CHARACTERIZING THE EFFECT OF NEW AND EMERGING TOBACCO PRODUCTS ON AIRWAY INNATE MUCOSAL DEFENSE

Sabri Abdelwahab

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Approved by:

Claire Doerschuk

Jonathon Homeister

Ilona Jaspers

Mehmet Kesimer

Robert Tarran

ABSTRACT

Sabri Abdelwahab: characterizing the effect of new and emerging tobacco products on airway innate mucosal defense
(Under the direction of Mehmet Kesimer)

Airway mucus/mucins serve as a barrier against smoke and other harmful substances that the respiratory tract is exposed to. In addition, airways exosomes have a role in the dynamic regulation of the airway tract response to a broad range of different possible environmental exposure of the body to such substances as smoke. This study ventured to characterize the effects of multiple brands of NETPs on the airway epithelia at multiple levels using in vitro model. Accordingly, the viability and integrity of tight junctions of smoke-exposed epithelia were evaluated. Apical secretions from NETP-exposed cultures were collected and subjected to label-free quantification mass spectrometric analysis. Additionally, chemical composition analysis of different cigarillo brands was also performed. Furthermore, part of the collected apical secretions from NETP-exposed culture secretions were processed for isolation of the exosome using sequential differential centrifugation. The airway exosomal miRNA profile was identified by using HTG EdgeSeq technology and next-generation sequencing platforms. The differential expression analysis was performed by using a bioinformatics tool.

Results showed that NETPs, in the form of little cigars, cigarillos, and waterpipe, collectively have greater effects than control air and cigarette smoke in terms of reduced cell viability and altered protein expression patterns. NETPs were also found to induce oxidative stress proteins and cause more profound alterations in the lung innate immune response.

Furthermore, the analysis of different cigarillo tobacco products revealed compositional differences and greater nicotine delivery to cells that may be linked to the differential effects of these products on cellular viability and protein expression profiles, which are associated with a range of health risks in the context of airway biology.

These study findings contradict the popular belief that NETPs are safer and less harmful than cigarettes. Instead, results indicated that NETP smoke leads to potential health risks and causes damage to the airways to an extent similar to or greater than that of cigarette smoke. These results could serve as a basis for the regulation of tobacco and NETPs and should inform considerations related to health risks and public perception.

To the souls of my parents who have inspired me to seek knowledge in life,
To my lovely family, my wife, and daughter who are the center of my life,
To all my teachers, professors and mentors who have taught me through my life.

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PREFACE

The research in this proposal seeks to establish a biological characterization of the effects of smoke from new and emerging tobacco products (NETPs) on human airways. Experiments were designed and executed to produce data on biological effects due to NETP smoke, not only to increase public knowledge and awareness about the health risks associated with the use of these products, but generate essential information to the legislative process and officials who regulate tobacco products. The data provide scientific evidence that may support decisions on tobacco product regulation. Furthermore, the studies produced hypothesis-generating results related to the pathobiological response of airways to NETPs smoke, which could be a jumping-off point for future mechanistic and functional studies.

Incredible and unlimited support provided by my mentor and his lab members was critical to accomplishing this work. However, much of the work was achieved in collaborative arrangements with strong support groups and tremendous provision from investigators and staff in the Marsico-Lung Institute and the Center for Tobacco Regulatory Science and Lung Health (TCORS) at the School of Medicine. One of many examples within this thesis is found in Chapter two, which includes the effects of new and emerging tobacco products (NETPs) on the airway mucin/mucus proteome. Part of this work, proteomic analysis of the apical secretions of HTBE cells exposed to little cigar smoke, was published in collaboration with other expert scientists from the Center for Tobacco Regulatory Science and Lung Health (TCORS). The article was published before the writing of this thesis with the following citation:

Ghosh, A., Abdelwahab, S. H., Reeber, S. L., Reidel, B., Marklew, A. J., Garrison, A. J., Lee, S., Dang, H., Herring, A. H., Glish, G. L., Kesimer, M., … Tarran, R. (2017). Little cigars are more toxic than cigarettes and uniquely change the airway gene and protein expression. *Scientific Reports, 7*, 46239. doi: 10.1038/srep46239.

Permission to include the proteomic analysis results of apical secretions of little cigar tobacco-exposed airway cultures in my Ph.D. dissertation was obtained from Arunava Ghosh, Ph.D., first author on the paper.

A manuscript related to cigarillo smoke exposure studies was generated from this thesis and submitted for publication. The title and contributing authors are as follows:

"Cigarillo smoke effects airway epithelia leading to altered protein expression. Sabri H. Abdelwahab [1,2] , Boris Reidel[1, 2], Jessica R. Martin[2], Arunava Ghosh[2], James E. Keating[3], Prashamsha Haridass[1,2] , Jerome Carpenter[1,2], Gary L. Glish[3], Robert Tarran[2,4], Claire M. Doerschuk[1, 2, 5], and Mehmet Kesimer[* 1, 2]" Submission in Process

The manuscript contains work completed in collaboration with other TCORS program laboratories. Dr. Arunava Ghosh from Dr. Rob Tarran's lab contributed in the cytotoxicity assay, and James Keating from Gary Glish's lab performed the chemical compound analysis. The manuscript also includes animal studies conducted by Jessica Martin from Dr. Claire Doerschuk's lab, which are not included in this thesis.

Chapter 3 includes studies related to airway exosomal miRNA profiling after exposure to smoke from new and emerging tobacco products (NETPs). In this section, Dr. Hong Dang from the Marsico Lung Institute assisted in analyzing the exosomal miRNA data and helped with the data interpretation. All the data related to the effect of waterpipe smoke exposure

[1] Department of Pathology and Laboratory Medicine
[2] Marsico Lung Institute
[3] Department of Chemistry
[4] Department of Cell Biology & Physiology
[5] Department of Medicine

on airway mucus in this thesis were completed and analyzed, and a manuscript is summarizing this work in preparation.

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2App	Two Apples Flavor only of Waterpipe Tobacco
AMDIS	Automated Mass Spectral Deconvolution & Identification System
ANOVA	Analysis of Variance
BALF	Bronchoalveolar lavage fluid
BPIFA1	Bactericidal/Permeability-Increasing-Fold-Containing Family A
CC10	Clara Cell 10 Kd Protein
CDC	Centers for Disease Control and Prevention
CLLO	Cigarillo
COPD	Chronic Obstructive Pulmonary Disease
ENDs	Electronic nicotine delivery devices
FDA	Food and Drug Administration
GBK	Garcia y Vega Game Black Cigarillo
GC-MS	Gas Chromatography-Mass Spectrometry
HMGB1	High-Mobility Group Protein-1
HTBE	Human Trachea-Bronchial Epithelial
HTT	Hi-Fi Tropical Tango Cigarillo
IgA	Immunoglobulin A
IQOS	Quit-Ordinary-Smoking (Philip Morris International).
KCS	Kentucky Research Cigarette
LCCB	Little Cigar Captain Black
LCCN	Little Cigar Cheyenne

LCs	Little Cigars
LCSS	Little Cigar Swisher-Sweets
MCC	Muco-Ciliary Clearance
miRNA WTA	miRNA Whole Transcriptome Assay
miRNA/miR	Micro-Ribonucleic Acid
MMWR	Morbidity and Mortality Weekly Report
NETPs	New and Emerging Tobacco Products
NGS	Next-Generation Sequencing
NTA	Nanoparticle Tracking Analysis
NYTS	National Youth Tobacco Surveys
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCL	Peri-Ciliary
QC	Quality Control
SAMHSA	Substance Abuse and Mental Health Services Administration
SPLUNC1	Short Palate, Lung, and Nasal Epithelial Clone 1
SSW	Swisher-Sweets cigarillo
TCORS	Center for Tobacco Regulatory Science and Lung Health
TEER	Trans-Epithelial Electrical Resistance
TLRs	Toll-like receptors
TOB	Waterpipe Tobacco with Two Apples Flavor
USHHS/USHS	U.S Health and Human Services
WP	Waterpipe

WTS	Whole Tobacco Smoke

CHAPTER 1: Characterizing The Effect of New and Emerging Tobacco Products (NETPs) On the Airway Innate Mucosal Defense

Overview and Specific Aims

According to the projections of Murray and Lopez (Murray & Lopez, 1997), mortality and morbidity rates associated with tobacco use will inflate to almost threefold within the next two decades, or "from 3.0 million deaths in 1990 to 8.4 million deaths in 2020" (Adkison et al., 2013). Most smoking-related mortality is due to atherosclerotic cardiovascular disease (CVD), lung cancer, and chronic obstructive pulmonary disease (COPD), where the latter includes chronic bronchitis and emphysema (Adhikari, Kahende, Malarcher, Pechacek, & Tong, 2008). Approximately 80% of chronic obstructive pulmonary disease (COPD) death is caused by smoking (United States Department of Health and Human Services [US-HHS], 2014).

In the United States (U.S.), it is estimated that tobacco products are used by over 20 percent of adults (Kasza et al., 2017). Use of tobacco products is, however, more common among younger adults aged 18 to 24 than among the older age group (Ahmed Jamal, 2017; Kasza et al., 2017). The Centers for Disease Control and Prevention, popularly known as the CDC (2016), identified tobacco as one of the critical causes of mortality in the United States, being responsible for 480,000 deaths annually (Arrazola et al., 2014). The aforementioned annual mortality rate is regarded as "preventable," but the glaring statistic is that one in five deaths in the US is attributable to tobacco smoking (Arrazola, Neff, Kennedy, Holder-Hayes, & Jones, 2014; CDC, 2016).

Tobacco smoking also increases the risk of many other acute and chronic diseases, including cancers at many body organs other than the lung (Hackshaw et al., 2004), diabetes mellitus or DM (Willi & Cornuz, 2007), and is a trigger for asthma symptoms (Gilliland et al., 2003). Additionally, smoking-related illness in the United States (US) results in burdensome economic consequences in the health system, including direct medical care and indirect costs attributed to lost productivity (US-HHS, 2014; Xu, Bishop, Kennedy, Simpson, & Pechacek, 2015).

Conventional cigarettes remain the most widely used tobacco product in the United States Unfortunately, tobacco use does not entail smoking just cigarettes. The recent years witnessed tobacco use in many different forms, including cigarettes and cigars, bidis, electronic cigarettes (e-cigarettes) also known as electronic nicotine delivery systems, hookah/waterpipe, and smokeless tobacco, among others (CDC, 2016), collectively termed new and emerging tobacco products (NETPs). These NETPs, including electronic nicotine delivery systems (e-cigarettes), were introduced in the United States market as alternative forms of tobacco use beyond traditional cigarettes (Adkison et al., 2013; Schick et al., 2017). Ironically, however, the public had been exposed not just to second-hand smoke, but to a considerable amount of myths and misconceptions surrounding these NETPs as being less harmful or 'safer' alternatives to the regular cigarettes. There is also a more substantial risk concerning NETPs other than health-related issues, including easy access to these products, as well (Radicioni et al., 2016; Schick et al., 2017).

New and emerging tobacco products (NETPs) are gaining popularity among the young population, particularly middle and high school students, where overall tobacco use is high. Furthermore, one of every five high school students report current tobacco use and about half of

them have experienced using a tobacco product (Arrazola et al., 2014). Sadly, there is a dearth of research-based information on NETPs, particularly with respect to its nature, the extent of usage, public health significance, and diseases attributable to NETPs compared to traditional cigarettes. This renders the potential health risks associated with the use of NETPs poorly understood, including their consequences to airways.

The airway epithelial barrier, including the peri-ciliary layer (PCL) and the mucus components are a multi-level layer that forms the structural basis of the local innate immune defense mechanism to protect the body's respiratory track. It is the first line of defense against inhaled biological and chemical substances including smoke (Radicioni et al., 2016; Radicioni et al.; Schick et al., 2017). Cognizant of the crucial role of the airway mucosal barrier in protecting the lung from inhaled smoke, the overarching aim of these studies was to examine the pathways by which the airway epithelial barrier responds and defends against inhalation of smoke from NETPs. Using the airways' epithelial secretory products (*i.e*, mucus and exosome-like vesicle samples) in response to such exposures, this investigation proposes to discover new mucosal biomarkers of harm for NETPs and compare them to traditional cigarette smoke. This study, therefore, advances the hypothesis that exposure to smoke from NETPs causes unique qualitative and quantitative changes in the airways including the proteome of the airway mucus and the cargo (micro-RNA) of secreted exosome-like vesicles that can be measured and used as biomarkers of harm and/or exposure of tobacco-induced changes in the lung's innate defenses **(Figure 1)**.

The specific aims are as follows:

Aim 1. **Assess the effect of NETPs on airway mucus/mucin biomolecules, including the expression of the proteome**. A label-free quantitative proteomics approach was used

to identify muco-proteome biomarkers associated with NETPs, including the following tobacco products:

Aim 1a. Little cigar

Aim 1b. Cigarillos

Aim 1c. Waterpipe shisha tobacco (hookah)

Aim 2. **Evaluate how these NETPs alter the composition of the extracellular vesicles in terms of the microRNA transcriptome**.

Aim 2a: Ascertain the quantity and frequency distribution of exosomal sizes post-NETP exposure by using Nanoparticle Tracking Analysis (NTA).

Aim 2b: Profile the micro-RNA exosomal composition in terms of NETP smoke exposure and non-exposure conditions by employing HTG EdgeSeq technology and bioinformatics tools.

In summary, the proposed research aims to investigate the impact of NETPs (little cigar, cigarillo, waterpipe shisha) on airway innate defense by discovering putative biomarkers of harm. The study contributes to better understanding the effect of those tobacco products on biology and pathophysiology of the human airway and provides additional information necessary to explain differences in tobacco-related risk outcomes among populations.

NETPs and the Scale of the Problem

New and Emerging Tobacco Products are defined as "tobacco and nicotine delivery products that have been introduced to the United States market in the past 15 years, products that have become significantly more popular in the past 15 years, or products that are being modified and used in new ways" (Schick et al., 2017). This includes any alternative forms of tobacco beyond conventional cigarette introduced and marketed under different brand names by different manufacturers and may be used by means other than smoking, such as smokeless tobacco **(Table 1)**. The tobacco industry continues to create new, modified and flavored tobacco products. These manufacturers established such tactics to keep the current consumers in the market, and at the same time attract nonsmokers particularly among the youth and young adult subpopulation.

New and emerging tobacco products or NETPs including cigarillo, little cigars, waterpipes, and e-cigarettes are becoming more popular among middle and high school students **(Figure 2)** (Ahmed Jamal, 2017; Gentzke et al., 2019; Singh et al., 2016). Data from the 2011–2018 National Youth Tobacco Surveys (NYTS) showed that approximately 4.8 million middle and high school American students are current tobacco product users, of whom, more than 2.3 million were current users of two or more tobacco products. An increasing proportion of tobacco users are using multiple products, and a combination of tobacco products is consumed by 40 percent of tobacco users. The most frequent combination is cigarettes and electronic cigarettes, used by almost 30 percent of those who use tobacco in more than one form (Kasza et al., 2017). E-cigarettes are the most popular NETP product among middle and high school students with 3.5 million users, followed by cigars with 1.2 million users. Hookahs and smokeless tobacco came next with 0.73 million and 1.0 million users from the same population, respectively (Gentzke et al., 2019).

Meanwhile, the prevalence of cigarette smoking among United States adults has declined from 42.4 percent in 1965 to 15.5 percent in 2016 (Ahmed Jamal, 2017). However, the period 2011-2015 saw a considerable increase in the use of e-cigarettes and hookah. In 2018, e-cigarettes topped the list of the most popular NETPs, found to be used by 4.9% of middle school students and 20.8% of high school students (Gentzke et al., 2019). As a comparison, the population of cigar users in the United States increased between 2000 and 2012 after which it began to decline (Wang, Kenemer, Tynan, Singh, T., and King, 2016) **(Figure 3)**. However, the tobacco industry promoted and enhanced the marketing of these products to focused groups in the 1990s, which increased the prevalence of use among adolescents (US-HHS, 2012). In 2016, an estimated 3.8 percent (12.3 million) of people in the United States aged 12 years or older were current cigar smokers, with the heaviest usage among young adults 18 to 24, at 14 percent (SAMHSA, 2015; Kasza et al., 2017). Likewise, the use of water pipe, also known as hookah, was found to be more prevalent among college students, with estimates of use ranging from 22% to 40%. (US-HHS, 2012; Dugas et al., 2010; Primack et al., 2008). Presumably, the increase in popularity of this tobacco product is at least partly due to the misconception that it is a safer alternative to traditional cigarette smoking although there is little, if any, scientific basis behind such claims.

