lines then the data cannot be compared.

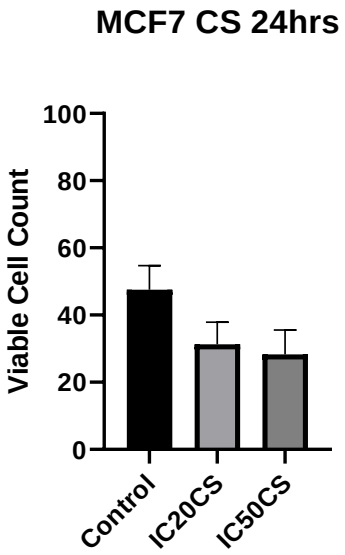
Figure 13: Cell viability (Trypan blue assay) of MCF-7 breast cancer cells exposed to **a.** waterpipe smoke extract with respect to the control at 24 and 48 hours and **b.** cigarette smoke extract with respect to the control at 24 and 48 hours.

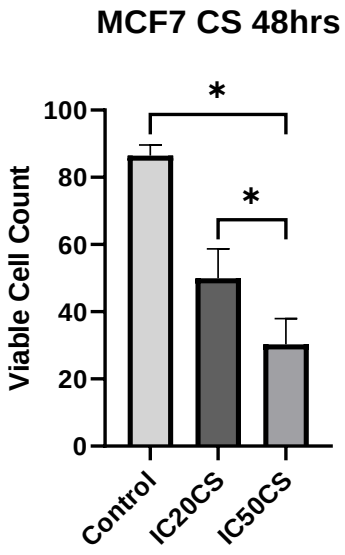
Cell viability was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test (*for $p<0.05$ and ** for $p<0.001$).

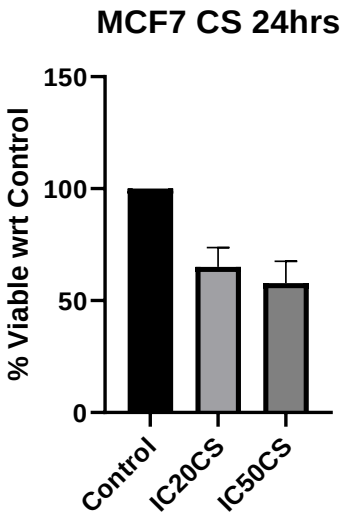
a.

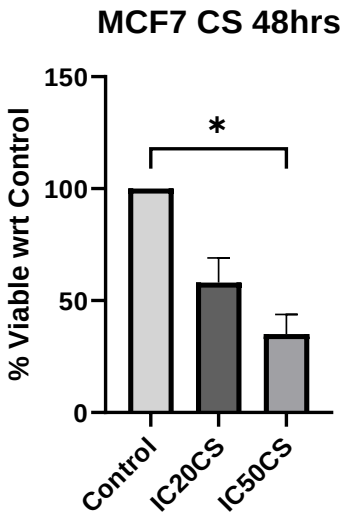


b.


MCF7 CS 24hrs
Viable Cell Count
100
80
60
40
20
0
Control
IC20CS
IC50CS


MCF7 CS 48hrs
*
*
Viable Cell Count
100
80
60
40
20
0
Control
IC20CS
IC50CS


MCF7 CS 24hrs
% Viable wrt Control
150
100
50
0
Control
IC20CS
IC50CS


MCF7 CS 48hrs
*
% Viable wrt Control
150
100
50
0
Control
IC20CS
IC50CS

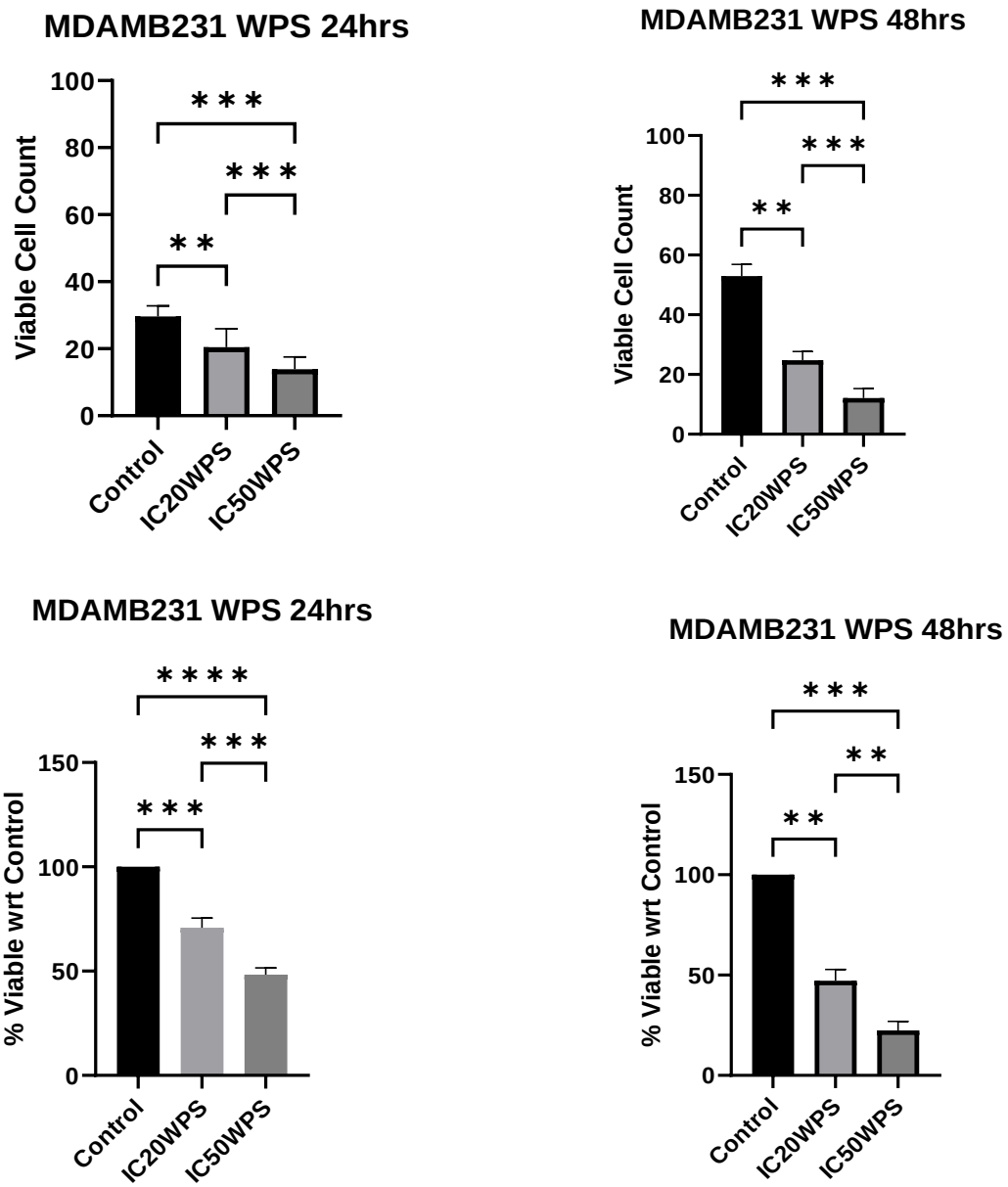
4. Cell Counting by Trypan Blue Assay for MDAMB-231 Breast Cancer Cells