NETP use might be on the rise among the youth, but there is a deficit of studies examining their effects, compared to traditional cigarette smoking. In this regard, much remains to be understood in terms of the long-term effects and possible adverse health effects of NETPs. On the basis that NETPs contain a group of different chemicals, toxins and nicotine also found in conventional cigarettes, it can be conjectured that these new tobacco products potentially pose adverse effects to users, including harm to the users' pulmonary function (Ghosh et al., 2017a),

respiratory illness, periodontal disease, as well as the potential for nicotine addiction (Akl et al., 2010; Eissenberg & Shihadeh, 2009).

Another source of concern is the flavors added to these new tobacco products. While flavors added to or intended to enhance cigarettes have been banned (with the exception of menthol) by the Food and Drug Administration (FDA, 2018a), NETPs such as cigarillo, little cigars and e-cigarette liquids contain flavors designed to appeal to the youth and vulnerable populations (Ambrose et al., 2015). At least one research study had shown that flavors in little cigar and cigarillo contributed to young adults' susceptibility and initiation to tobacco use (Sterling, Fryer, Nix, & Fagan., 2015). These products offer a wide range of flavors that presumably appeal to different subpopulations of middle and high school students based on the different percentages of their use across subpopulations of American high school and middle school students.

Using data from the 2014 National Youth Tobacco Survey (NYTS), it was estimated that 70.0% (3.26 million) of all current youth tobacco users have used at least one flavored tobacco product in the past 30 days. Among current users, 63.3%, (1.58 million) have used flavored e-cigarettes, and about 60.6% (1.02 million) have used flavored hookah tobacco. Of the students who used cigars, 63.5% reported using a flavored cigar (Corey et al., 2015). Given that over two-fifths of this population of American students use flavored NETPs, it is alarming that despite the absence of scientific research on their health effects, permission is given to add such chemical flavors to these products.

Until such time that the health effects of chemical flavors have been cleared of safety issues, public health interest dictates that the youth and other consumers be dissuaded from using NETPs. The alternative action is to prohibit the additives (Corey et al., 2015). It is, thus,

important that research into NETPs be conducted to explore the health impacts of these new tobacco products.

Airway Mucus

Airway mucus is a structured, multi-layer gel matrix acting as the structural foundation of the respiratory pathway's immunity defense mechanism (Bonser & Erle, 2017; Fahy & Dickey, 2010). The mucus that constitutes the mobile mucus layer covering the human airways is a complex mixture of mucins, globular proteins, antimicrobial proteins and peptides, sugar, salts, lipids, minerals, and water (Fahy & Dickey, 2010). The gel-forming mucins such as MUC5B and MUC5AC, are the major macromolecular contributors to the properties of mucus responsible for the transport of this layer, in combination with cilia-covered epithelial cells and an airway surface to maintain the lungs in sterile or semi-sterile condition (Bustamante-Marin & Ostrowski, 2017; Dickson & Huffnagle, 2015). The airway mucus and the ciliated epithelium together provide what is called the mucociliary clearance (MCC) that facilitates clearing of obstructions or pathogens and toxins in the airway tract through coughing (Dickey, Fahy, Kesimer, Evans, & Thornton, 2016; Fahy & Dickey, 2010).

Airway mucus hyperconcentration and hypersecretion are important pathophysiological and clinical manifestations of chronic obstructive pulmonary disease (COPD), bronchial asthma (asthma), bronchiectasis, pulmonary cystic fibrosis, and other chronic airway inflammatory diseases (Anderson et al., 2015; Henderson et al., 2014; Henson, 2005; Kesimer et al., 2017). In such diseases and other hypersecretory conditions, the airway mucus develops qualitative and quantitative abnormalities that contribute to the morbidity and mortality of chronic airway disease such as COPD (CDC, NCCDP, & OSH, 2010). Research studies have also shown that inflammation and oxidative stress mechanisms are involved in the pathogenesis of chronic

airway inflammatory diseases and that they trigger excessive mucus production and secretion by glands and goblet cells (Curran & Cohn, 2010).

Tobacco exposure, particularly form cigarettes, is one of the most influential risk factors for various respiratory diseases, including COPD. Among tobacco users, the chronic bronchitis component of COPD can result from dysfunctional clearance of thick obstructive mucus, wherein subsequent exposure to tobacco smoke results in goblet cell metaplasia, hypersecretion of mucus co-occurring with mucus dehydration, with the composition and biophysical features of the mucus itself becoming abnormal (Anderson et al., 2015). Furthermore, tobacco smoke exposure compromises the clearance of mucus, which leads to colonization by bacterial pathogens and increases susceptibility to respiratory tract infection and recurrent airway infection (Bagaitkar et al., 2008; Murphy, 2006).

Since the airway mucus serves as the front line of defense against tobacco smoke ingested through inhalation, this research proposal assesses the impact of cigarette smoke and smoke from the use of NETPs on the airway mucus in terms of biomolecules, other aspects of its barrier, and biophysical properties. This study, therefore, hypothesizes that inhaled cigarette and NETP smoke induces a unique response effect on changes in the airways' mucus barrier, which can be measured and quantified by proteomic approaches, and can be used as an assessment and/or biomarker of harm.

Exosome-like Vesicles

Cell-derived extracellular vesicles called exosomes are small, 50-150 nm organelles present in many biological fluids, and secreted by different cell types including epithelial, hematopoietic and some tumors cell (Bobrie, Colombo, & Raposo, 2011; Thery, Amigorena, Raposo, & Clayton, 2006). Exosomes contain an array of proteins, mRNA, and microRNAs, and

are thought to play a role in regulating the immune system (Bobrie et al., 2011; Valadi et al., 2007). They contribute to the immune response, and communication between cells. The molecular features of these structures depends mainly on the cellular source from which they are derived (Valadi et al., 2007).

Research has shown that airway tract cells are responsible for secreting highly organized exosome/exosome-like vesicles **(Figure 6)** (Kesimer et al., 2009b). Exosome-like vesicles perform various functions such as transport of both non-coding RNA and proteins to enable the different immune cells and epithelia cells of the airway tract to communicate with each other, the delivery of complex intercellular messages, and removal of toxic or excess molecules from cells (Harischandra et al., 2017; Kesimer et al., 2009b).

Exosomes, thus, play an important role in the dynamic regulation of airway tract response to the widely different possible internal biological processes and environmental conditions or substances the body is exposed to, such as smoke (Alexander et al., 2015; Harischandra et al., 2017; Russ & Slack, 2012). This is especially true if the ensuing pathological processes result in changes in exosome protein or miRNA cargo. The function of miRNAs, which belong to the broad group of micro-sized noncoding RNA, is mainly the silencing of RNA and controlling of the post-transcriptional expression of genes and, additionally, the inhibition of protein translation (Ambros, 2004). During conditions such as inflammation and immune responses, the exosomes in miRNA are altered. These small nucleotide polymers serve various roles during inflammation that, in turn, are thought to be able to alter the progression of many conditions affecting the lungs (Alexander et al., 2015; Kesimer et al., 2009b).

Considerable research on the usefulness of exosomes as biomarkers have been conducted and the outcomes suggest that they could potentially serve as an excellent biomarkers

to conditions resulting from tobacco exposure (Alexander et al., 2015; Kesimer et al., 2009b; Russ & Slack, 2012). Exosomes secreted by the epithelia cells of the airway tract can be effective biomarkers owing to the direct exposure of the airway tract to tobacco smoke and resulting inflammation and immune response (remodeling).

In this research proposal, we hypothesized that tobacco smoke from cigarettes and NETPs effect changes in the exosomal cargo (miRNA) of the airway epithelial cell. This study, therefore aims to determine the potential of miRNAs and vesicular proteins as biomarkers following exposure to tobacco smoke. As earlier noted, there is a dearth of information about the effects of exposure to NETP smoke, particularly, little cigar, cigarillo, and waterpipe, on the health of the airway. This thesis provides unique data and generate crucial information on the biological effects of NETPs that could form the basis for regulating these tobacco products.

The study contributes to the understanding of the effects of the use of tobacco products on the barrier function of the airway tract epithelial tissues and helps to form a more integrated concept of the mucus/mucin clinical biomarkers that could provide evidence to disprove the safety claims of NETP manufacturers. The knowledge in these studies may result in the creation of regulatory measures and development of preventive strategies to address the public health issues pertaining to the use of all tobacco products, including the NETPs that are particularly popular among young people.

CHAPTER 2: Effect of New and Emerging Tobacco Products (NETPs) on Airway Mucin/Mucus Proteome

Overview

Extensive research has shown that cigarette smoking has multiple adverse effects on the airways, such as chronic obstructive pulmonary disease (COPD) and cancer. A variety of new and emerging tobacco products (NETPs) have aroused the interest of tobacco consumers who use these products because of flavoring that appeal to their senses and satisfy their fascination for tobacco. Forms of NETPs include, among others, the little cigars, cigarillo and waterpipe, which are commercially available on the market and are gaining popular patronage among cigarette and other tobacco-product enthusiasts. Nevertheless, despite the emerging popularity among its patrons, the health risks of smoking NETPs have not been sufficiently investigated.

While there are claims that smoking NETPs is safer than traditional tobacco products and that NETPs have fewer associated health risks than traditional cigarette smoking, there is a dearth of research-based evidence to support these claims. Hence, a gap in knowledge exists concerning the conjectural advantage of NETPs as safer alternatives to smoking traditional tobacco products. This study ventured to characterize the effects of multiple brands of NETPs on the airway epithelia at multiple levels using *in vitro* models. Accordingly, the viability and integrity of tight junctions of smoke exposed in the epithelia were evaluated. Apical secretions from NETP-exposed cultures were collected and subjected to label-free quantification mass

spectrometric analysis. Additionally, chemical composition analysis of different cigarillo brands was also performed.

Results showed that NETPs, in the form of little cigars, cigarillo and waterpipe, collectively have greater effects than air (control) and cigarette smoke in terms of reduced cell viability, and altered protein expression patterns. NETPs were also found to induce oxidative stress proteins and cause more profound alterations in the lung innate immune response. Furthermore, the analysis of different cigarillo tobacco products revealed compositional differences and greater nicotine delivery to cells that may be linked to the differential effects of these products on cellular viability and protein expression profiles. These differences may be associated with a range of health risks, in the context of airway biology.

These study findings contradict the popular belief that NETPs are safer and less harmful than cigarettes. Instead, results indicated that NETP smoke leads to potential health risks and causes damage to the airways to an extent similar to or greater than that of cigarette smoke. These results could serve as a basis for the regulation of tobacco and NETPs and should inform considerations related to health risks and public perception.

Introduction

Airway mucus

The airway mucus is a structured, multilayer protective gel matrix that acts as the structural foundation of the innate defense mechanism of the respiratory pathway (Fahy & Dickey, 2010). The mechanism of mucociliary clearance utilizes the unique properties of mucus and its movement via the cilia to facilitate the clearance of obstructions, pathogens, and toxins from the airway tract (Dickey et al., 2016; Fahy & Dickey, 2010). Some studies have shown that a reduction in clearance of the airway caused by altered mucus or mucin composition, as well as the inherent biological and physical properties of the airway precede airway-related pathology and clinical symptoms **(Figure 3)** (Churg & Wright, 2009; Kesimer et al., 2017; Reidel et al., 2018).

In the case of chronic airway diseases, the airway mucus develops qualitative and quantitative abnormalities leading to morbidity and mortality (Bonser & Erle, 2017; Dickey et al., 2016; Kesimer et al., 2017). It should be noted that early abnormal airway functioning principally results from rational and other changes in the gel-forming mucins MUC5B and MUC5AC (Kesimer et al., 2017). A condition referred to as mucin hypersecretion is a feature in many chronic inflammatory airway tract issues, such as those which result from cigarette smoking (Churg & Wright, 2009; Fahy & Dickey, 2010; Kesimer et al., 2017; Ramos, Krahnke, & Kim, 2014), as well as in respiratory illnesses like asthma, chronic bronchitis, and cystic fibrosis (Ramos et al., 2014). Consequently, excessive mucus in the airway causes an obstruction that, in turn, lowers pulmonary functioning, which can heighten morbidity and mortality risks (CDC et al., 2010; Ramos et al., 2014).

Airway mucus/mucin serves as a barrier against smoke and other harmful substances that the respiratory tract is exposed to (Cao et al., 2018; Reidel et al., 2018; Yoshida & Tuder, 2007). Tobacco smoke tends to cause irritation in the airway tract as it is inhaled down from the mouth towards the upper part of the airway (Kesimer et al., 2017; Reidel et al., 2018). Consequently, continuous exposure to tobacco smoke results in goblet cell metaplasia, mucus hypersecretion, and mucus dehydration, which eventually leads to abnormal mucus composition **(Figure 7)** (Anderson et al., 2015; Fahy & Dickey, 2010).

Fundamental research into the respiratory system, toxicity studies, and experiments involving exposure to smoke inhalation generally utilize *in vitro* airway tract models. The *in vitro* airway tract culture consists of tightly-junctioned, polarized epithelial cells with basolateral and apical membranes, and culture fluid restricted to the surface of the basolateral membrane while the apical surface is air-exposed (Kesimer et al., 2009a; Baginski et al., 2006; Karp et al., 2002). Cilia and microvilli grow on the surface of the apical members as forms of mucin covering, given that the cells have been previously subjected to laboratory experimentation on mucin production. This *in vitro* model is, therefore, comparable to the human mucociliary differentiation of a pseudostratified epithelium *in vivo* (Kesimer et al., 2009a; Aufderheide et al., 2015; Karp et al., 2002).

In this research, the effects of new and emerging tobacco products were characterized after exposing little cigar (LC), cigarillo and hookah/ waterpipe smoke to the airway epithelial mucosal barrier. A primary, well-differentiated human bronchial epithelial cell culture was utilized and was exposed to the NETPs to investigate key issues relevant to the airways and subsequently, on the respiratory health of the cell culture exposed to tobacco smoke.

Little Cigar & Cigarillo

Cigar and cigarettes were defined as follows. Cigar refers to "a roll of tobacco wrapped in leaf tobacco or any substance that contains tobacco" (World Health Organization [WHO] – International Agency for Research on Cancer [IARC], 2004, p. 56). Meanwhile, cigarettes are "any roll of tobacco wrapped in paper or in any substance not containing tobacco" (as cited in Lempert & Glantz, 2018, p. s120). Cigars and cigarettes also differ in the manufacturing process, where cigars consist of a binder and wrapper which are both made with air-cured and fermented tobaccos (Garner et al., 1934). Comparatively, cigarettes comprise of a blend of heat-cured and air-cured tobaccos as major components with a small portion of sun-cured (oriental) tobaccos. However, cigarettes do not contain fermented tobacco (WHO – IARC, 2004). The dried and fermented tobacco leaves in cigars are smoked by drawing smoke from the cigar into the mouth, whereas cigarettes are meant to be inhaled (Darkis & Hackne, 1952; Garner et al., 1934; IARC Working Group on the Evaluation of Carcinogenic Risk to Humans, 2004) **(Table 2**).

Cigars are categorized by size, from smaller cigars, which include little-filtered cigars or cigarillos, to larger cigars, such as large premium cigars (Cohn A, 2015; Shopland, 1998/2012). The Alcohol and Tobacco Tax and Trade Bureau - US Department of Treasury (ATTTB - USDT 2014) further classifies cigar products as little cigars if they weigh less than 1.36 gram each (i.e., >3 lbs. /1000 units), whereas cigars that weigh more than 3 lbs. per 1,000 units are considered large cigars.