The data in the bar graphs of **Figure 14** show that the number of MDAMB-231 cells exposed to subtoxic concentrations of waterpipe and cigarette extracts like MCF-7 cells decreased with higher concentrations and with respect to the control.

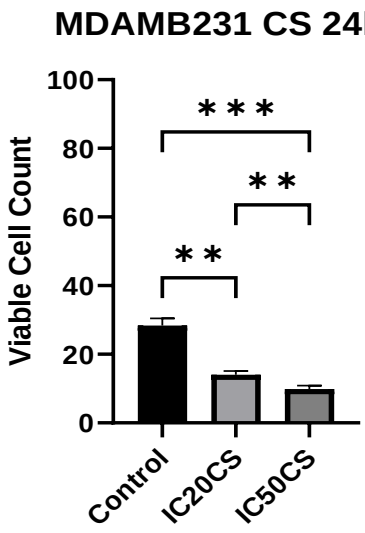
Figure 14: Cell viability (Trypan blue assay) of MDAMB-231 breast cancer cells exposed to **a.** waterpipe smoke extract with respect to the control at 24 and 48 hours and **b.** cigarette smoke extract with respect to the control at 24 and 48 hours.

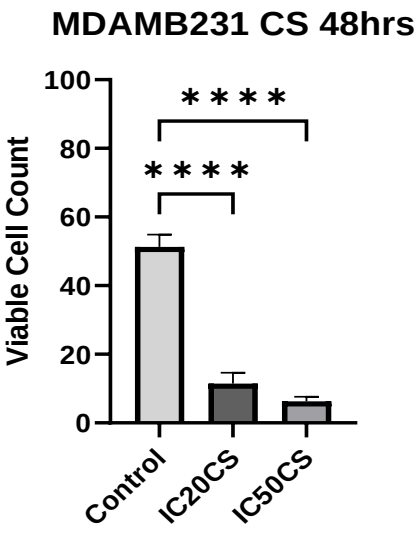
Cell viability was calculated as % relative to control, and data are presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test (** for p<0.005, *** for p<0.0005 and **** for p<0.0001).

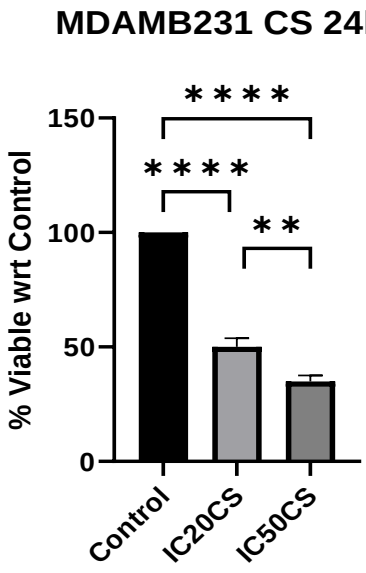
a.

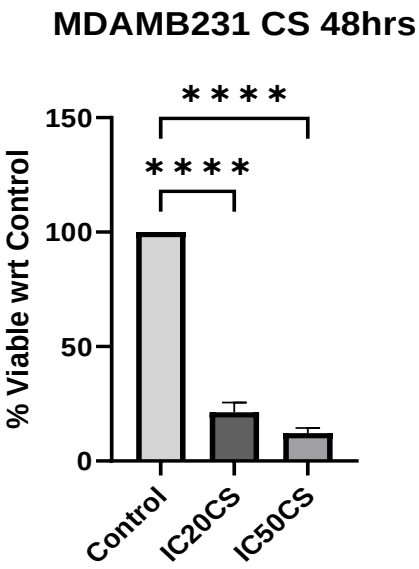


b.


MDAMB231 CS 24hrs
Viable Cell Count
100
80
60
40
20
0
***
**
**
Control
IC20CS
IC50CS


MDAMB231 CS 48hrs
Viable Cell Count
100
80
60
40
20
0
****
****
Control
IC20CS
IC50CS


MDAMB231 CS 24hrs
% Viable wrt Control
150
100
50
0
****
****
**
Control
IC20CS
IC50CS

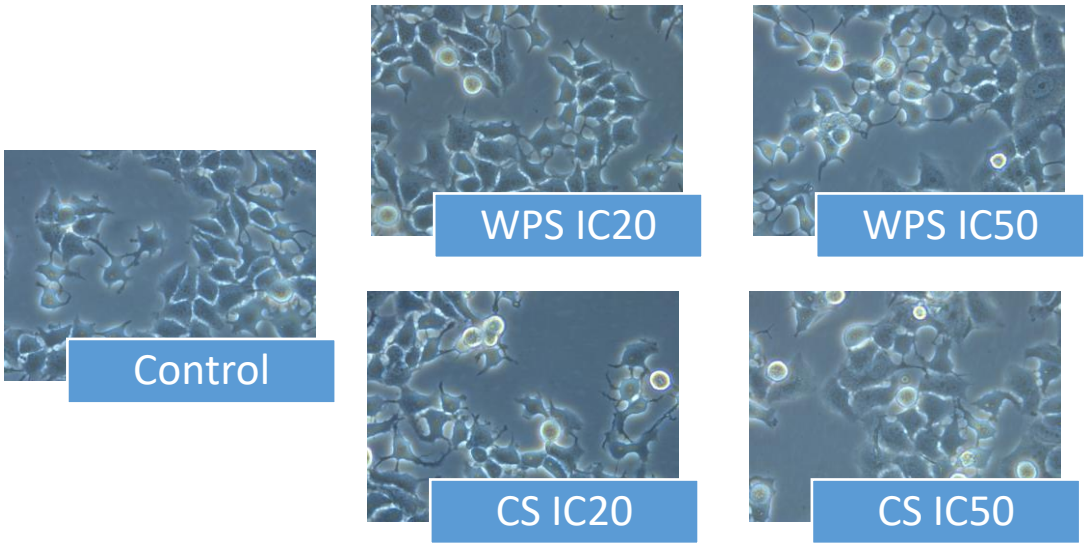

MDAMB231 CS 48hrs
% Viable wrt Control
150
100
50
0
****
****
Control
IC20CS
IC50CS