Little cigars. Physically, little cigars are almost identical to cigarettes in both shape and size. These products are sold in larger packs of 20 and usually come with filtered tips, designating that they are intended to be inhaled like cigarettes (SAMHSA, 2015). Research shows that people do inhale smoke from little cigars (Messer et al., 2015). It is important to note

that the little cigars, many of which look almost exactly like cigarettes can also be manufactured with fruit and candy flavors that are banned from ordinary cigarettes (Ambrose et al., 2015; Messer et al., 2015).

Cigarillo. The cigarillo is a form of NETP which is smaller than a regular cigar but is usually larger than cigarettes or little cigars. Cigarillos are usually made without filters, although these can be smoked using a mouthpiece made of plastic, glass or wood, which act as filter tips to facilitate inhalation when used (Kong et al., 2017). Cigarillos may be sold individually, instead of solely by the pack, and packs contain between one to five pieces each. Therefore, they are inexpensive. Furthermore, they are also taxed at a lower rate, and as a result, cigarillos are generally more affordable to teens (FTC, 2001; Ambrose et al., 2015; Corey et al., 2014; ATTTB - USDT 2014)

Waterpipe (Hookah)

Waterpipe (WP) smoking refers to a social style tobacco use known by various names: qalyan, goza hookah, hubble-bubble, narghile and shisha. The name usage mainly depends on the location or the country where this smoking style is practiced. Waterpipe smoking was invented in India in the fifteenth century and has long been popular in the Middle East. Over the past decade, however, smoking waterpipes have also emerged as a trend in the US, particularly near college campuses and among high school students. WP is now a popular tobacco smoking method among adolescents and young adults. According to the CDC, 20.2% of high school students in the US reported they are currently using any tobacco product in 2016. Meanwhile, 5.8% of these high school students reported they are currently using hookah (Ambrose et al., 2015; Kasza et al., 2017; Maziak, Ward, Afifi Soweid, & Eissenberg, 2004).

It was also observed that hookah café business operations increased within campus perimeters or in predominantly Middle-Eastern communities in the US (Lyon, 2008; Smith-Simone et al., 2008). A possible explanation for popular hookah café businesses is a common misconception that waterpipe smoke is a "safer" alternative to cigarette smoking and entails "fewer" health risks (Lyon, 2008; Maziak et al., 2004; Smith-Simone, Maziak, Ward, & Eissenberg, 2008). It should also be emphasized that aa a form of social smoking behavior, the waterpipe is usually smoked and enjoyed in groups. Thus, from the health perspective, WP group smoking may also be practiced around nonsmokers, which increases the health risk to non-smokers who are exposed to secondhand smoke.

Waterpipes (WPs) are generally manufactured in different dimensions and appearance, but they function in a similar manner for smoking flavored tobacco. The main components of a waterpipe are the head, body, water bowl, and hose(s) **(Figure 9**) along with other accessories, such as the purge valve, grommets, plate and vase gasket.

Historically, WPs were used for the consumption of various derivatives of tobacco, along with other substances, such as cannabis or opium (Balfour, 1885; Goodwin et al., 2014). The most popular tobacco used in a WP is called muaasel, also sometimes referred to as shisha in locales where the WP (hookah) are not alternately called shisha. Muaasel is a sticky, thick mixture of molasses, vegetable glycerol, and shredded tobacco leaf flavored with dried fruit. Typical flavors of muaasel include apple, cola, coconut, grape, guava, lemon, mint, peach, as well as many other fruit-based mixes (Ambrose et al., 2015; Maziak, 2008; Primack et al., 2012). Some sweetened and flavored non-tobacco-based muaasel are also available, as it has been advertised in certain areas where tobacco smoking is either not allowed and/or smokers prefer to use WPs instead. (Shihadeh et al., 2012).

The WP smoking style entails a particular setup **(Figure 9) .** As depicted in the illustration in the top right of **Figure 2**, the bowl at the top of the WP holds the sticky mixture of tobacco covered with aluminum foil and heated by the burning charcoal on top. The charcoal heats the tobacco through the foil to produce smoke, which travels through the body of the waterpipe, and passes through a hose which could be a single, as shown on the right, or multiple, like the lower most right image, for the purpose of inhalation (Kasza et al., 2017; Neergaard, Singh, Job, & Montgomery, 2007).

Experimental Design and Materials & Methods

Cell Culture

Donor human tracheo-bronchial epithelial (HTBE) cells were collected and cultured on Transwell column supports measuring 24 mm in diameter (Genesee Scientific Crop, Research Triangle Park, NC, USA). HTBE cells were cultured on an air-liquid interface for four to six weeks to ensure the generation of well-differentiated, polarized cultures that simulated the *in vivo* pseudostratified mucociliary epithelium (Fulcher, Gabriel, Burns, Yankaskas, & Randell, 2005; Kesimer et al., 2009). Differentiated HTBE cultures typically secrete mucus containing about 1000 µg/ml of total protein on average. To obtain mucus secretions, the apical surface of the culture is washed using 500 µl of phosphate-buffered saline (PBS) solution for each Transwell column (Holmen et al., 2004). Each wash was collected after incubation for one-hour post-exposure at a temperature of 37°C, with centrifugation of 3000 g of the apical secretions for 10 minutes to eliminate shed cells and debris. Demographic characteristics of the airway primary epithelial cell primary cultures used found in **Table 3.** These cells and their apical secretions were exposed to tobacco product smokes in an exposure chamber as described below.

HTBE cell exposure to whole tobacco smoke (WTS)

In the little cigars and cigarillo studies, an LM1 smoke engine (Borgwaldt, Hamburg, Germany) was used to generate smoke according to the manufacturer's protocol (Clunes et al., 2012). The smoke generated from cigarette, little cigar or cigarillo was applied to cultured human tracheal bronchial epithelial (HTBE) cells that were transferred to the smoke apparatus. The control cells were exposed to ordinary air in an equivalent paradigm that matched the number of puffs obtained from the tobacco products in the investigated culture. Sterile Ringer's solution (120 mM NaCl, 5.2 mM KCl, 1.2 mM MgCl2, 1.2 mM CaCl2.2H2O, 12 mM NaHCO3, 24 mM HEPES, and 10 mM glucose, pH 7.4) was used to perfuse the HTBE cells during exposure. **Figure 8** illustrates the experimental design set-up to investigate the effect of little cigar and cigarillo on airway epithelia mucus barrier. Following exposure, the culture was then placed in the culture medium and returned to 5% CO_2 for incubation at 37°C. The cells were washed using 0.5 ml of PBS for each Transwell column at one-hour post exposure. The collected washes were stored in the -20°C freezer until analysis. The cells were exposed to smoke or air once per day for five consecutive days. The basolateral media was changed every day to keep the cultures sterile for the entire five-day duration. The machine was run for five to ten empty puffs between the smoking sessions to avoid cross exposure of different tobacco products during smoke exposure (Ghosh et al., 2017a). All tobacco products were stored at ambient laboratory temperature for 24-hours before use.

Little cigar brands/flavors and exposure paradigms. For comparison of the effects from conventional cigarettes, smoke was generated from Kentucky Research Cigarettes (KCS) (CODE 3R4F, Class A cigarettes, weight 1.01 ± 0.01 gm, and 8.4 cm per unit). Three commercial tobacco products that mentioned, "These Cigars are predominantly natural tobacco

with non-tobacco ingredients added" were investigated. The following brand names were collectively termed as little cigars (LC) in this study: Swisher Sweets Little Cigars (Filtered Little Cigars) (weight 1.34 ± 0.01 gm, length 10 cm per unit), Captain Black Little Cigars (weight 1.13 ± 0.01 gm, length 9.8 cm per unit), and Cheyenne Cigars (Full Flavor) (weight 1.38 ± 0.02 gm, length 9.8 cm per unit). For all studies, one puff was equivalent to 35 ml per 30 seconds using a butt length of 36 mm without covering the ventilation holes (unless otherwise mentioned). The above-mentioned smoke parameter produced 14-15 puffs for KCS, 18-19 puffs for Swisher Sweets (LCSS), 16-17 puffs for Captain Black (LCCB) and 20-21 puffs for Cheyenne (LCCN) (Ghosh et al., 2017a).

Cigarillo brands/flavors and exposure paradigms. The exposure paradigms of whole tobacco smoke (WTS) for conventional cigarettes (i.e., KCS) and CLLO were 14 x 35 ml puffs and 30 x 35 ml puffs, respectively, at a rate of one puff every 30 seconds. The cells were exposed to whole CLLO smoke. Another group of CCLO were exposed to 14 puffs only at the same rate, similar to the KCS exposure pattern. Three CLLO tobacco products were evaluated, which were collectively referred to as cigarillos: Swisher-Sweets cigarillo (SSW) (Swisher International, Inc.), Garcia y Vega Game black cigarillo (GBK) (Swedish Match USA, Inc.) and Hi-Fi Tropical Tango cigarillo (HTT) (Unitabac LLC, NH).

Waterpipe (hookah) smoke exposure (Figure 10): An S1000 shisha smoker machine (Borgwaldt KC, Hamburg, Germany) was used to generate waterpipe tobacco smoke as per the described Borgwaldt KC ISO protocol in order to obtain standardized WP tobacco smoking. To prepare the waterpipe, the head was filled with 15 grams of poplar shisha tobacco "Two Apples" flavor Al-Fakher brand (Al-Fakher Tobacco Trading, Ajman, United Arab Emirates). The other products tested were: Shiazo Steam Stones Two Apples flavor only without tobacco component

(Shiazo© Germany, Europe) as it was advertised as a form of tobacco-free smoking, and Two Apples Melon Flavor Hookah Herbal Sheesha. Shiazo Steam Stones is a premium quality shisha that has no tobacco, no nicotine and no tar. Tobacco or the flavor covered using aluminum foil has been earlier perforated (Ø 3 inch, 74 holes) for air passage. To start the smoking session, a rapidly lighting charcoal (40mm) was lit and put on top of the waterpipe head. Distilled water, measuring 750 ml, was poured over the bowl and the stem was placed 30 mm beneath the surface of the water (Eissenberg & Shihadeh, 2009; Neergaard et al., 2007; Schubert et al., 2015; Shihadeh, 2003). The HTBE cells were subjected to one smoking session every day for 5 days, beginning with a warm-up, wherein the cells received 20 puffs. The cells received 20 puffs at an interval of 60 seconds, which lasted from 3.62-3.70 seconds at 0.530 L volume per puff (Shihadeh, 2003). The same set up without tobacco or flavoring product was prepared using the shisha smoker machine to generate air to expose HTBE cells, which was represented as the air-sham group. As earlier described, PBS washes and apical secretions were collected and the basolateral media were changed on a daily basis.

In Vitro Trans-epithelial Electrical Resistance (TEER) Measurement

To assess the impact of NETP smoke exposure on airway monolayer epithelial cell integrity and permeability, the TEER technique (EVOM2™ Epithelial Volt/Ohm Meter, World Precision Instruments, Inc., Sarasota, FL) was utilized. STX2 manual electrodes were used according to the manufacturer's protocol, as described previously (Ghosh et al., 2017b; Srinivasan et al., 2015); (Ghosh et al., 2017a).

In Vitro Calcein AM/Propidium Iodide Assay

To characterize the effects of cigarillo and little cigars (CL) smoke exposure on the human airway epithelium, cellular viability was assessed using the calcein AM/propidium iodide

assay (live and dead cell staining assay). The calcein AM/propidium iodide assay method was performed as previously described (Ghosh et al., 2017). Briefly, one-hour post-exposure, the HTBE cells were washed with PBS, stained apically with 3-µM calcein AM (Life Technologies) in PBS and incubated for 30 min at 37°C. The cells were then washed with PBS, stained again with 150 µM propidium iodide (Sigma-Aldrich, St. Louis, MO) for 15 minutes at 37°C and then washed with PBS. Culture replicates from three donors were used. Afterwards, 10-20 random images per culture were captured using a Leica SP5 confocal microscope (Leica Biosystems) with a 63X glycerol immersion objective in XYZ scanning mode. ImageJ software was applied to quantify the stained cells. HTBE cells exposed to waterpipe smoke were stained similarly. The Infinite® M1000 PRO microplate reader (Tecan Trading AG, Switzerland) measured the fluorescence intensities

Label-free Quantitative Proteomic Analysis

A Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer coupled to an Ultimate 3000 nano HPLC system (LC-MS/MS system) (Thermo Fisher Scientific, Thermo Electron North America LLC) was used for label-free quantitative proteomic analysis of the apical secretions of HTBE cell cultures exposed to tobacco smoke (CLLO, LC and waterpipe). A volume of 250 µl (containing ~7.5 µg total protein as determined by BCA) were collected as starting volume apical secretions from six HTBE cells cultures for each exposure group and analyzed. Proteomic sample preparation was performed using the filter-aided sample preparation (FASP) method (Keller, Nesvizhskii, Kolker, & Aebersold, 2002). The cysteine residues were reduced and alkylated using 10 mM dithiothreitol (Sigma-Aldrich) and 50 mM iodoacetamide (Sigma-Aldrich), respectively.

The next step involved the addition of trypsin solution (25 ng/µl) to the sample, followed by overnight incubation at 37°C. The tryptic-digested peptides were vacuum freeze-dried and then dissolved in a solution containing 25 µl of 2% acetonitrile and 0.1% trifluoroacetic acid. The mass spectrometry runs were performed using peptide material from the samples, with each run using peptide materials that corresponded to one µg of total protein, as described previously (Kesimer et al., 2015).

Proteomic data analysis. The Proteome Discoverer 1.4 software (Thermo Scientific) was used to process the raw data and to search against the Universal Protein Resource (UniProt) protein sequence database. Validation of MS/MS based proteomic data was conducted using Scaffold 4.4.8 (Proteome Software Inc. Portland, OR, USA). The Scaffold Local FDR algorithm was used to establish peptide identifications at greater than 95.0% probability, where identifications were rejected at < 95.0% probability using the Protein Prophet Algorithm (Keller et al., 2002; Ma, Vitek, & Nesvizhskii, 2012; Kesimer et al., 2015). The latter algorithm was also applied for protein identification, with the added condition of identifying at least two peptides per protein. Proteins were annotated with GO terms from the geneassociation.goa_ human.gz file.

The next steps involved protein free-label quantification and generation of a heatmap using the Heatmapper web-server graphical interface by employing the average total precursor intensity and calculating the *Z*-score using the program application (Babicki, 2016). Principal Component Analysis (PCA) was performed using PerSPECtives 2.0.6 (Proteome Software Inc. Portland, OR, USA) by summarizing the intensities of the identified precursor ions for each protein as protein intensities. The protein intensities were normalized to the total intensity of all identified proteins in each sample. A Venn diagram was prepared in the Venny 2.1 online

reference interactive visionary tool (Oliveros, 2007). Analysis of variance (ANOVA) was performed to determine the significance of differences between the air and smoke exposure groups for culture replicates from six donors.

One-way ANOVA was then performed, and multiple comparisons using the Tukey method were conducted using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). The STRING algorithm was used for functional enrichment pathway analysis of proteins (STRING v10.0) (Szklarczyk et al., 2015).

Chemical Compound Analysis

Gas chromatography-mass spectrometry (GC-MS) was employed to perform chemical compound analysis of cigarillo tobacco products. The same smoking parameters were used, and the smoke was directed through Cambridge filter pads (44mm) (Borgwaldt, Hamburg, Germany). All filter samples were extracted with 5 ml of methanol (Optima grade), vortexed for one minute, filtered through a 0.2-µm syringe filter (filter-pressed dry) and then dried under nitrogen for approximately 30 minutes. The scintillation vials were weighed before and after extraction to determine the total extracted mass. The dried extracts were then reconstituted in pyridine (200 µL) and derivatized with 99:1 BSTFA: TMCS (300 µL) [BSTFA = N, O-bistrifluoroacetamide; TMCS =trimethylsilyl chloride] for two hours in a water bath at 50°C. The derivatized solutions were stored at -20°C until analysis.

GC-MS analysis was performed on a Bruker EVOQ 456 gas chromatograph-triple quadrupole mass spectrometer using an Agilent DB-5MS capillary column (30 m, 0.25 mm ID, 0.25 µM film) and helium carrier gas (99.999% purity). Derivatized filter extracts were diluted 10-fold in acetonitrile (Optima grade), and injections (1 µL) were performed with a Bruker CP-8400 autosampler with an injector temperature of 290°C. Samples were prepared and analyzed in

triplicate (diluted three times from the same derivatized solution). The GC oven was programmed with a 30-minute temperature gradient (60-300°C), with the carrier gas split ratio at 1:10, the transfer line at 250°C and the EI source held at 200°C. Full-scan mass spectra were acquired from *m/z* 40-600.