5. Cell Morphology Changes Under the Microscope

Images of MCF-7 and MDAMB-231 cells exposed to the inhibitory concentrations (IC20 and IC50) of waterpipe and cigarette after 24 and 48-hour respectively were taken by Leica Microscope at 40x magnification as shown in **Figures 15 and 16**. Comparing cells exposed to waterpipe and cigarette with control cells, the exposed cells of the MDAMB-231 cell line displayed a long, thin, spindle-like shape with boundaries resembling those of loosely adhered cells, though the findings necessitate further evaluation and validation. No morphological changes were seen with MCF-7 cells when exposed to smoke extract. Also, there is a decrease in the number of cells noticed in both MCF-7 and MDAMB-231 breast cancer cell lines after exposure to smoke extract as shown by the presence of floating cells that means more dead cells and as confirmed by counting cells in the trypan blue results section.

Figure 15: Cell morphology at 40X of **a.** MCF-7 24-hour exposure to IC20 and IC50 of waterpipe and cigarette compared to unexposed control cells and **b.** MCF-7 48-hour exposure to IC20 and IC50 of waterpipe and cigarette compared to unexposed control cells.

a.



b.

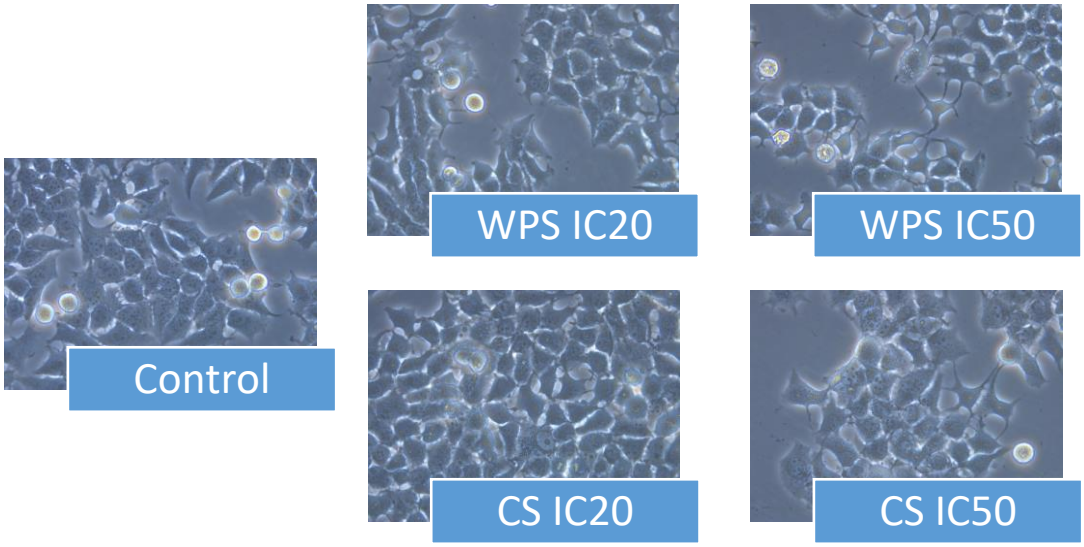
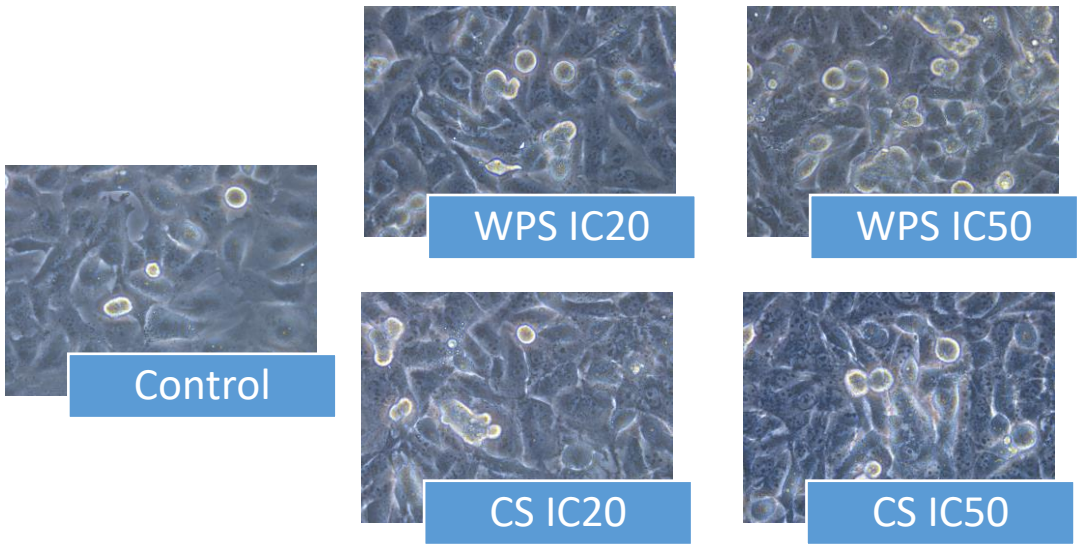
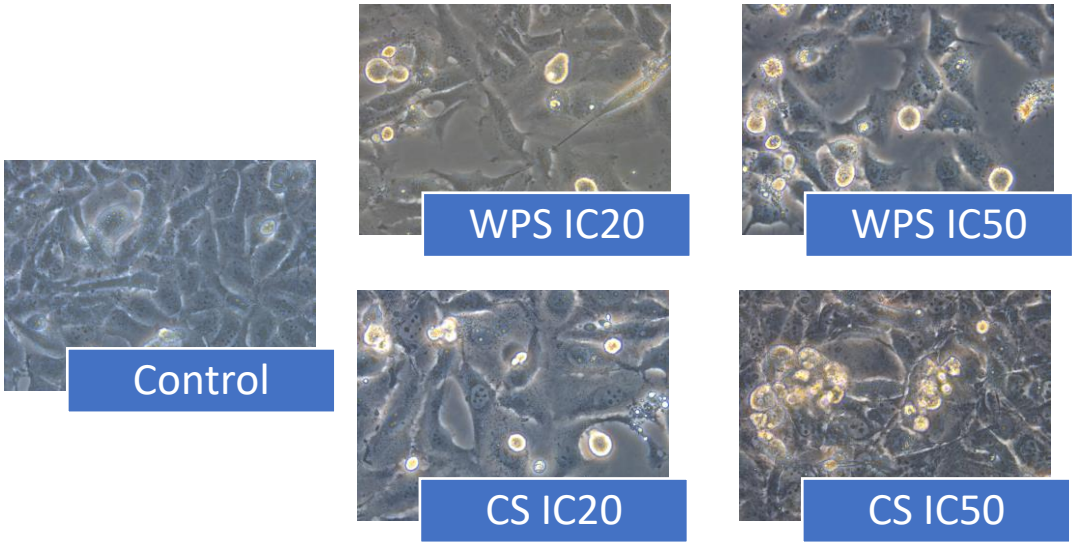