Compound identification was performed using the NIST 2014 mass spectral database and AMDIS chromatography software (Mass Spectrometry Data Center). Reports were exported from AMDIS to Excel based on library matching. In library matching, it was ascertained how well the experimental mass spectrum matched the database mass spectrum. All matches (across samples) were organized by compound name, and matches with inconsistent retention times were removed. The glycerol, which peaked at approximately a nine-minute retention time and was present in all samples was used as an indicator to confirm that there were no retention time shifts that could account for errant matches.

After removing inconsistent retention time matches, the data were sorted by retention time, and every match, except the highest scoring match, was deleted. AMDIS reports all potential matches for each GC-MS peak, although only a single compound is expected to be found, leaving only the best match for each peak in the final data. GC-MS was also employed to measure nicotine concentrations in the apical secretions. Concentrations were determined using a 1/x weighted calibration curve (10-500 ng/mL range). Three replicates per exposure group were measured, and the averages and standard deviations were compiled.

Results

Effect of New and Emerging Tobacco Product (NETP) Smoke on Primary Airway Epithelial Cells

The effects of cigarillo (CLLO), little cigars (LCs) and waterpipe (WP) smoke on viability of the human airway epithelia were evaluated by conducting live and dead cytotoxicity assays.

Meanwhile, trans-epithelial electrical resistance (TEER) measurement was performed to assess the cellular viability and dynamic tight junction integrity of the airway epithelial cells. The experiment revealed that new and emerging tobacco products (NETPs) from cigarillo-, little cigar-, and waterpipe-smoke exposure decreased cellular viability and airway epithelial cell tight junction integrity. Comparatively, given that the cigarillo is a whole tobacco product with a bigger size, and little cigars as their name implies are smaller as demonstrated in **Table 4**. The results showed that HTBE cells exposed to 14 puffs of either cigarette or cigarillo smoke were similarly affected **(Supplement Figure 1)**. However, in cells smoked to the whole cigarillo (30 puffs), the data showed that cigarillo tobacco products caused more epithelial cell death than exposure to air or cigarette smoke. Accordingly, a significant increase in the number of propidium iodide-positive cells in the CLLO and in LC smoke groups were observed as shown in **Figure 11A and B** and **Figure 12A and B** as compared to cells exposed to cigarette (KCS) smoke or room air. These results are similar to the findings of Ghosh et al. (2017), which were limited to LCs.

In waterpipe smoke-treated cells, the propidium iodide intensity increased after the exposure as indicated by fluorescence measurement using Tecan microplate reader. In WP smoked cells, both flavored only Two Apples (2App) and shisha tobacco with Two Apples flavor (2App+ TOB) groups manifested decreased cellular viability compared to air-sham. However, decreased cellular viability was statistically significant only in the latter group (i.e., 2App+ TOB group) as shown in **Figure 13A and B**.

Additionally, all investigated NETPs demonstrated statistical significance at the .05 level ($p < 0.05$) of reduced viability from TEER measurements after smoke-exposure of HTBE cells to CLLO **(Figure 11C)** and LCs **(Figure 14)** compared to those of air- and cigarette (KCS) smoke-

exposed HTBE cells. Likewise, the significant reduction was associated with WP smoked 2App+ TOB compared to those of the air-sham exposure group **(Figure 13C)**. These results are also similar to the findings of Ghosh et al. (2017), which were limited to LCs.

Label-free Quantitative Proteomic Analysis of Cell Secretions after NETP Smoke Exposure

Little Cigars Smoke Exposure

In the proteomic analysis of the apical secretions collected from little cigars (LCs) smoke-exposed HTBE, approximately ~200,000 spectra were acquired, leading to the identification of about ~4000 peptides which could be assigned to 1300 proteins. Around ~930 of these proteins were assigned at least 2 peptide identifications and subsequently included in the label-free proteomic quantitation using total intensities as the sums of individual precursor peak areas. A complete list of all proteins used in the quantification can be found in **Supplement Table 2.** Significant changes in expression of proteins displaying an ANOVA with p-value below 0.001 are shown in a heat map subsequent to chronic tobacco exposure **(Figure 15A).** One hundred thirty four proteins were altered quantitatively after chronic tobacco exposure, 84 proteins were significantly and uniquely upregulated after little cigar exposure while only two proteins were uniquely altered after cigarette (KCS) exposure **(Figure 15B).** The vast majority of proteins were involved in cellular metabolism **(Figure 15C).** However, proteins involved in immune response, antioxidants, secretory granules and apoptosis were altered **(Table 5).** Bar graphs of some affected proteins can be found in **Supplement Figure 2.** Pathway analysis was performed on these proteins. The analysis revealed that vesicle-mediated transport, detoxification of reactive oxygen species, metabolism of xenobiotic and cell migration/wound healing pathways were all altered by exposure to little cigar smoke **(Figure 15D)**.

Cigarillos Smoke Exposure

The apical secretions from cigarillo smoke-exposed HTBE cells presented qualitative and quantitative protein expression changes compared to those of air- or cigarette smoke-exposed HTBE cells. In secretions from all cells, approximately 118725 spectral peaks were obtained from the mass spectrometer and cross-referenced to about ~5200 identified peptides that were assigned to 727 proteins. The aforementioned criteria were also applied to identify these proteins in term of the number of peptides assigned and their signal intensities. Significant changes in the expression of approximately 389 proteins out of 727 proteins were observed **(Figure 16A1).** Proteomic analysis revealed that statistically significant differential expression of proteins occurred at both the quantitative and qualitative levels. Protein changes were observed across the smoke-exposed groups, but unique response profiles were seen among the groups exposed to smoke from different cigarillo brands **(Figure 16A2).** For example, the principal component analysis (PCA) illustrated clustering of protein expression in apical secretions from smoke-exposed HTBE cells compared to air-exposed cells as shown in **Figure 16B**. Furthermore, there was segregation between the KCS cluster and those generated by the cigarillo-exposed groups. Importantly, unique clustering patterns also occurred among cigarillo brands, such as HTT vs SSW and GBK, with overlap between the cigarillo brands GBK and SSW **(Figure 16B)**.

Hierarchical clustering of 44 differentially expressed proteins revealed a unique pattern with clustering of the cigarette smoked-exposed group and the cigarillo smoke-exposed group, as shown in the heatmap **((Figure 16C)**. The full list of proteins with statistically significant differential expression that were used in the quantification analysis can be found in the supplementary material **(Supplement Table 3)**. Based on enrichment analysis using GO terms

(Gene Ontology Consortium) of the identified proteins in the apical secretions from smoke-exposed HTBE cells, many biological processes were affected by cigarillo smoke, such as the innate immune response, the response to stimulus, and cell killing

The proteins that presented statistically significant differential expression in the cigarillo groups were subjected to pathway analysis using the string DB tools. Functional enrichment analysis showed the overexpression of pathways affecting various biological processes, including innate immunity, immune response elements **(Figure 16D)**, oxidative stress and the cell killing process **(Figure 16E)**.

Cigarillo smoke also mediated altered mucin expression. Significant decreases in the membrane-bound airway mucins MUC1, MUC4 and MUC16 were observed **(Figure 17A-C)**. Surprisingly, while MUC5B was significantly upregulated in the KCS cigarette smoke group, it was downregulated in the cigarillo smoke group **(Figure 17D)**. These observations indicate that exposure to cigarillo smoke altered innate immune processes, such as mucus clearance, antimicrobial responses and the complement system. Alterations in protein expression following exposure to cigarillos included downregulated BPI fold-containing family A and B, neutrophil gelatinase-associated lipocalin, complement C3 **(Figure 18A-D)**, and polymeric immunoglobulin receptor proteins **(Figure 18E)**. Significant increases in oxidative stress and reactive oxygen species levels were observed after exposure to cigarillos, including genes involved in the peroxiredoxin-1 and 5 **(Figure 19A-B)**, aldehyde dehydrogenase-3A **(Figure 19C)**, and alcohol dehydrogenase-1 **(Figure 19D)** oxidative stress pathways.

Waterpipe Smoke Exposure

To determine the effect of waterpipe smoke exposure on airway mucus proteome, the label- free quantitative analysis proteomic of HTBE cell apical secretions identified more

than 1700 proteins among all exposure groups. Approximately more than 300 proteins underwent statistically significant differential expression changes after waterpipe smoke exposure. Sufficient details of this finding can be found in **Supplement Table 4**

The data analysis also revealed that about 275 proteins were upregulated in the 2App+TOB exposure group, whereas 168 proteins were upregulated among the group exposed to the flavor only without tobacco (2App) **(Figure 20A)**. Furthermore, 16 and 45 proteins uniquely and differentially expressed in 2App+TOB and 2App exposure groups, respectively, were also found **(Figure 20B)**. As illustrated by the principal components analysis (PCA) **(Figure 20C)**, protein expression in apical secretions from waterpipe tobacco smoke-exposed HTBE cells were clustered differently compared to air-sham-exposed cells. The PCA also demonstrated similarity overlaps between the flavors and tobacco groups, as depicted in the hierarchical heatmap, which displayed the pattern of protein expression for each exposed group **(Figure 20D)**. Volcano plot analysis was also performed to enumerate distinct exposures between the tobacco and flavor groups. The results not only revealed that the protein expression profile changed after exposure to flavor or tobacco **(Figure 20E and F)** compared to the air-sham control, but the protein profile also changed between the flavor and tobacco exposures as shown in **Figure 20G**.

The experiment on waterpipe smoke exposure also indicated biological process alterations **(Figure 20)** such as adhesion, cell killing, immune system, and in the metabolic process. The result exhibited that waterpipe-smoked HTBE altered processes in the immune system and innate immune responses. For example, BPI fold-containing family A1 **(Figure 22A),** Galectin-3 **(Figure 22B),** neutrophil gelatinase-associated lipocalin **(Figure 22C)** and complements factor B **(Figure 22D)** and w C3 **(Figure 22E)** were downregulated in both, flavor and tobacco exposed groups, while matrix metalloproteinase-9 (MMP-9) **(Figure 22F)** and high

mobility group protein-B1 (HMGB1) **(Figure 22G)** were upregulated. However, gamma-interferon-inducible lysosomal thiol reductase (IFI30) **(Figure 22H)** downregulation was associated with 2App+ tobacco group only.

It is evident that waterpipe smoke exposure increased proteins that are involved in oxidative stress and detoxification process, such as glutathione reductase **(Figure 23A)**, thioredoxin reductase-1 **(Figure 23B)**, thioredoxin **(Figure 23C)** and aldehyde dehydrogenase 1A3 **(Figure 23D)**, in addition to aldo-keto reductase family-1 C1 and C3 **(Figure 23E and F)**. Furthermore, enrichment pathway analysis indicated the significant differential expression protein change after waterpipe smoke displayed pathways were involved in activation of the immune response signaling pathway **(Figure 24)**, oxidative stress, and cell death (**Figure 25**).

Chemical Compound Analysis of Cigarillo Smoke and Comparison to Cigarette Smoke

The chemical compounds of little cigar smoke were published previously indicating that LCs produced more chemicals than cigarettes (Ghosh et al., 2017a). The current study ventured to associate the effects of cigarillo tobacco products to the toxicity of their chemical components by investigating the characteristics of these chemical compounds in of their gas and mainstream smoke extracts generated by the smoking machine. The gas chromatography-mass spectrometry (GC-MS) analysis revealed that various cigarillo brands (SSW, GBK and HTT) and cigarettes shared similar chemical compounds. The analysis, however, further demonstrated that the chemical profiles of extracts from cigarillo tar particles were different from those of KCS cigarettes **(Figure 26A-B)**. In this analysis, 22 (29.7%) chemical compounds were common among the investigated tobacco products, including KCS while 12 compounds (16.2%) were common among the SSW, GBK, and HTT groups but absent from KCS **(Figure 26C)**. The list of

all compounds identified from the analysis is provided in the Supplementary Material **(Supplement Table 5).**

Moreover, the analysis revealed marked differences in chemical profiles among the different cigarillo brands. For example, 3-ethoxy-4-hydroxybenzaldehyde, 4-methoxybenzaldehyde, 2, 2-dimethyl-2-sila-1, 3-dioxacyclohexane, ethyl propanoic acid, and 2-ethylhexyl hexyl ester sulfurous acid were exclusively detected in the GBK brand, whereas dihydro-5-pentyl-2(3H)-furanone, 3-ethoxybenzaldehyde, and hexanoic acid were identified only in the HTT brand. Meanwhile, 2-(2-(2-Ethoxyethoxy) ethoxy) ethanol and mannitol were detected only in SSW cigarillos.

The data also showed that cigarillos deliver more nicotine to cells. Nicotine is well known as the primary addictive component in tobacco products (US-HHS, 2010). The nicotine levels in the apical secretions of smoke-exposed HTBE cells were measured to estimate the amount of nicotine delivered to cells from each of the tobacco products. In the apical secretions of smoke-exposed HTBE cells, the peak nicotine levels after smoking were detected at one-hour post-exposure. We observed that the secretions from 14 puffs pattern cigarillo smoke had relatively high nicotine level compared to cigarette **(Supplement Figure).** The data show that SSW and HTT delivered 47 % more and GBK delivered 119% more nicotine to the cells compared to cigarettes **(Supplement Table 1)**. However, the results show that the secretions from the whole cigarillo smoke-exposed group contained higher nicotine levels after one hour of exposure, with the potential to deliver more nicotine to the cells at an average of 203.6 ng/ml than cigarettes, at an average of 45.8 ± 20 ng/ml **(Figure 26D)** Notably, the different brands of cigarillo products delivered different levels of nicotine to the cells. The GBK brand was found to deliver the highest nicotine levels (264.9 ± 61 ng/ml), whereas the SSW brand delivered the

lowest nicotine levels (137.4 ± 30 mg/ml). Meanwhile, HTT delivered a nicotine concentration of 207.0 ± 32 ng/ml.

Discussion

Airway epithelia and mucus are in direct contact with tobacco smoke and provide easily accessible pool of proteins that are required for lung health. They can also be used as biomarkers of tobacco exposure and in the assessment of toxicity from tobacco smoke (Aguiar et al., 2019; Shields et al., 2017). Extensive research has shown that cigarette smoking has adverse effects on the respiratory and airway mucociliary systems, including but not limited to alterations of the mucus clearance system (Aufderheide et al., 2015; Ramos et al., 2014; Yoshida & Tuder, 2007), a proinflammatory process (Lee et al., 2012), increased oxidative stress elements (Isik et al., 2007; Reidel et al., 2018; Tavilani et al., 2012), apoptosis (Ghosh et al., 2017b), and dysregulation of the innate immune/immune response (Jaspers, 2014; Qiu et al., 2017). However, the public health risk of NETPs including little cigars (LCs), cigarillo (CLLO), and waterpipe (WP) smoking have not been sufficiently studied compared to that of regular cigarettes, despite an increasing number and variety of these products in many different brands and flavors becoming available on the market, according to CDC's Morbidity and Mortality Weekly Report (Hu et al, 2016). It is, therefore, an important and escalating need to better understand the risks associated with these new and emerging products of tobacco smoking.

Little cigars. The investigation on little cigars in this research demonstrated that compared to cigarettes, LCs, which are smaller in size than the typical cigarillo, result in decreased cell viability and increased deleterious effects on the airways, similar to the findings of Ghosh et al. (2017). Moreover, the cellular cytotoxicity and apical secretion protein profile of LCs were significantly greater than the Kentucky traditional experiment cigarettes. Many altered

proteins are involved in key pathways that would be used to ameliorate the increased toxic burden, including proteins involved in the detoxification of xenobiotics, e.g. aldehyde dehydrogenase (Jang et al., 2014) or carbonyl reductase (Kalabus et al., 2012) and proteins like peroxiredoxin and glutathione reductase (Bazzini et al., 2013) that are required to metabolize reactive oxygen species. Reactive oxygen species contribute to the toxicity of tobacco exposure (Valavanidis et al., 2009) and upregulation of these proteins is likely a contributory response to this process.