Figure 16: Cell morphology at 40X of **a.** MDAMB-231 24-hour exposure to IC20 and IC50 of waterpipe and cigarette compared to unexposed control cells and **b.** MDAMB-231 48-hour exposure to IC20 and IC50 of waterpipe and cigarette compared to unexposed control cells.

a.



b.



B. PART II. MOLECULAR EXPERIMENTS

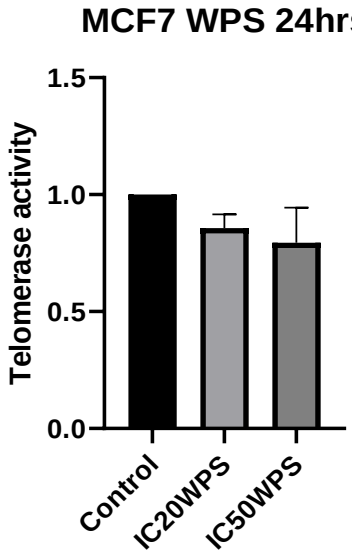
1. Telomerase Activity and Telomerase Expression in Breast Cancer Cells Exposed to Waterpipe and Cigarette Smoke Extracts after 24 hours.

MCF-7 cells exposed to subtoxic concentrations of waterpipe at the IC20 and IC50 of the 24-hour exposure showed a non-significant trend of decrease in telomerase activity with waterpipe that was significant with cigarette at the IC50 **(Figures 17.a.1 and b.1)**. On the contrary, for telomerase expression, there was a trend, though nonsignificant, of increase in expression at all exposures. **(Figure 17.a.2 and b.2).** Unlike MCF-7, MDAMB-231 cells exposed to subtoxic concentrations of waterpipe at the IC20 and IC50 of the 24-hour exposure showed a decrease in telomerase activity that is significant with both waterpipe and cigarette **(Figures 18.a.1 and b.1)** and an increase in telomerase expression that is only significant with cigarette **(Figures 18.b.1 and b.2)**.

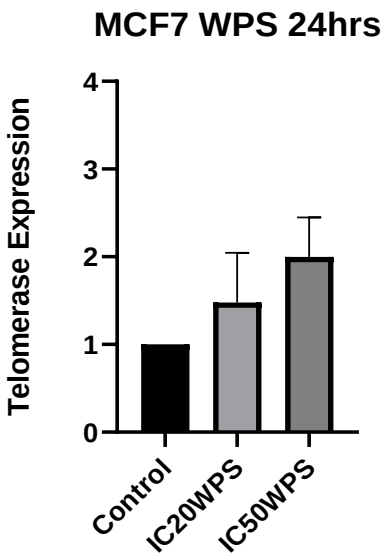
Figure 17: Telomerase activity and RNA expression in MCF-7 cells after 24 hours of exposure to **a.** WPS and **b**. CS, respectively

Results were calculated as relative to control and presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test (*for $p<0.05$).

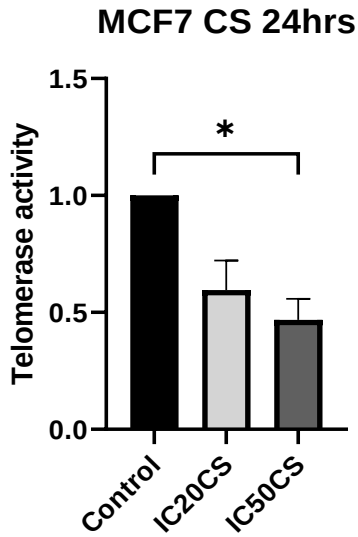
a.1.



a.2.



b.1.



b.2.

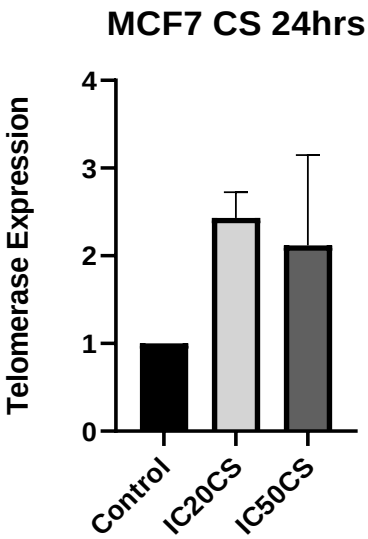
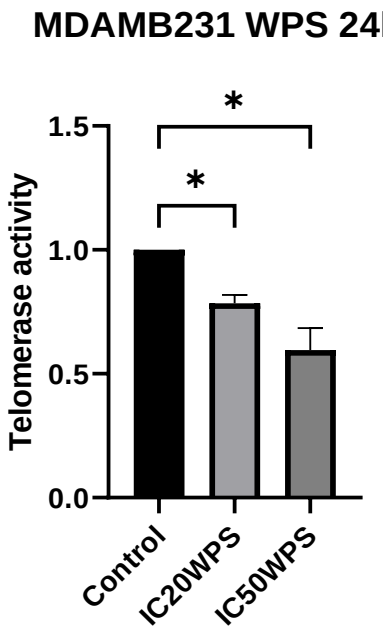


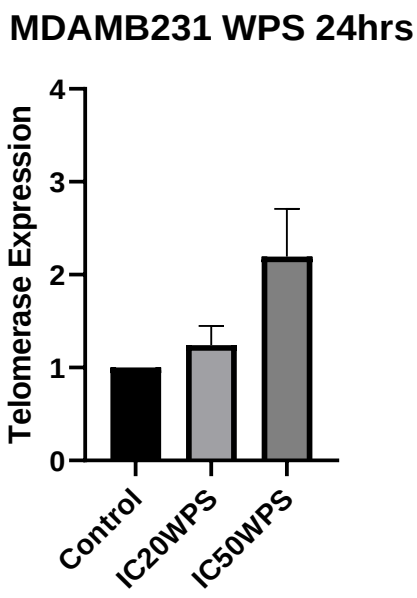
Figure 18: Telomerase activity and RNA expression in MDAMB-231 cells after 24 hours of exposure to **a.** WPS and **b**. CS, respectively

Results were calculated as relative to control and presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test (*for $p<0.05$ and ** for $p<0.005$).

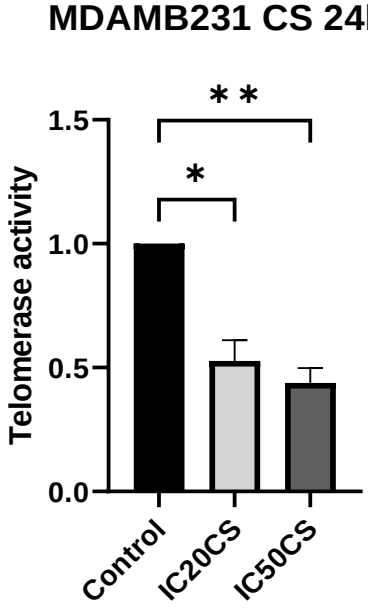
a.1.



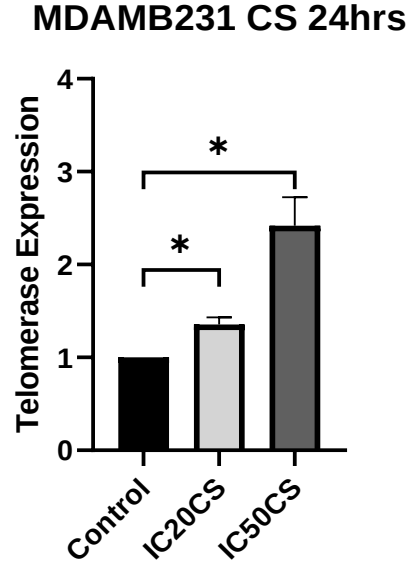
a.2.



b.1.



b.2.



2. *Relative Telomere Length (RTL) in Breast Cancer Cells Exposed to Waterpipe and Cigarette Smoke Extracts after 24 and 48 hours.*

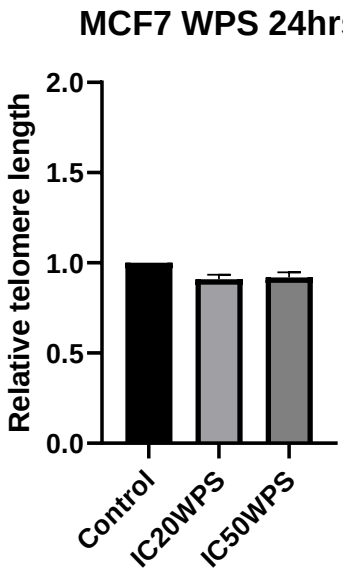
There was no marked change in RTL in MCF-7 cells exposed to subtoxic concentrations IC20 and IC50 of waterpipe and cigarette for 24 and 48 hours respectively **(Figure 19).**

However, MDAMB-231 cells exposed to IC20 and IC50 waterpipe and cigarette concentrations showed a significant increase in RTL only at IC20 of waterpipe and IC50 of cigarette after 24 hours while no significant change after 48 hours of exposure **(Figure 20).**

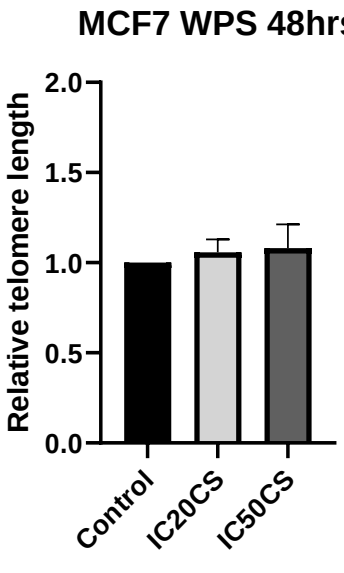
Figure 19: Relative telomere length (RTL) in MCF-7 cells after 24 and 48 hours of exposure to **a.** WPS and **b**. CS, respectively

Results were calculated as relative to control and presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test.

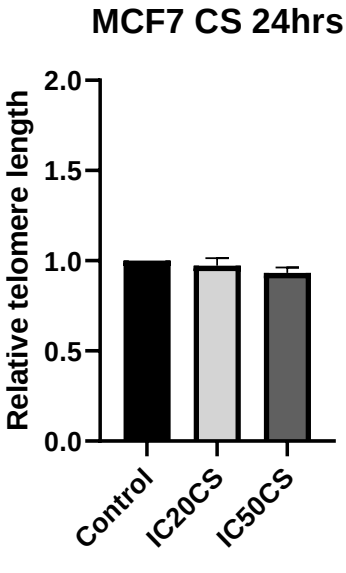
a.1.



a.2



b.1.



b.2.