Airway mucus, a key component of the lung's natural defense system, contains over a thousand proteins including mucins, anti-microbial proteins, and proteases that are required for innate fortification and the appropriate regulation of inflammation (Bartlett et al., 2008; Candiano et al., 2007; Kesimer et al., 2009a; Parker & Prince, 2011; Travis et al., 2001). Dysregulation of the airway mucosal defense proteins were observed after LC smoke exposure, which suggests that pulmonary protection may be altered by exposure to LC smoke. There are a host of studies which supported a common conclusion that cigarette users bear an increased susceptibility to both viral and bacterial infections (Bagaitkar et al., 2008; Brook & Gober, 2005; Givon-Lavi et al., 2006). Comparatively, LC users may be bearing the same or heightened susceptibility to these infections as well. However, further studies will be needed to confirm this hypothesis.

Although these research data provide scientific evidence that exposure to LC smoke significantly altered the airway epithelial proteome, the result was derived from an *in vitro* model, where a living system from a human being in the form of isolated cells was utilized to enable focused investigation of the effect(s) of compounds. However, results from *in vitro* model research are only as reliable as the degree to which such *in vitro* or isolated system "replicates

the biology of the human target cells and their responses to toxic substances" (Institute of Medicine & National Research Council, 2005, p. 218). Thus, further research is essential to fully understand the mechanism of action, in this case, of NETP smoke on the airway mucus and lung epithelial cells. It should be clear at this point that in the present research an *in vitro* sample of a relatively pure population of bronchial epithelial cells were examined, whereas *in vivo*, immune cells and alveolar epithelia all together contribute to the dynamic airway mucus proteome. Overall, data from this study evaluation indicate that LCs exert significantly more toxic effects than regular cigarettes and elicited a greater biological response from the epithelia as they adapted to the noxious environment caused by chronic LC tobacco exposure.

Cigarillos. The portion of the present study on cigarillos helped to fill the health information gap on this form of NETP by characterizing their effects on the airway mucosal barrier at multiple levels using an *in vitro* model. Several novel findings are evident from these studies. Results indicate that the effect of whole cigarillo smoke on primary epithelial cultures was greater than that of cigarette smoke, as measured by cytotoxicity, cell integrity, and protein expression. Cigarillo smoke contains more potentially toxic chemicals than cigarette smoke, which could be linked to the observed increase in cytotoxicity and alterations in cell integrity and protein expression. These findings contradict the popular misconception that cigarillos are safer and are associated with fewer health risks than cigarettes. Cigarillos are also bigger in size compared to cigarettes and little cigars **(Table 2)**, thus, a whole cigarillo results in twice as many puffs than a cigarette (KCS)

In the HTBE cells exposed to the cigarillo smoke protocol for five days, there was an increasing in propidium iodide-positive cells. This provides evidence that cigarillos are associated with more toxic effects than cigarettes. Furthermore, different cigarillo products

exhibited a range of cytotoxicity levels. While cigarillo products are categorized as one tobacco entity, the present study showed that cigarillo products vary in physical characteristics, chemical components and the amount of nicotine levels delivered to cells. Accordingly, variations in the magnitude of the risks to airway health associated with different cigarillo brands or tobacco products were demonstrated. To illustrate, the analysis showed that greater quantitative protein expression dysregulation was associated with the cigarillo group, particularly the GBK brand, which delivered the greatest amount of nicotine to the cells. Notably, chemical analysis showed that Garcia-y-Vega Game black cigarillo (GBK) brand contains five (6.8%) unique chemical compounds that were not identified in other cigarillo brands **(Figure 26C).**

Studies have also shown that low-nicotine content cigarettes reduce dependence and increase the chances that a user will quit smoking (USHHS, 2000). The FDA reacted by recommending a reduction in the nicotine level of cigarettes during manufacture. The aim of the FDA pronouncement was to reduce the risks to health and tobacco dependency among smokers ((FDA), 2017). However, as demonstrated, cigarillo tobacco products used in this study, which are not even regulated by the FDA, contain more chemical compounds than cigarettes and delivered greater amounts of nicotine. In addition, the differences in physical characteristics and chemical composition among cigarillo products provide insights into their harmful effects on airway biology.

The accumulation of oxidative damage has been implicated in both acute and chronic cell injury (Van Eeden & Sin, 2013). Furthermore, oxidative stress may participate in the pathogenesis of some airway diseases, such as in COPD (Tavilani et al., 2012). The oxidant/antioxidant imbalance caused by cigarette smoke may also contribute to the inflammatory process (Rahman, 1999). The results of our investigation of protein expression in

the secretions of HTBE cells support the observation that cigarillos induce more oxidative stress and cause more profound alterations in the lung's innate immune response than air and conventional cigarette smoke. Thus, the oxidative burden produced by cigarillo smoke can be worsened in the lungs by the addition of dysregulated and altered expression of immune response proteins and the activation of inflammatory leukocytes in the lungs of cigarette smokers (Lee et al., 2012). These effects increase and prolong the insult to the lungs with chronic exposure to tobacco and other tobacco product smoke. It should also be noted that various lung diseases are associated with and mediated by chronic inflammation (Chen et al., 2018).

Cigarette smoking is well documented to cause changes in innate immune responses (Qiu et al., 2017). Smoking has been implicated in the production of many immune or inflammatory mediators, including both proinflammatory and anti-inflammatory cytokines. Numerous studies have shown that cigarette smoking has adverse effects on the respiratory system, the immune system, and the natural host defense mechanisms of the body, such as mucociliary clearance and antimicrobial activity (Jaspers, 2014; Mall, 2008; Radicioni et al., 2016). Dysregulation of immune proteins alters immunological homeostasis and restores immune tolerance, and can, therefore, contribute to the development of diseases and increase susceptibility to secondary microbial infections (Bals et al., 1999; Boucher, 2007; Stämpfli & Anderson, 2009; Voynow & Rubin, 2009).

In a similar manner, exposure to cigarillo smoke altered proteins that are important in the body's inherent immune processes, such as mucus clearance, antimicrobial responses and the complement system. For instance, the decrease in the short palate, lung, and nasal epithelial clone 1 (SPLUNC1) protein, which is also known as bactericidal/permeability-increasing-fold-containing family A, member 1 (BPIFA1), was associated with cigarette and cigarillo smoke.

BPIFA1 is a protein that is secreted into the airway lumen, where it maintains airway hydration via interactions with the epithelial sodium channel and contributes to airway surface liquid homeostasis and proper clearance of mucus (Garcia-Caballero et al., 2009; Moore et al., 2018). Other proteins that were altered and decreased in response to cigarillo smoke include neutrophil gelatinase-associated lipocalin, which contributes to bacterial overgrowth (Bartlett et al., 2008).

Similarly, the levels of polymeric immunoglobulin receptor proteins were decreased, which suggested alterations in the transcytosis of soluble dimeric IgA antibodies in epithelial cells (Kaetzel, 2013). Secretory IgA antibodies represent the first line of antigen-specific immune defense at mucosal surfaces. Dysregulation of polymeric immunoglobulin receptor protein expression and subsequent alteration of transcytosis and immune complexes from the basolateral to the apical mucosal epithelial cell surface result in profound consequences for the pathogenesis of infections and inflammations (Hunziker & Kraehenbuhl, 1998; Kaetzel, 2013; Moore et al., 2018; Ohlmeier et al., 2012).

Another compound, uteroglobin is an important innate defense/ immunomodulatory protein that is highly expressed in the lower airways, mainly in nonciliated Clara cells (also known as CC10-positive cells) (Rokicki et al., 2016). Studies have demonstrated that CC10 plays an anti-inflammatory role in the lung (Hay et al., 1995; Mantile et al., 1993). The expression of this protein was highly suppressed after cigarillo exposure in mice, suggesting one mechanism through which immunosuppressive effects may occur in the airways.

As previously mentioned, multiple studies have demonstrated that cigarette smoke has the potential to induce respiratory mucins via proinflammatory stimuli that are relevant to COPD pathogenesis and, thus, contribute to mucin hyperproduction status in COPD patients (Nikota & Stampfli, 2012; Ramos et al., 2014; Seys et al., 2015; Yoshida & Tuder, 2007)).

Similarly, the effects of cigarillo smoke involve alterations in mucin homeostasis and subsequent mucociliary clearance in the airways. In the present study, relevant tissue responses were measured to evaluate the adverse health effects of tobacco products. The findings were consistent with those of previous studies, in which respiratory mucins, such as mucin MUC5B and mucin 1, were elevated following exposure to cigarette smoke. However, mucin-related proteins, including mucins MUC5B, MUC16, MUC4, and MUC1, were downregulated after cigarillo smoke exposure.

Alteration of MUC5B concentrations had been linked to pathologies of the lung. Increased MUC5B is related to chronic bronchitis/airway obstruction (Kesimer, et al., 2017), whereas decreased MUC5B is associated with impaired mucociliary clearance in aged mice (Grubb et al, 2016). The observation in this study regarding decreased mucin concentrations is a surprising result, which may be due to reduced expression and/or enhanced clearance of these molecules. In this respect, future studies are essential for further clarification.

Taken together, the study findings indicate that cigarillo exposure leads to potential health risks and causes damage to the airways. These results could potentially serve as the basis for the regulation of tobacco and cigarillo products and weigh heavily on considerations related to health risk and the redirection of public perception. However, this study is somewhat limited by the fact that among adolescent cigarillo users, only approximately 38.2% use cigarillos as marketed, whereas the majority of users make alterations of the product for their enjoyment. To illustrate, 40.3% of cigarillo smokers mix cigarillo tobacco with marijuana, and an additional 28% use other manipulation methods, such as adding or removing tobacco from the wrapper (Kong et al., 2017; Blank, Cobb, Eissenberg, & Nasim, 2016; Blank, Nasim, Hart, & Eissenberg, 2011; Kostygina, Huang, & Emery., 2017).

Qualitative study findings showed that cigarillos are generally manipulated and used as blunts by young adults (Giovenco et al., 2017; Koopman Gonzalez, Cofie, & Trapl, 2017). "Blunts" are defined as cigar shells (e.g., large cigars, little cigars, or cigarillos) mixed with marijuana after some or possibly and/or alternatively all of the tobacco has been removed (Yerger, Pearson, & Malone, 2001; Koopman Gonzalez et al., 2017). Thus, smoking blunts can expose users to high levels of nicotine, as well as toxicants. This increases the odds of users developing dependence on cannabis and nicotine (Timberlake, 2009). It is quite alarming that cigarillo product characteristics are traditionally associated with blunt use because this may shape the tobacco market regions where marijuana usage is legal (Giovenco et al., 2018). For instance, the Splitarillos brand of cigarillos uses marijuana flavors to promote cigarillos to consumers (Kostygina et al., 2017).

Cognizant of the foregoing concerns, future research directions point towards the necessity of understand the various features of cigarillo products which are subject to consumer manipulation. Such information is vital to inform the regulation with respect to product design and attributes, with the goal of reducing cigarillos' appeal to young consumers, and habitual users in general. Another critical pathway for future studies would be examination of co-use of tobacco and cannabis among adolescents and the its contribution to health and nicotine dependence outcomes. Thus, on the whole, research on how adolescents are using cigarillos is essential to inform regulators on both product design and attributes can discourage use among youth by reducing appeal to youth consumers.

There are wide variations among the cigarillo tobacco flavors/brands commercially available on the market, which are expected to exert varying health effects on the airways. Clustering or categorizing these tobacco products might be necessary to enable

authorities to carry out a more comprehensive analysis of their short-term and long-term health effects on the airways. With more comprehensive analysis of these products, adequate regulation, and if necessary, control measures can be put in place.

Waterpipes. The waterpipe now serves as a favored method for the consumption of various derivations of fruit-flavored tobacco among young adults (Ambrose et al., 2015; Maziak et al., 2004; Strulovici-Barel et al., 2016). In fact, it has become a common belief among waterpipe users that the water in the equipment used in waterpipe smoking filters out "toxins" from the smoke, making the waterpipe a far less harmful habit and providing a safer smoking alternative to cigarettes (Akl et al., 2010; Noonan, 2013; Smith-Simone et al., 2008). Nevertheless, studies have shown that waterpipe smoke contains high levels of toxins and chemicals, and that waterpipe tobacco users inhale large quantities of the potentially harmful toxins that induce tobacco-related disease (Eissenberg & Shihadeh, 2009; Jawad et al., 2018; Rammah, Dandachi, Salman, Shihadeh, & El-Sabban, 2012, 2013; Shaikh, Vijayaraghavan, Sulaiman, Kazi, & Shafi, 2008; Primack et al., 2016).

Despite the increasing prevalence of waterpipe smoking, there is lack of data on the airway health effects of waterpipe smoking and there are less or no federal regulations regarding its use (WHO, 2005). The study findings on waterpipe smoking show that tobacco exposure through waterpipe smoke decreases cellular viability and integrity of the airway epithelial barrier, which is associated with alterations in the protein expression in apical secretions of smoked HTBE cells, similar to cigarette smoke exposure. This suggests that waterpipe smoke can pose a potential health risk in the airways and challenges the concept that waterpipe smoking, as an emerging way of smoking tobacco, is a "healthier" alternative to cigarette smoking.

Through animal studies, *in vivo* research have shown the association between waterpipe smoke and lung injury by promoting changes in inflammatory and oxidative stress biomarkers (Khabour et al., 2015; Khabour et al., 2018; Khabour et al., 2012; Khan et al., 2018). In a more recent study, results revealed that mice exposed to waterpipe smoke had increased the levels of total protein in their bronchoalveolar lavage fluid (BALF) as compared to unexposed mice (Khabour et al., 2018). In particular, waterpipe smoke exposure induced airway inflammation. Inflammatory cells such as lymphocytes, macrophages, and neutrophils increased in the BALF of mice exposed to tobacco smoke (Khabour et al., 2015; Khabour et al., 2018; Khan et al., 2018; Rammah et al., 2013).

Studies also found evidence that exposure to waterpipe smoke increased the levels of TNFα, IL-6, and IL-1β in the lung tissue and BALF of mice (Khabour et al., 2012). The cytokines IL-6, TNFα, and IL-1β, are considered as biomarkers of ongoing inflammation. It should also be noted that TNF-α is an essential inflammatory mediator that exerts a major role in the development of such illnesses as asthma, chronic bronchitis, chronic obstructive pulmonary disease, acute lung injury, and acute respiratory disease syndrome (Rammah et al., 2012; Shaw et al., 2014).

The analysis shows that the quantitative protein expression changes in waterpipe smoked-HTBE cells, resulted in altered expression of proteins that play an important role in the innate immune processes, such as mucus clearance, antimicrobial responses and the complement system. As an illustration, the BPI fold protein A1 (BPIFA1) is involved in the airway inflammatory response and contributes to airway surface liquid homeostasis, as well as proper clearance of mucus (Garcia-Caballero et al., 2009; Moore et al., 2018). In this study, BPIFA1 was significantly decreased due to waterpipe smoke exposure. Moreover, gelatinase-associated

lipocalin also plays an important role in the body's natural immune response to bacterial infection (Bartlett et al., 2008). However, in this study, waterpipe smoke exposure induced a downregulation of this protein (i.e., the gelatinase-associated lipocalin).