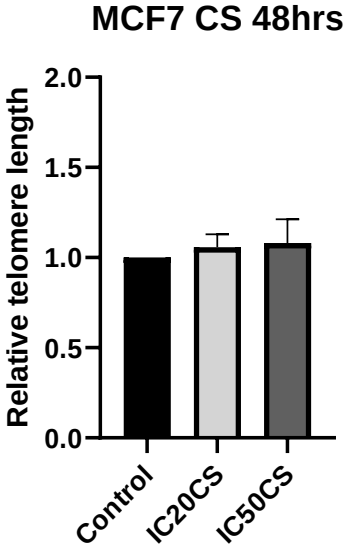
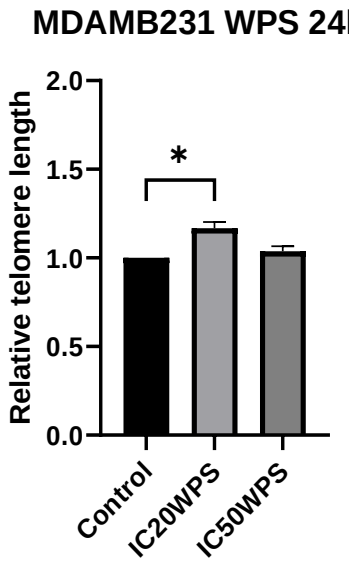


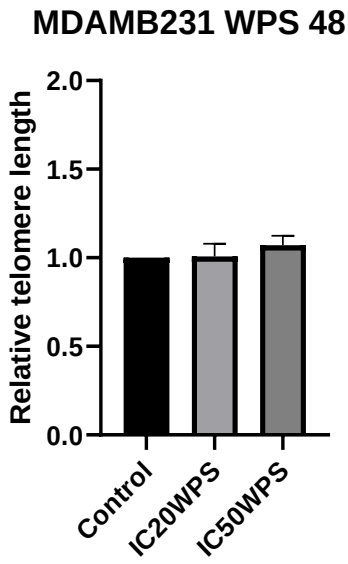
Figure 20: Relative telomere length (RTL) in MDAMB-231 cells after 24 and 48 hours of exposure to **a.** WPS and **b**. CS, respectively

Results were calculated as relative to control and presented as mean + SEM of at least three independent trials. Comparisons were performed between each treatment condition and control using one-way ANOVA followed by Tukey HSD *post-hoc* test (*for $p<0.05$).

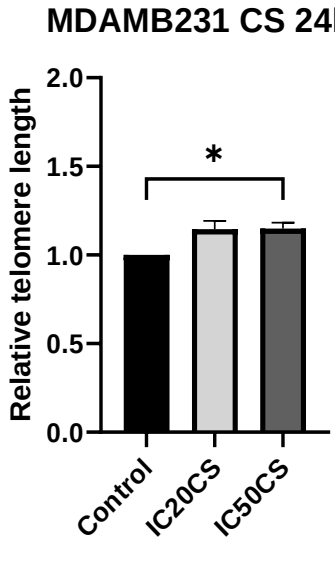
a.1.



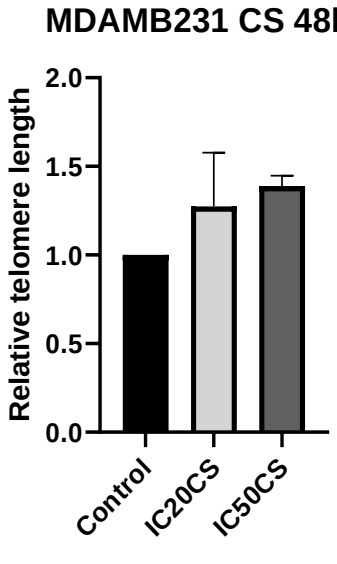
a.2.



b.1.



b.2.



CHAPTER V

DISCUSSION

Breast cancer ranks first worldwide contributing to 11.7% of newly diagnosed cases with 2.3 million cases diagnosed in 2020. Thus breast cancer was declared by the International Agency for Research on Cancer (IARC) as the leading cause of cancer globally [36, 42]. In addition, the phenomenon of smoking, one of the contributing factors of cancer, is widely spread in the society taking different forms from cigarette, cigar, e-cigarettes, waterpipe and other vaping methods. In this thesis, we are tackling for the first time the effects of acute subtoxic exposure of breast cancer cells to waterpipe and cigarette smoke extracts on relative telomere length (RTL), telomerase activity and expression.

Compared to cigarette, very few studies evaluated the effects of exposure of cancerous cell lines to waterpipe extracts. Hence, the data shown in this thesis is unique. Search for articles was done on PUBMED using the keywords “waterpipe”, “WPSE”, “waterpipe condensate” with breast cancer cell lines and other cells. Only one article by Sadek *et al.* [59] included MCF-7 breast cancer cell line exposed to waterpipe. This exposure promoted epithelial-mesenchymal transition and caused the invasion of breast cancer cells highlighting one important effect of smoke on breast cancer that is cell metastasis and carcinogenic potential. Other articles included waterpipe exposure with other cell lines including A549 and Beas2b [6, 62, 63, 65, 68]. For instance, Khalil *et al.* [63] studied 6 different flavors of tobacco with A549 cell line and found the Double Apple as the most potent. Since the Double Apple tobacco brand was commonly used and proved to be toxic on A549 cells, it was similarly used in our experiments on breast

cancer cell lines following the Beirut smoking protocol in the lab of Dr. Shihadeh [110]. In a similar way, we exposed breast cancer cell lines MCF-7 and MDAMB-231 to waterpipe using a range of concentrations from the lowest (1mg/ml) to the highest (30mg/ml) and cigarette from the lowest (0.5 mg/ml) to the highest (2mg/ml), evaluated the effect on the cells using MTT assay and determined the inhibitory concentrations IC20 and IC50 of waterpipe and cigarette with each cell line.

Despite that waterpipe contains far more carcinogenic chemicals that renders it more toxic per session than a single regular cigarette session, it is proposed that waterpipe contains lower quantities of biologically active constituents and these constituents are potentially resulting in a considerably more dilutant toxicant mixture. Besides, the contribution of the charcoal in the TPM formation is lower than expected especially that the toxicants resulting from charcoal burn are being diluted by the TPM produced from the tobacco. This process renders waterpipe less mutagenic and less toxic per unit mass of condensate. Therefore, a delivered constituent of 1500mg of particulate matter with waterpipe is greater than a delivered constituent of 30mg of particulate matter with cigarette, yet the higher particulate matter is considered a more dilute toxicant mixture [6]. Thus, this clarifies the fact behind using in our initial experiments higher concentrations **(30, 20, 15, 12, 10, 7, 5, 3, 1mg/ml)** with waterpipe as they are more diluted compared to the lower concentrations **(2, 1.5, 1, 0.7, 0.5mg/ml)** with cigarettes justifying our results that showed cytotoxicity from cigarettes at lower concentrations while waterpipe at higher ones.

We identified by cell counting that the number of breast cancer cells decreased after exposure to subtoxic concentrations of smoke from waterpipe and cigarette, and the reason behind this decrease is attributed to the toxic effect of smoke on some cells

resulting in dead cells confirmed under the microscope by the presence of floating cells. The remaining alive breast cancer cells seen under the microscope showed morphological changes post-exposure to the IC20 and IC50 of waterpipe and cigarette at two endpoints, 24 and 48 hours, but further evaluation and validation are required. MDAMB-231 cells, for instance, formed many spindle-like shapes. Similar changes were identified by Sadek *et al.* with MCF-7 cells [59], and those changes were attributed to epithelial mesenchymal transition (EMT), a phenomenon associated with extensive carcinogenic potential by an increase in cell invasion and migration. In our experiment, some potential changes in the cell shape are suggestive of an enhanced carcinogenic effect and even metastasis. Of course, this effect can be seen with increased invasion and migration. As a matter of fact, Sadek *et al.*, Sun *et al.*, Yu *et al.* and Jeon *et al.* [59, 125-127] have previously shown that exposure to smoke including waterpipe increases cell invasion and migration of breast and other cell types when compared to unexposed cells suggesting an enhanced tumorigenic effect via the involvement of epithelial mesenchymal transition (EMT). We plan to verify this finding by performing invasion and migration assays.

Concerning the effect of waterpipe and cigarette on telomere length, telomerase activity and expression, our results together with the findings in the literature suggest that a decrease in telomerase activity was compensated by an increase in the expression of *h*TERT, and hence no change in RTL. We are the first to investigate the effect of waterpipe and cigarette on RTL and telomerase in breast cancer cells. Since telomerase strictly correlates with the growth rate and gets repressed when cells exit the cell cycle [80, 81], then to understand the genetic effect of smoke on telomerase and RTL, cell cycle analysis is required. Of interest, many *in-vitro* studies showed cell cycle arrest at

G0/G1 phase after exposure to waterpipe and cigarette as in **Table 6**. For example, Rammah *et al.* [6] showed that exposing alveolar cancer cells (A549) to waterpipe induced cell cycle arrest and cells became most likely quiescent. This justifies similarly that breast cancer cells also underwent cell cycle arrest at G0 phase after exposure to smoke thus cells entered a state of senescence where there is no cell division thus a decrease in telomerase activity. Nevertheless, more studies with cell cycle on breast cancer cells are warranted to explain our results.

Cells that enter a state of senescence are no longer proliferating since there is no cell division. Therefore, to overcome the two barriers of proliferation M1 growth arrest and M2 crisis, the expression of telomerase *h*TERT, the rate-limiting determinant of telomerase activity, is required [83]. This hence explains the increase in the expression of telomerase in our results as a compensation to the decrease in telomerase activity. When telomerase expression increases, cells overcome the barriers of proliferation by entering a cycle of infinite cell division resulting in enhanced carcinogenicity and possible metastasis. Hence, the increased telomerase expression may be a marker of enhanced carcinogenic potential of MCF-7 and MDAMB-231 cells.

Our results showed no change in RTL with MCF-7 cells exposed to waterpipe and cigarette after 24 and 48 hours respectively compared to a significant lengthening of RTL with MDAMB-231 cells only with the IC20 of waterpipe and the IC50 of cigarette after 24 hours, and no change after 48 hours. In the literature, studies showed that maintenance of RTL can be either through telomerase or by an ALT mechanism required in case of cell immortalization [97, 128]. This alternative lengthening of telomere method is attributed to homologous chromosomal recombinations that lead to telomere elongation. Recently, one study identified that DNA damage can trigger these

recombinations to mediate elongation [129]. In addition, *in-vitro* studies showed that waterpipe and cigarette exposure can affect the integrity of the DNA by either causing its damage or inhibiting its repair pathways [130-133]. DNA damage can also contribute to cell cycle arrest mediated by p53 induction [134]. Therefore, the maintenance or lengthening of telomeres in our breast cancer cells may have occurred via an ALT mechanism triggered by DNA damage and not by telomerase due to its inactivity (**Figure 21**). Nevertheless, more studies with genotoxicity on breast cancer cells are warranted to confirm the presence of DNA damage. We also plan to do RTL at 72 hours to see whether the maintenance or elongation trend continues.

Table 6: In-vitro studies showing effects of waterpipe and cigarette exposure on cell cycle.

Type of smoking exposure	Reference	Model	Tissue Type	Effect on Cell Cycle
Waterpipe	Rammah *et al.* 2012 [6]	A549	Human alveolar epithelial cells	Induces cell cycle arrest at the G0/G1 phase of the cell cycle and cellular senescence mediated by the p53-p21 pathway
	Rammah *et al.* 2013 [131]	HAEC	Human endothelial cells	Causes accumulation of the cells in G0/G1 phase and a decrease in the percentage of cells in the S phase, suggesting a cell cycle arrest at G0/G1
	Shihadeh *et al.* 2014 [63]	A549	Human alveolar epithelial cells	Induces cell cycle arrest at G0/G1 and increased cell doubling time
	Sadek *et al*. 2018 [60]	MCF7 and BT20	Breast cancer cells	Deregulates cell cycle progression and increases colony formation (flow data not shown)
Cigarette	Kim *et a*l. 2017 [132]	JEG-3	Human placental cancer cells	Deregulates the cell cycle by altering the expression of PCNA as a cell proliferation marker (flow data not shown)
	Henderson *et al*. 2008 [133]	HUVEC	Human umbilical vein endothelial cells	Leads to G1 phase cell cycle arrest after 24 hours compared to control cells
	Esakky *et al.* 2014 [134]	GC-2spd(ts)	Mouse spermatocyte cells	Induces S-phase arrest by inhibiting DNA replication and downregulating G1-S phase cyclins
	Krayzler *et al*. 2015 [135]	SCC-25 and SCC-15	Oral cancer cells	Increases pre-G1 fraction and reduces G2/M fraction
	Salem *et al.* 2013 [136]	hTERT-BJ1	Telomerase-immortalized human foreskin fibroblasts	Activates p53 and p21 and decreases RB phosphorylation, which are markers for senescence and cell cycle arrest (flow data not shown)

CHAPTER VI

FUTURE PERSPECTIVES & EXPERIMENTS

In future, we plan:

- To expose breast cancer cell lines MCF-7 and MDAMB-231 to chronic subtoxic doses of waterpipe and cigarette *in-vitro* mimicking the lifetime exposure of smokers *in-vivo*.
- To determine the genotoxicity effect of WSE and CSE on these immortalized mammary epithelial cells, and to evaluate cell cycle and apoptosis.
- To evaluate potential for enhanced carcinogenicity by performing cell invasion and migration assays.
- To perform RTL at 72 hours and look for the elongation trend.
- To evaluate the effect of waterpipe and cigarette synergism on the cells.
- To evaluate the effect and behavior of smoking exposure on RTL and telomerase of cells other than the breast such as alveolar, bronchial, bladder and colon cancer cells etc.