Analysis form the *in vitro* study showed that downregulation of metalloproteinase 9 (MMP9) is associated with waterpipe exposure. This is consistent with an animal study which revealed an association between waterpipe smoking and lung injury. After exposure to waterpipe smoke, the levels of metalloproteinase (MMP) proteases in the lungs of mice, changed. Particularly, the expression of MMP-1, MMP-9, and MMP-12 proteins increased significantly. (Khabour et al., 2015). Thus, inflammatory response after the exposure is attributed and mediated by the release of MMPs (Navratilova et al., 2016; Segura-Valdez et al., 2000)

Another class of molecules are complement proteins, which are known to regulate immune cell functions (Andoh et al., 1993; Andoh et al., 1998; Gonzalez-Begne et al., 2011). In the waterpipe experiments, some complement proteins decreased after waterpipe smoke exposure. The expression of these proteins were highly altered after *in vitro* waterpipe smoke exposure, suggesting mechanisms through which immunosuppressive effects may occur in the airways. Another interesting protein that was altered by waterpipe smoke exposure is the high-mobility group protein-1 (HMGB1), which exhibits pro-inflammatory activities involved in regulation of the inflammatory response and oxidative stress-mediated autophagy (Sims et al., 2010). The HMGB1 protein also activates inflammatory cells through multiple surface receptors (Andersson et al., 2000; Sims et al., 2010). It also regulates inflammatory responses and interacts with component of the adaptive immune response, such as TLRs and cytokines (Andersson et al., 2000). One study argued that HMGB1 contributes to the pathogenesis of various chronic inflammatory and autoimmune diseases (Urbonaviciute et al., 2008). In this context, it is being

suggested that alterations in the HMGB1 may be a mechanism through which waterpipe smoke may trigger inflammation.

Collectively, altered expression of these three proteins may point towards mechanisms by which waterpipe smoke exposure may contribute to enhanced susceptibility to lung inflammation and infection. Certainly, further studies are warranted to fully understand the impact of waterpipe smoking on the function of these immune system proteins. Elucidating the functions of these sets of immune proteins in lung immune homeostasis, will contribute to a better understanding of the pathogenesis of airway diseases, including COPD.

Animal studies demonstrated that oxidative stress is induced by waterpipe smoke (Javed et al., 2017; Khabour et al., 2018). It was also evident that enzymes responsible for oxidative stress, such as glutathione peroxidase and superoxide dismutase (Khabour et al., 2018), and myeloperoxidase levels (Khan et al., 2018) significantly increased in the BALF and lungs of exposed mice. These findings contributed to a significant understanding of mechanisms by which exposure to waterpipe smoke lead to lung inflammation and oxidative stress among mice.

Additionally, it is also important to realize the effect of using flavors only without tobacco in the waterpipe smoking instrument. The present study revealed that the flavors of the NETPs used in the experiments, by themselves, cause a harmful effect observed through increased oxidative stress, as seen on the detoxification process-related proteins. The latter proteins may be increased to help defend and detoxify the airway against the burden of exposure to the flavor used on the NETP (Isik et al., 2007; Kaur et al., 2018; Macnee & Rahman, 1999).

There are many flavoring chemicals used in manufacturing NETPs to increase its appeal to consumers. These flavors include apple, berry, fruits pineapple mango, etc. (Primack et al., 2012; Smith-Simone et al., 2008) . These flavors have been approved by the FDA as food

additives, meant to be ingested and digested (FDA, 2018b). However, when these food additives are used as ingredients for other products, like tobacco products for inhalation, the stamp of safety by the FDA may be compromised. The logic is simple: food additives may be safe for ingestion, but those studies do not justify the approval of such food additives for smoking and inhalation.

There are currently few, if any studies that scrutinized the safety of food flavoring chemicals for inhalation or breathing. Thus, the scientific community, health care professionals, and regulatory bodies have practically no knowledge about the health risks associated with the inhalation of food flavor additives. Additionally, most of the tobacco used in waterpipes are flavored to render them more appealing among youth and young adult consumers and further increase WP popularity among patrons (Primack et al., 2012; Smith-Simone et al., 2008; Ambrose et al., 2015). The above arguments, therefore, necessitate investigation and examination of the specific heath impact of NETP products, marketed under the flavors only category and the tobacco with flavoring category, in the airway, with special attention to the complexity of combining these two categories or combining one or both categories with other mixes that waterpipe aficionados concoct to enjoy social smoking more. Such combinations and mixing practices may have a different and adverse health effects and consequences.

This study concludes that waterpipe smoke exposure in an *in vitro* model leads to decreased cellular viability and alteration of their apical secretion proteins, as well as the innate immune response system and tends to increase the occurrence of oxidative stress. Nevertheless, these result from an *in vitro* model may not be similar to the dynamic smoking behavior or smoking pattern involved in an *in vivo* model. Researchers should also take into consideration that previous studies revealed the duration of a session for smoking waterpipe ranges

approximately from 45 to 60 minutes. The same studies identify the tobacco consumption in one sitting to be about 171 puffs (Morris et al., 2012; Shihadeh et al., 2004; Neergaard et al., 2007). In this respect, therefore, more studies are warranted to elucidate how the natural human smoking behavior and exposure and its consequences affect *in vivo* human airways in the biological and healthcare context.

Other challenges to research characterizing the adverse health effects of waterpipe tobacco are the complexity of the setting, including the user exposure to volatilized tobacco products, flavors, carbon monoxide and charcoal components. Additionally, the contextual factors become more complex as consumption of multiple tobacco products come into play. This scenario is more real than hypothetical because a recent study indicated that approximately 40% of youth and adult tobacco users in the US consume multiple tobacco products, otherwise termed as polytobacco usage (Lee et al., 2014; Kasza et al., 2017; Trapl et al., 2016). Given, therefore, that many waterpipe users consume multiple tobacco products, there is an urgent need to further evaluate the health risks associated with waterpipe smoking within the context of multiple tobacco product use, and perhaps even, concurrent substances use.

Conclusions

This chapter presents the study, its rationale and objective to evaluate the effects of new emerging tobacco products (NETPs) in the form of little cigars, cigarillos and waterpipe smoking on the airway mucin/mucus proteome. Particularly, the investigation evaluated the potential harm emanating from the use of these tobacco products and the potential health risks associated with their use in smoking. The findings showed that the tobacco products examined in the present study pose similar or even more adverse health effects compared to regular cigarettes.

In particular, little cigars, cigarillo and waterpipe smoke were found to cause reduced cell viability and integrity, alter protein expression patterns, and induce oxidative stress proteins.

The results of the study suggest that cigarillo tobacco products may be associated with a wide range of health risks in terms of airway biology. Currently, cigarillos form one broad category of tobacco products, but the data derived from three distinct brands revealed that different cigarillo products presented significant differences in terms of health risks, chemical compounds, effects on cellular viability, and protein expression profiles. This result further suggests that it may be useful to create more specific tobacco product subcategories to better inform users and the public, in general, about the nature and effects of these products.

The present study also indicates that acute tobacco exposure through waterpipe smoke changes the integrity of the airway epithelial barrier and alters the expression of the protein in the secretions from HBE cells, comparable to cigarette smoke exposure. This is highly suggestive that waterpipe smoke can pose potential health risks in the airways and challenge the concept that waterpipe smoking, as an emerging way of smoking tobacco, is a “healthier” alternative to cigarette smoking.

CHAPTER 3: Airway Exosomal miRNA Transcriptome Profiling Post-Exposure to New and Emerging Tobacco Products (NETPs) Smoke

Overview

Exosome-like vesicles are small membrane vesicles secreted by the epithelial cells of the airway tract. They play an important role in the lung's innate immunity, in the remodeling of the epithelium, in airway biology, and in intercellular communication through vesicular cargo, including the miRNA. There is strong evidence that exosomal miRNA (circulating miRNAs) participate in the biological response to environmental exposure and have a role in the dynamic regulation of the airway tract response to a broad range of internal biological processes and environmental conditions or exposures of the body to such substances such as smoke. Thus, profiling exosomal miRNAs may contribute to the identification of tobacco exposure biomarkers that predict airway biological effects. The exosomal miRNA profile may also be used in the evaluation of the consequences of harm from tobacco products. Cognizant of the utility of the exosomal miRNA profile to understand how tobacco smoke harms the airway, this study investigated the potential role of exosomal miRNAs to evaluating the effect of smoke from New and Emerging Tobacco Products (NETPs). We hypothesized that tobacco smoke from NETPs in the form of little cigar, cigarillo and waterpipe changes the cargo, the miRNA, in the exosomes derived from airway epithelial cells.

Cultured human primary airway epithelial cells were exposed to little cigar, cigarillo and waterpipe smoke. Afterwards, apical secretions were collected and processed for isolation of the exosome using sequential differential centrifugation. The exosomal miRNA profile was

identified by using HTG EdgeSeq technology and next generation sequencing platforms. The differential expression analysis was calculated by the statistical method using DeSeq2 software.

The comparative exosomal miRNA analysis revealed that exposure to smoke from NETPs in the form of little cigar, cigarillo and waterpipe resulted in alterations in the HTBE cells, in which dysregulation set of exosomal miRNA expression was observed. The sets of miRNAs were predicted to be involved in mechanisms related to bacterial invasion of epithelial cells, immune response, gene-regulated membrane organization, response to stress, regulated cell death, and regulation of catalytic activity. The data generated directly assessed relevant changes in the airways which may be biologically associated with tobacco use and may contribute to the science base to inform the authorities in the regulation of these tobacco products.

Introduction

Exosome-like vesicles are small membrane vesicles which are secreted from multi-vesicular endosomes by most cell types, including epithelial cells, immune cells, reticulocytes, and tumor cells (Bobrie et al., 2011; Soo et al., 2012). They are found in many biological fluids such as plasma (Bonnerot et al., 2005), urine (Pisitkun et al., 2004), bronchoalveolar lavage fluid (BALF) (Kim et al., 2018), mucus, and saliva (Kesimer & Gupta, 2015; Kesimer et al., 2009b; Taylor & Gercel-Taylor, 2013). Exosome like-vesicles potentially contribute to intercellular communication through their vesicular cargo (Harischandra et al., 2017; Valadi et al., 2007). As a consequence of their regulation of gene expression, the components of exosomes (DNA proteins, mRNA and micro-RNA) have the ability to influence multiple pathophysiological processes in recipient cells (Harischandra et al., 2017; Russ & Slack, 2012). The airway tract exosomes are involved in the innate immunity of the lungs, in the remodeling of the epithelium, and in airway biology (Kesimer et al., 2009b).

There is research evidence that HTBE cell exosomes formed after cigarette smoke exposure-related stress are responsible for airway remodeling pathogenesis in COPD (Sessa & Hata, 2013; Szymczak et al., 2016). One study also concluded that the biology of exosomal miRNAs, particularly in the context of the airway tract, is reflective of the lungs being constantly exposed to different stressors ranging from cigarette smoke to noxious chemicals (Alexander et al., 2015). Exosome-like vesicles play an important role in intercellular communication through vesicular non-coding RNA cargo (Gupta et al., 2018). These transported non-coding RNA, also known as miRNA, can enable different immune cells and epithelial cells of the airway tract to communicate with each other (Bobrie et al., 2011; Choi et al., 2012; Neudecker et al., 2017). Exosome-like vesicles perform various functions, such as, the delivery of complex intercellular

messages and the removal of toxins or excess molecules from cells (Harischandra et al., 2017; Kesimer et al., 2009b). These functions provide an important mechanism for mediating different stress-induced cellular responses (Alexander et al., 2015; Benedikter et al., 2017).

In general, microRNA (miRNA) belongs to the diverse group of a micro-sized noncoding RNA molecules, is approximately 22 nucleotides in length, and negatively regulates gene expression (Ambros, 2004). The function of microRNA mainly involves silencing of the RNA and managing the post-transcriptional expression of genes by interacting with the targeted mRNA, thus, inhibiting protein translation (Ambros, 2004). There is established evidence that the mature miRNAs can also move into extracellular vesicles and be exported out of the cell. These microRNAs have been identified in exosomes, where they are encapsulated and, hence, protected from degradation (Bobrie et al., 2011; Zhang et al., 2015). These circulating miRNAs within exosomes (exosomal miRNAs) can regulate the gene expression of target cells both locally and systemically. As such, they could be attractive sources for peripheral biomarkers (Bobrie et al., 2011).

Exosomes and their cargo miRNA (circulating miRNAs) represent a major component of natural airway defense (Kesimer et al., 2009b; Radicioni et al., 2016). During pathophysiological conditions such as inflammation and immune responses, the miRNAs in exosomes are altered (Kumarswamy et al., 2011; Pua & Ansel, 2015; Rajasekaran et al., 2016). These small nucleotide polymers serve various roles during inflammation that, in turn, are thought to be able to alter the progression of many conditions affecting the lungs (Alexander et al., 2015; Kesimer et al., 2009b). There is also strong evidence that exosomal miRNA (circulating miRNAs) participate in the biological response to environmental exposure (Harischandra et al., 2017; Simpson et al., 2008; Taylor & Gercel-Taylor, 2013).

Exosomes, thus, play an essential role in the dynamic regulation of airway tract response to a broad range of possible internal biological processes and environmental conditions, or bodily exposure to substances such as tobacco smoke (Alexander et al., 2015; Harischandra et al., 2017; Russ & Slack, 2012). Researchers are increasingly realizing the potential utility of exosomes as biomarkers for a host of conditions that result from tobacco exposure. Thus, interest in research along this line of inquiry is increasing, particularly on exosomes as very good biomarkers of a plethora of adverse health conditions as a consequence of injury of the airway epithelia from tobacco exposure (Alexander et al., 2015; Kesimer et al., 2009b; Russ & Slack, 2012). Exosome-like vesicles secreted by the epithelial cells of the airway tract can be effective biomarkers owing to the tract's direct exposure to tobacco smoke and the inflammation and subsequent remodeling of the resulting immune response (Kesimer et al., 2009b; Russ & Slack, 2012).

It has been established that exosomal miRNAs play an essential role in environmental exposure and this can potentially facilitate efficient monitoring of cellular response of the airway tract due to tobacco smoke exposure. Thus, profiling exosomal miRNAs may contribute to identification of tobacco exposure biomarkers that predict the biological effects on the airway tract. Consequently, the exosomal miRNA profile may be used for evaluating the harmful effects of tobacco products. The present research, as discussed in this chapter, also investigated the potential role of exosomal miRNAs in NETP-related exposure. Accordingly, it was hypothesized that tobacco smoke from NETPs, in the form of little cigar, cigarillo and waterpipe, change the exosomal cargo (i.e., the miRNA) of airway epithelial cells. The study mainly focused on the potential of circulating exosomal miRNAs to serve as a source of tobacco product-related exposure vesicular biomarkers for use in health risk measurement. A better understanding of the mechanism of tobacco exposure-related injury biomarkers can help in

developing more efficient evaluation strategies, tobacco product regulation, and a preventable risk approach to health care.

Materials and Methods

Cell Culture and Whole Tobacco Product Exposure

The Marsico Lung Institute - Center Tissue Culture Core of the University of North Carolina at Chapel Hill provided the primary human trachea-bronchial epithelial (HTBE) cells which were collected and cultured in the preparation of an air-liquid interface for a four to six week period to form well-differentiated, polarized cultures that resemble *in vivo* pseudo-stratified mucociliary epithelium (Kesimer et al., 2009b; Fulcher, 2005; Randell, 2011). Mucus secretions were obtained by performing 500 µl phosphate-buffered saline (PBS) solution washes on the apical surface of the cultures (Holmen et al., 2004; Kesimer et al., 2009a). Each wash was collected following 30 min of incubation at 37°C. Culture washings were subjected to centrifugation at 3000 *g* for 10 min in order to remove debris and dead cells.

As previously described in Chapter 2, human trachea-bronchial epithelial (HTBE) cells were exposed to little cigars, cigarillos, and waterpipe smoke by using an LM1 smoke engine and an S1000 shisha smoker machine (Borgwaldt KC, Hamburg, Germany), respectively. Smoking machines were used to generate smoke according to the manufacturer's protocol (Clunes et al., 2012). Smoking exposure paradigms of whole tobacco smoke (WTS) was performed as described in Chapter 2. Briefly, the patterns for conventional cigarettes, Kentucky research cigarette (KCS) and little cigar were 14 x 35 ml puffs, whereas for cigarillo, the cells were exposed to whole cigarillo smoke comprised of 30 x 35 ml puffs, at a rate of one puff every 30 seconds. The brands investigated were little cigar Swisher-Sweets (LCSS) and Swisher-Sweets cigarillo (SSW) (Swisher International, Inc.). In the waterpipe experiment, the equipment

head was filled with 15 grams of poplar shisha tobacco "Two Apples" flavor using the Al-Fakher brand (Al-Fakher Tobacco Trading, Ajman, United Arab Emirates) or Two Apples flavor only without tobacco component (Shiazo© Germany, Europe). The cells received 20 puffs at an interval of 60 seconds lasting from 3.62-3.70 seconds at 0.530 L volume per puff (Shihadeh, 2003).