Some work in progress was already started in the lab that would also help in explaining the results of the telomere length in this thesis like cell cycle by using flow cytometry assay allowing the differentiation of cells in G0/G1, S phase and G2/M and locating the specific cycle to which changes were first identified and relating this to the data with telomere length and telomerase activity and expression. Also, we are currently working in the lab on showing the genotoxic effect of WSE and CSE by using the gamma H2AX assay, a sensitive molecular marker of DNA damage and repair. If

smoking exposure contributed to DNA damage, then this would also explain our results obtained here with telomere and open the horizon into explaining a new phenotypic change called alternative lengthening of telomeres (ALT) that is triggered by DNA damage.

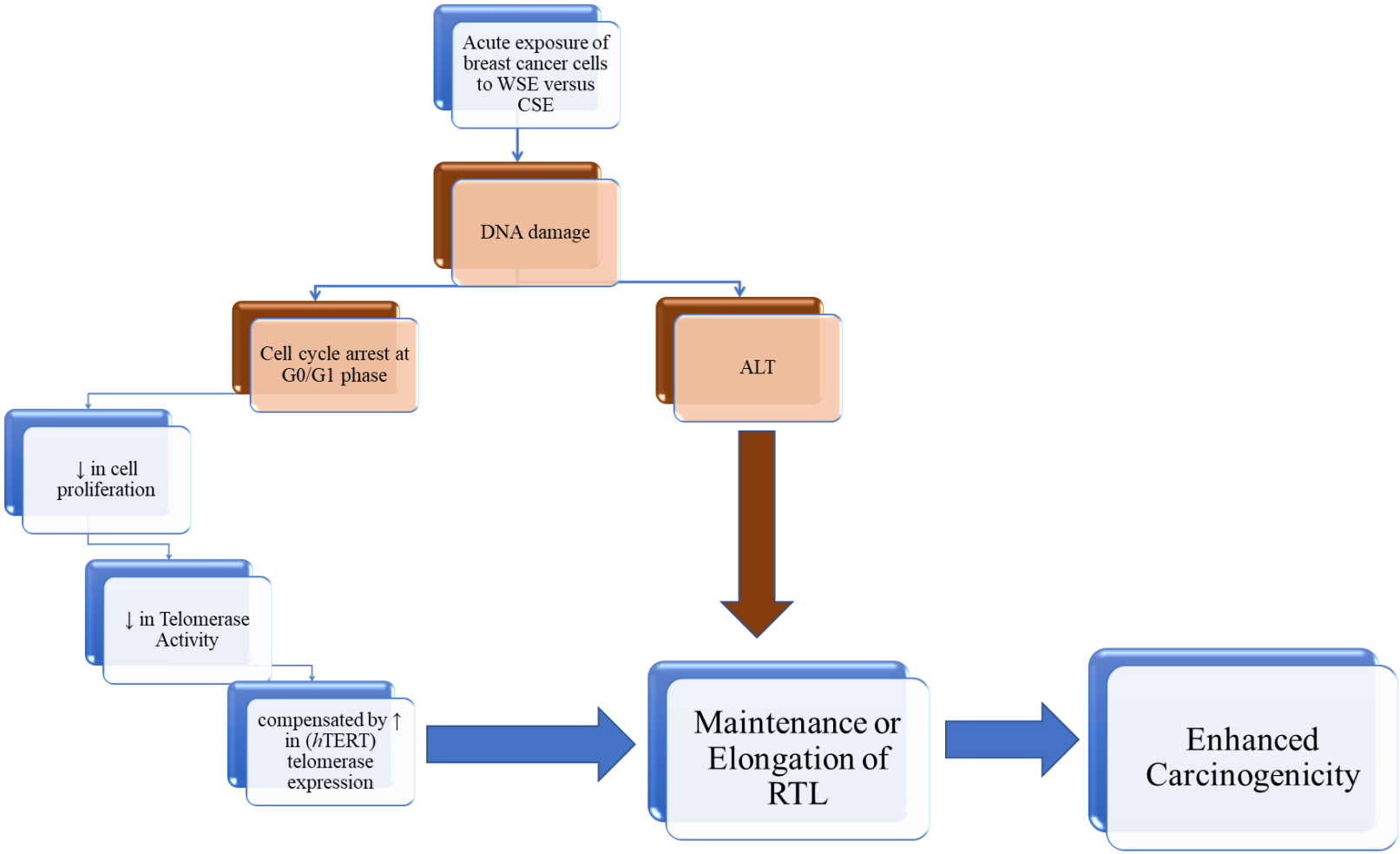
CHAPTER VII

CONCLUSIONS

Despite the epidemiologic evidence showing the association of cigarette smoking with shorter RTLs and the strong correlation between increased telomerase activity with ever-smokers compared to never-smokers, acute exposure of waterpipe and cigarette (24 and 48 hours) *in-vitro* was not associated with shorter RTLs, on the contrary telomere length was maintained in MCF-7 and elongated only with the IC20 of waterpipe and the IC50 of cigarette after 24 hours in MDAMB-231 breast cancer cells. This may be attributed to the fact that waterpipe and cigarette induce cell cycle arrest at G0/G1 phase resulting in cell senescence and consequently a decrease in cell proliferation.

Our results went a step further in elucidating a telomerase-linked mechanism between telomerase activity and its rate-limiting determinant *h*TERT expression. Our findings showed a decrease in telomerase activity that was compensated by an increase in *h*TERT telomerase expression to escape the proliferation barriers in cell senescence resulting in enhanced carcinogenesis. Since MDAMB-231 is known to be more invasive than MCF-7, then the noticed elongation with MDAMB-231 correlates with a greater extensive carcinogenic potential compared to MCF-7. Further studies on cell cycle, genotoxicity, apoptosis, invasion, and migration are required to explain our results, in addition to exposing cells to chronic subtoxic concentrations that would mimic the lifetime exposure of smokers *in-vivo*.

Figure 21: Summary of the potential genetic effect of waterpipe and cigarette on breast cancer cells



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