With all the investigated tobacco products above, the cells were exposed to smoke once per day for five consecutive days. Daily, apical secretions were collected as described after the one-hour of smoking exposure. The collected samples were subjected to differential sedimentation for exosome-like vesicle isolation process as described below. **Figure 27** shows the methods for exosome isolation & miRNA analysis.

Isolation and Characterization of Exosome-like Vesicles

Exosome like-vesicles were derived and isolated from human trachea-bronchial epithelial (HTBE) cell cultures exposed to NETPs in the form of little cigar, cigarillo and waterpipe smoke. The apical secretions of the smoked-HTBE cells were isolated by using sequential differential centrifugation (Kesimer et al., 2009b; Thery et al., 2006) as described previously based on two studies (Gupta et al., 2018; Kesimer & Gupta, 2015). Briefly, the protocol pooled volumes of apical secretion material together. The samples were then subjected to multistep centrifugation, at 3000 *g* for 10 min and 10,000 *g* for 30 min to eliminate cell debris and other extraneous particles. The vesicles were subsequently pelleted at 65,000 *g* and 100,000 *g*. Afterwards, the isolated vesicles were resuspended in 30-μl PBS volume. The Nanoparticle Tracking Analysis (NTA) method was performed with a NanoSight version NS300 (Malvern instrument, United Kingdom) equipped with the NTA 3.0 analytical software (Malvern Panalytical Ltd, UK) to characterize exosome-like vesicle sizes and concentrations.

Samples were diluted in PBS in the ratio 1:500 and then loaded into the sample chamber of the NanoSight instrument. Triplicate recording videos of 60 sec each were performed per sample. The protocol was optimized to accurately focus and track the vesicles. Point scattering was accomplished using the NTA analytical software through an unlabeled micro-vesicular path. A 635-nm laser was beamed to a 0·25-ml chamber and Brownian motion was determined from the video recording sequence, with the quantification of each possible particle by determination of the mean squared displacement. After the aforementioned procedures, the NTA software analyzed the videos and reported the vesicle size together with an estimate of the concentration for each sample, as previously described (Dragovic et al., 2011).

Exosomal miRNA Purification and Next-Generation Sequencing (NGS) Analysis

To perform exosomal purification, the HTG-EdgeSeq automated technology was employed. The miRNA library was generated by following the HTG EdgeSeq miRNA Whole Transcriptome Assay (miRNA WTA) ILM kit protocol (HTG Molecular Diagnostics, Inc. Tucson, AZ, US) (Danilin S., 2017). The WTA protocol enabled the automation of the nuclease protection stage in the process of library preparation to facilitate use of this platform for next-generation sequencing (NGS). This assay was constructed to measure approximately 2083 human miRNA as miRbase version 20. The miRNA WTA ILM kit protocol was followed as briefly described earlier (Gupta et al., 2018).

Accordingly, 15 ml of lysis buffer was added to the 15-ml exosome sample. Tubes were then heated to 958°C for 15 min. afterwards, 1.5 ml of proteinase K was added, and the sample was mixed well by pipetting and incubating for 30 minutes at 508°C in an orbital shaker. A total of 25 ml of working lysate was transferred to each well of the HTG EdgeSeq scanning plate. The HTG EdgeSeq program was started after appropriate kit components for preparing miRNA

libraries were loaded into the system platform. Upon completion of the HTG EdgeSeq run, the sequencing adaptors and barcodes were added to the sequencing libraries. The samples were then amplified using the Polymerase chain reaction (PCR) method. After the PCR step, sequencing libraries were concentrated, pooled, and then sequenced on a MiniSeq or HiSeq2500 rapid run (RR) Illumina sequencing system using the Single End 50 cycles setting.

Bioinformatics Analysis

The Bioconductor R package was applied to analyze the raw count data of the exosomal miRNA differential expression. DeSeq2 was utilized in this part of the experiment as an analysis model to estimate variance-mean dependence in differential gene expression data based on the negative binomial distribution based on the inputs from a comparable study and the website, Bioconductor. It offers tools for analysis of high-throughput genomic data powered by the statistical programming language R. Additionally, the website also facilitates comprehension of genomic information (Huber et al., 2015). DeSeq2 calculates a normalization factor for each gene and the correction factor is applied to library size (Jagla et al., 2012; Reddy, 2015). Standard statistical analysis was implemented for quantitative expressions of miRNA and to conduct unsupervised data clustering analysis. Pathway analysis for miRNA was also performed using Diana miRPath v2.0, a web based software (I. S. Vlachos, 2012).

Results

Characterization of Exosome-like Vesicles

The Nanoparticle Tracking Analysis (NTA) used in the experiments for the characterization of exosome-like vesicles derived from HTBE cells smoke exposure reported that the average size in smoke-exposed groups was 245 nm for little cigar **(Figure 28A)** and 292.8 nm for cigarillo **(Figure 29A)**, whereas their concentrations were 1.19 e^{+11} particles/ml **(Figure 28B)**

and 6.94 e $^{+11}$ **(Figure 29B)** particles/ml, respectively. Measurements of the sizes of exosome-like vesicles in the waterpipe-smoked group was 269.5 nm for 2App and 276.4 nm for TOB **(Figure 30A)**, whereas the concentrations were 6.26 $^{e+1}$ particles/ml and 5.73 $^{e+11}$ particles/ ml, respectively **(Figure 30B).** The characterization of exosome-like vesicles for whole tobacco groups are summarized in **Table 6**

Exosomal miRNA Analysis Profile

Global discovery of exosomal miRNA analysis was performed to investigate the potential role of exosomal miRNAs in NETP exposure. The HTG EdgeSeq technology and next-generation sequencing were applied to generate the genomic library construction, and the differential expression analysis was calculated using the DeSeq2 statistical software.

Little cigar smoke exposure. Over 2000 miRNAs were detected among the experimental groups. Approximately 98 miRNAs were increased expression in LCSS smoked-HTBE cells compared to the air group **(Figure 31A)**, and 42 miRNAs compared to the cigarette (KCS), with another six downregulated miRNAs **(Figure 31B)** The differentially expressed miRNAs were involved in many pathways such, as NF-kappa B signaling, chemokine pathway, and apoptosis pathways, in addition to other biological processes and functional pathways as shown in **Figure 32A** and **Supplement Table 6** (I. S. Vlachos, 2012). The list of the top 25 significant differentially expressed miRNA is illustrated in **Figure 32B**

Cigarillo smoke exposure. Similar to little cigar smoke exposure, over 2000 miRNAs were detected and a partial list was presented by the heatmap showing their expression patterns among air, KCS and SSW smoke-exposed groups **(Figure 33A)**. When the cut off in the experiment was set to a p-value < 0.05 and a fold change of > 2, data from the cigarillo study elicited about 85 significant differentially expressed miRNA when SSW was compared to air

(Figure 33B) or 53 miRNAs compared to KCS **(Figure 33C)**. Among these miRNAs in the SSW smoke-exposed group, upregulation of miRNA-1303 was observed **(Figure 33D)**. Upregulation of miRNA-1303 was involved in the NF-kappa B signaling pathway and, in the Mucin,-N-Glycan biosynthesis. Similarly, miRNA-4655-5p **(Figure 33E)** was upregulated and it is involved in TGF-β signaling and MyD88-independent toll-like receptor signaling pathways. Furthermore, the downregulation of miRNA-561-3p **(Figure 33F)** was associated with SSW smoke exposure. As illustrated in the pathway analysis, the downregulation of miRNA-561-3p may be traced to membrane organization, its response to stress, regulated cell death and regulation of catalytic activity (I. S. Vlachos, 2012). The predicted list of affected pathways can be found in **Supplement Table 7**

Waterpipe smoke exposure. After changes in the differential expression were observed in raw data, quality control was performed on the processed samples. Unfortunately, the air-exposed group failed to pass quality control (QC) **(Figure 34)** and were eliminated. Most air samples were out of range from the expected value, likely due to technical background noise. Recent studies suggest that this noise originates from multiple sources, including an increase in the signal ratios, transcriptional noise, variation in the process of expression, or possibly, inappropriate quantity of starting molecules (Kim et al., 2015; Saliba et al., 2014). All air samples were excluded from the differential expression analysis. The Two Apples flavor (2App) was compared to waterpipe tobacco with Two Apples flavor.

In the implementation of DeSeq2 statistical procedure for the differential expression of miRNAs, a difference in miRNA expression pattern among the flavor and tobacco groups **(Figure 35A)** was observed in the overview analysis. The principal components analysis (PCA) also revealed unique clustering between waterpipe tobacco and its flavor, with sub-clustering among the flavor groups **(Figure 35B)**. The results also showed that approximately 442

upregulated and downregulated miRNAs were identified in the exposure groups ($p < 0.05$) as shown in **Figure 36A.** To increase the reliability of the observation and eliminate some of the background noise, the potential power of the statistical test was increased by limiting the statistical significance level to $p < 0.00005$. This adjustment resulted in about 136 miRNAs with altered expression.

Among the waterpipe tobacco smoke-exposed group, it was observed that miR-23b-3p, miR-23a-3p, miR-221a-3p, miR-34c-5p, miR-26b-5p, miR-449b-5p, miR-27a-3p, miR-224-5p, miR-191-5p and miR-31-5p were up-regulated **(Figure 36B)**. The top downregulated miRNAs after waterpipe smoke exposure were miR-937-5, miR-1273c, miR-6807-5p, miR-6765-5p, miR-3197, miR-1238-5p, miR-1224-5p, miR-4725-3p, miR-6790-5p and miR-663b **(Figure 36C).** Pathway analysis indicated that the highly significant differentially expressed miRNAs were involved in many biological processes and functional pathways such as mucin type O-Glycan biosynthesis, proteoglycans in cancer, pathways in cancer, ECM-receptor interaction, rap1 signaling pathway, regulation of actin cytoskeleton and endocytosis, etc. (I. S. Vlachos, 2012) **(Table 7).**

Discussion

Airway exosomes are involved in the lung's natural immunity and in modulating the immune response. They play an important role in intercellular communication through vesicular cargo, the molecular constituents of which contribute to the remodeling of the epithelium and of airway biology (Gupta et al., 2018; Kesimer et al., 2009b). There is also research evidence that HTBE cell exosomes formed after cigarette smoke exposure-related stress are responsible for airway remodeling pathogenesis in COPD (Sessa & Hata, 2013; Szymczak et al., 2016) . Likewise, the biology of exosomal miRNAs, particularly in the context of the airway tract and

the lungs being constantly exposed to different stressors ranging from cigarette smoke to noxious chemicals, constitute the airway responses to this exposure by modulating the immune response (Ryu et al., 2018) (Alexander et al., 2015).

This research attempts to increase knowledge about NETPs smoke effects on the airway biology effects by investigating the exosomal miRNA transcriptome following NETP-smoke exposure. The study presents a global analysis of exosomal miRNA expression post-exposure to NETPs in the form of little cigar, cigarillo, and waterpipe. Results suggest that exosomes derived from NETP-exposed cells affect changes in the exosomal miRNA expression quantitatively, with the alterations being mediated by exposure to these particular tobacco products.

Based on these investigations, as to the differentially expressed miRNAs of the exosomes derived from NETP-exposed cells, it was observed that miR-3675-3p, miR-7111-5p, miR-214-5p, miR-323b-3p, miR-449c-5p, miR-92b-3p, miR-503-5p, miR-370-3p, miR-744-3p, miR-1269b, miR-4452, miR-4283, miR-6886 were upregulated after exposure to the little cigar group. Using pathway analysis , the aforementioned set of miRs were predicted to be involved in the mechanisms related to the bacterial invasion of epithelial cells (I. S. Vlachos, 2012; Liu et al., 2009; Maudet et al., 2014) and the process of endocytosis (Janas et al., 2015; Morelli et al., 2004). The study also found that the Mucin type O-Glycan biosynthesis pathway was affected by exposure to little cigar smoke because of changes in the set of miRNA expressions (I. S. Vlachos, 2012), such as: miR-613, miR-214-5p, miR-520a-5p, miR-1183, miR-1236-3p, miR-6859-5p, miR-6847-5p, miR-130a-5p, miR-8085, miR-4476 and miR-8060. This set of miRNA expressions were significantly upregulated. The alterations in miRNA suggest that little cigar exposure may alter mucin biostructure, which contributes to the alteration of the immune defense

system against bacterial pathogen colonization and invasion in the airways. However, mechanistic studies are needed to confirm this possibility.

The result also showed that cigarillo exposure upregulated a set miRNAs involved in the NF-kappa B signaling pathway and Mucin-N-Glycan biosyntheses, including miRNA-1303, miR-6782-5p and miR-937-5p (I. S. Vlachos, 2012; Ma et al., 2011). Likewise, the exposure also upregulated miR-4566-5p, which is involved in TGF-β signaling and in MyD88-independent toll-like receptor signaling pathways (Guo et al., 2015). Meanwhile, the downregulated miR-561-3p is involved in gene-regulated membrane organization, response to stress, regulated cell death, and regulation of catalytic activity (I. S. Vlachos, 2012).

Additionally, waterpipe tobacco smoke exposure was associated with downregulation miR-21. The miR-21 is thought to target IL-12 and mitigate pathology by alleviating the immune response to allergies (Pua & Ansel, 2015). Furthermore, miR-21 can possibly regulate negative feedback in the airway epithelium's mucin secretion by acting on MARCKS mRNA expression (Lampe et al., 2013).

Nicotine is commonly known as the primary addictive component in tobacco products (US-HHS, 2010). Studies have also shown that low-nicotine cigarettes reduce dependence and increase the chances that a user will quit smoking (US-HHS, 2000). A number of research studies have shown the relationship between miRNA regulation and nicotine addiction. For example, miR-21 was found to be upregulated in the chronic nicotine abuse model (Cai et al., 2009; Huang & Li, 2009). Meanwhile, miR-21 is a critical master regulator of the immune system (Kumarswamy et al., 2011). Additionally, miR-21 and miR-335 were up-regulated by 100-μM of nicotine exposure (Huang & Li, 2009). However, miR-146a was

significantly down-regulated in placentas exposed to cigarette smoke as compared to controls (Maccani et al., 2010).

The present study demonstrated that NETPs upregulated miR-4440, miR-3934-5p, miR-92b-3p, miR-4664-5p, miR-4732-3p and miR-1183. Alteration of these miRNAs expression associated with NETP smoke may contribute to addictive behaviors. The implications of this finding should direct healthcare and tobacco regulation authorities towards strategies and initiatives to address the harms posed by NETPs because the aforementioned set of miRNAs are known to be involved in nicotine addiction mechanisms (Zhang et al., 2016) (I. S. Vlachos, 2012).

The experiments also demonstrated that circulating miRNA cargo in the exosome-like vesicles changed after smoke exposure to NETPs. These changes may also contribute to up-regulation or down-regulation of expression in related genes after exposure to tobacco smoke. Subsequently, the exosome-like vesicle changes may also result to variations in the protein expression level.

A prior study, where this researcher was involved, showed that circulating miRNA in the exosome-like vesicles play critical roles in airway intercellular communication through their cargo (Gupta et al., 2018). Thus, it is reasonable to speculate that exosomes may carry not only miRNA, but potentially, exposure residue chemicals in the form of nicotine, acrolein and other similar by-products from tobacco exposure, as well. In this respect, further studies, specifically the chemical compound analysis of exosome-like vesicles, will provide insights into the direct identification of chemicals in the cargo. Alternatively, or in tandem, cell cultures may also be treated with these exosome-like vesicles to elucidate the biological effects derived from tobacco exposure.

Many of the miRNAs altered in this study were associated with pathways in cancer attributable to NETP-smoke exposure. These include miR-6886-3p, miR-8085, miR-3064-5p, miR-6792-3p, miR-1236-3p, miR-1269b, miR-4510, miR-449a, miR-449b-3p, miR-8054, miR-4422, miR-34a-5p and miR-503-5 (Barros et al., 2018; I. S. Vlachos, 2012; Lages et al., 2012).

The comparative analysis of exosomal miRNA analysis also revealed that exposure to smoke from NETPs in the form of little cigar, cigarillo and waterpipe caused alterations in the HTBE cells in which dysregulation was observed in sets of exosomal miRNA expression. This finding should point towards continued adoption of comprehensive measures to fully understand which miRNA-based biomarkers can best predict health risks associated with the tobacco products examined in the present study. In particular, scientific evidence provides support to the view that different pathogenetic stages in pulmonary conditions result from deregulated expression of protein-coding genes in response to abnormal miRNA expression (Sessa & Hata, 2013; Szymczak et al., 2016).

The study featured a global discovery technique for exosomal miRNA associated with tobacco exposures. However, isolation of exosomes by the ultracentrifugation method is a multi-step procedure and is time-consuming in comparison to other methods like Exosomal RNA Extraction Kits. Nevertheless, it cannot be denied from this study and from existing literature that the ultracentrifugation approach yields a high purification rate (Kesimer et al., 2009b; Thery et al., 2006). The speed of NTA also shows distinct advantages over other currently popular methods of microvesicle analysis such as absorption to latex beads. The detection of exosomes and microvesicles by NTA, however, cannot differentiate between the types of particles being

detected. Thus, discrimination between true exosomes and other membrane microvesicles, would be beneficial.

Fortunately, a recent advance in the ability to incorporate fluorescence detection into NTA might provide an answer to the challenges in detection mentioned in the last part of the preceding paragraph (Kesimer et al., 2009b; Thery et al., 2006). Using fluorescence, either via coupled antibodies or antibody-conjugated quantum dots might allow the detection of subsets of microvesicles within a sample (Kesimer et al., 2009b; Thery et al., 2006). Purifying miRNA from HTBE cell-derived apical secretions is a somewhat challenging process. To address this challenge, implementation of an automated HTG EdgeSeq system for miRNA analysis without the need for RNA extraction or enzymatic sample processing, and a fully automated nuclear protection assay to produce reproducible and reliable profiles of miRNA expression will encourage more research within this line of inquiry (Danilin S., 2017).

The platform of an automated HTG EdgeSeq system only requires a low material volume of exosomes (15-25 µl) to provide a quantitative measurement of over 2083 miRNAs in the sample. The study used *in vitro* exosome-like vesicles derived from a relatively pure population of bronchial airway epithelial cells and identified those miRNAs that are differentially expressed with smoking. However, in an *in vivo* model, other cells may contribute to this process such alveolar epithelia, macrophages, and other immune cells. Therefore, future investigations should consider the importance of determining the original cell sources of the exosome-like vesicles to be used as study samples.

Integrated airway exosomal miRNA and proteomic data after HTBE cell smoked to NETPs

In order to learn more about the airway biological changes brought about by exposure to smoke generated from the NETPs, we experiment attempted to integrate airway

exosomal miRNA, and proteomic data of the apical secretions from the smoked-HTBE cell to the NETPs evaluated. Due to the complexity of the proteomic and miRNA transcription, the vast quantities of data generated per experiment posed a blend of various statistical, computational and informatics-associated challenges to make sense and understand the sheer volume of the information output at hand. Further analysis of selected proteins, particularly, matrix metallopeptidase 9 (MMP9) and polymeric immunoglobulin receptors (PIgR) were carried out to predict the potentially targeted miRNA of these proteins and attributed their change to these miRNAs. **Figure 37** illustrates the miRNAs predicted to target MMP9 and PIgR. A similar analysis may be applied to any interesting molecules identified in this study data set.

As explained in previous work in biochemistry, miRNAs in the form of short single-stranded RNAs recognize sequences in the 3′ untranslated region (UTR) of mRNAs and cause post-transcriptional silencing of the target mRNA. Silencing occurs with the suppression of protein synthesis and induced mRNA degradation (Fabian et al., 2010). Typically, each miRNA regulates more than one gene, which in turn, may lead to modification of the expression and function of other downstream genes. However, it is also possible that one gene can be targeted by multiple miRNAs (Fabian et al., 2010) (Andres-Leon et al., 2016; Zhang et al., 2016). Exosomal miRNA are the key players influencing the host innate immune response during exposure (Harischandra et al., 2017; Russ & Slack, 2012). The innate immune system is characterized by its responses to tobacco smoke (Qiu et al., 2017). It should be clarified at this points that mRNA expression due to tobacco exposure can modulate immune system responses (Harischandra et al., 2017; Momi et al., 2014).

Prior research studies conducted during the new millennium have identified the critical contribution of miRNAs to the development and function of innate immune cells (Gomez et al.,

2017; Zhang et al., 2016; Taganov et al., 2006). To illustrate, among the miRNAs that influence the innate immune system, such as miR-146a, miR-155, and miR-132, have been the most intensively studied. The miRNA, miR-146a, is NF-κB-dependent and targets the NF-κB pathway, the latter regarded as the central pathway in innate immunity. It was also reported that miR-146a directly targets and represses several downstream signaling molecules, including IL-1 receptor-associated kinase 1 (IRAK1), IL-1 receptor-associated kinase 2 (IRAK2), and TNF receptor-associated factor 6 (TRAF6) (Taganov et al.,2006). Meanwhile, miR-155 and miR-132 are up-regulated in response to lipopolysaccharide (LPS). In the case of miR-155, depending on the nature of the stimulation, it can either strengthen or suppress innate immune responses in macrophages and DCs (Taganov et al., 2006). Meanwhile, increased levels of miR-132 expression induces stimulation in monocytes by directly targeting interleukin-1 receptor-associated kinase 4 (IRAK4) (Nahid et al., 2013).

Meanwhile, another protein, the BPI fold containing family A member 1 (BPIFA1), affects the innate immune responses of the upper airways. Its functions consist of binding bacterial lipopolysaccharides (LPS) and inhibiting the formation of biofilm by pathogenic bacteria. BPIFA1 plays its role in the airway inflammatory response after exposure of the airways to irritants, which may attract macrophages and neutrophils (Bingle et al., 2007; Campos et al., 2004; Sayeed et al., 2013). In the experiment performed for this study, BPIFA1 was significantly downregulated in the apical secretions in all HTBE cell smoked using NETPs **(Figure 38A1-3).** According to the miRDB database, the BPIFA1 gene is targeted by 24 miRNAs (Liu & Wang, 2019; Wong & Wang, 2015).

To investigate the potential mechanism by which these tobacco products decrease the level of BPIFA, the outcome miRNA expression profiles data from different independent

exposure experiments were overlaid to predict potential miRNA target genes from the database **(Figure 38B).** Accordingly, four common miRNAs were identified in the experiments to target BPIFA1. The miRNA, miR-4726-5p **(Figure 38C1-2),** was upregulated in all three experiments, whereas, miR-15a-5p **(Figure 38D1-2)**, miR-15b-5p **(Figure 38E1-2)** and miR-16-5p (F1-2) **(Figure 38F1-2)** were downregulated. Findings showed that miRNA 4726-5p is negatively correlated with BPIFA1 protein expression in the data sets. Meanwhile, miR-4726-5p and protein expression were upregulated parallel to BPIFA1 after smoke exposure from the NETPs. Thus, miR-4726-5p may target and contribute towards the regulation of BPIFA1, but it is not yet clear how BPIFA1 is regulated by this miRNA.

To attribute direct correlation and validate the foregoing observation, it is necessary to determine the mRNA level. Furthermore, recognition of potential miRNA molecular targets may identify relevant mechanisms in NETP-induced changes in airway biology. This will facilitate a better understanding of how exposure to smoke up-regulates or down-regulates the expression of related genes and how smoke exposure contributes to these biological changes

Conclusions

To conclude, the study analyzed the exosomal miRNA alterations in an *in vitro* model to formulate miRNA signatures which may be used as biomarkers to assess health risks associated with NETP exposure. The data provided directly assessed the biologically relevant changes in the airways associated with tobacco use and may contribute to the science base to inform authority regulation of NETPs. Thus, more studies to justify stricter regulations for NETPs should catalyze the realization of reforms designed to investigate the mechanistic function of tobacco-smoking related airway biological changes.

CHAPTER 4: Overall Conclusions and Implications on Tobacco Regulation

Overall Conclusions

A mash-up of history, legend, human struggle, and nescience wrought the curse of tobacco on humanity. At the outset, tobacco use appeared to be recreational rather than medicinal, as the evil and powerful Aztec sorcerer Acayatl was fabled to have enticed the Native Americans "to chew, to roll and smoke, and to dry and sniff tobacco leaves 5000 years BCE … to assuage hunger pangs from lack of food … [to inhale] smoke through a reed for medicinal purposes … as a symbol of goodwill ('peace pipes') and for ceremonial purposes … [or] to induce a stuporous state" (Slaby & Cocores, 1991). Nicotine, which is the active constituent of tobacco "interacts with the nicotinic acetylcholine receptors and stimulates the dopaminergic transmission" (Mishra et al., 2015). This internal activity excites the brain's reward center and results in mood elevation. Nevertheless, no reputable research publication *en masse* had been found to document any medicinal or health advantages of tobacco smoke inhalation.

Amidst a sea of research evidence, cigarette smoking or the inhalation of practically all tobacco products, have been found to cause adverse health effects. Since 1964, the US Surgeon General, through official health reports and published literature, offered ample communication to the public for their awareness and subsequent guidance. With definitive clarity, smoking in general, has been causally linked to a plethora of illnesses and to other antagonistic effects on the respiratory system (Centers for Disease Control and Prevention [CDC], 2010)). The biological and behavioral mechanisms responsible for smoking attributable diseases are, therefore, made known to the general population, with the necessary warnings.

As the public began to take heed of the caveats against cigarette smoking and planed the role of these warring's in reducing tobacco smoking-attributable diseases, leading tobacco use reduction targets of the Healthy People 2020 initiative have been met among adolescent smokers and children, where the latter pertains to exposure to secondhand smoke. Meanwhile, the Health People 2020 target for adult smoking is presently improving (Office of Disease Prevention and Health Promotion, 2019). Although the foregoing statements are positive and much welcomed developments, this is not yet the time to rejoice and allow a hiatus in strategies to curb the consumption of tobacco products. An emerging threat is gaining ground among younger consumers.

A variety of new and emerging tobacco products (NETPs) have aroused the interest of tobacco consumers who patronize these products through flavors that appeal to their senses and satisfy their fascination for tobacco. Many forms of NETPs are commercially available on the market and are gaining popular patronage among cigarette and other tobacco-product enthusiasts. This study is, however, limited to three NETP forms: the little cigar (CL), cigarillo and waterpipe (WP). The researchers consider the looming popularity of NETPs as largely due to a reliance on fallacious misconceptions that NETPs are 'safer' alternatives to cigarette smoking, and that adverse health effects, if any, are lesser than cigarettes. These two aforementioned misconceptions may be regarded as idiosyncratic beliefs by individuals or groups who are inclined to cigarette smoking, but are hindered in their passion because of the widely known injurious consequences of tobacco smoking.

Using *in vitro* models, Chapter 2 of the present study found that NETPs, in the form of LCs, CLLO and WP, collectively have greater effects than cigarette smoke in terms of reduced cell viability and altered protein expression patterns. Furthermore, NETPs tend to induce oxidative

stress proteins and to cause more profound alterations in the lung's innate immune response. These findings challenge the popular misconception that NETPs are safer and less harmful than cigarettes. Rather, NETP smoke leads to potential health risks and causes damage to the airways to an extent similar to or even greater than that of cigarette smoke.

In Chapter 3 of the present study, experiments were also performed using *in vitro* models grounded on the hypothesis that tobacco smoke NETPs, in the form of LC, CLLO, and WP, alter the exosomal miRNA cargo in airway epithelial cells. Results revealed dysregulation in a set of exosomal miRNA expression. This finding suggests that exposure to these particular tobacco products affects quantitative changes in the exosomal miRNA expression with the alterations being mediated by the exposure. Finally, the study revealed that the set of altered miRNAs were associated with pathways in cancer attributable to NETP-smoke exposure.

In retrospect, the study findings challenge the present misconception that NETPs are 'safer' and cause 'lesser' adverse effects than cigarettes. With the advent of new advertising schemes campaigning that NETPs are better choices over cigarettes, the scientific evidence contributed by this study is a contradiction of the NETP 'safe claims. It appears that NETPs are not necessarily safer alternatives. As 15 BC Roman fabulist remarked: "Things are not always what they seem; the first appearance deceives many; the intelligence of a few perceives what has been carefully hidden" (Behr, 2011)

Scientific Evidence and Tobacco Control

The results from this research could be used by the authorities charged with regulation of tobacco products to more seriously restrict the rather loose reign on NETPs. To illustrate, it appears at first glance that the WP or the more popularly known term, hookah, is regulated by the Food and Drug Administration (FDA). However, the regulations are quite lax and are geared only to

discourage WP use for individuals under the age of 18. Nevertheless, the threat to health is not covered, except for mandatory warnings and listing of ingredients/components on the label/packaging ((FDA), 2019)). The same is true with the LC and the CLLO, and all other NETPs and tobacco products ((FDA), 2018)). Simple logic should make one realize that access restrictions cannot ensure deterrence among resourceful and determined users. Even the Institute of Medicine is aware of the 'substitution of sources' and 'use of social sources' strategies to circumvent the restrictions imposed by authorities (Committee on the Public Health Implications of Raising the Minimum Age for Purchasing Tobacco Products, Board on Population Health and Public Health Practice, & Institute of Medicine, 2015).

The premise of this section of the study rests not on access restrictions on users/ consumers, but on more stringent restrictions for manufacturers. This study lists pathways dysregulation and biological alterations that exposure to NETP smoke may trigger. These may be involved in early stages of many pathological conditions including, among others, COPD, cardiovascular diseases and cancer. Additionally, health economics are burdened by smoking-related illness. Notably, there is $300 billion per year associated with direct and indirect medical costs related to smoking-related illness (US-HHS, 2014; Xu et al., 2015).

Given that the scientific evidence presented in this study is obtained in *in vitro* models from human specimen, scientists and public health authorities are in the best position to validate the results of this and other studies using *in vivo* models. The most opportune time is now. The authorities cannot delay imposition of more stringent regulations directed at the manufacturers of tobacco products. If the findings of *in vitro* model studies are not good enough for ground sweeping and draconian reforms on tobacco regulation, validation studies using *in vivo* models need to be a

public health priority by the government. The burden of disease should be a strong catalyst for policy change.

Reform in Tobacco Control Legislation and Policy

considering the premise articulated in the preceding page that the significance of the findings in this study support more stringent restrictions for manufacturers, and the limitations inherent on possible regulations that may be imposed by the FDA, solid support from the legislative arm of the government is crucial. From the healthcare perspective, the health and well-being of the general population should be a top priority to enact legislation. While it is not being insinuated in this study that the tobacco manufacturing business should cease operations, stiffer terms need to be legislated to discourage manufacturing of tobacco products. One of the simplest approaches to discourage manufacturing of tobacco products, in general, is to impose higher taxes. This can be coursed through legislation. However, this might disrupt the form of market structure known as competition.

There is, however, a more technical and focused approach that can be established through legislation – a regulated market model, as envisioned by an Australian researcher (Borland, 2003). As explained in Borland (2003), the overall effect of a regulated market model will be the elimination of a range of incentives and opportunities to launch and operate commercial marketing of tobacco products. Additionally, new incentives will have to be created to foster research and development of non-harmful products.

This approach addresses the threat to a healthy market competition, but directs the business enterprises to innovate their products. In principle, the essence of control in this novel approach is to control sales promotions by reducing the creation of extra social value to consume tobacco. Another ingenious feature of the Borland (2003) approach is for the government to be granted

open and ongoing access to the engineering of tobacco products, and all the other aspects, such as manufacture, promotion, and distribution. Although the legislative process, as well as the crafting of the implementing rules and regulations of such a regulated market model will be complex and tedious, it will be worth the effort. The gains of such legislation towards creating a truly safer alternative to cigarette smoking can only be bought at the price of commitment and resolve.

APPENDICES

Note: The tables and figures are numbered consecutively from Chapter 1 to Chapter 3. They are however separated by Chapter.

APPENDIX A: TABLES AND FIGURES FOR CHAPTER 1

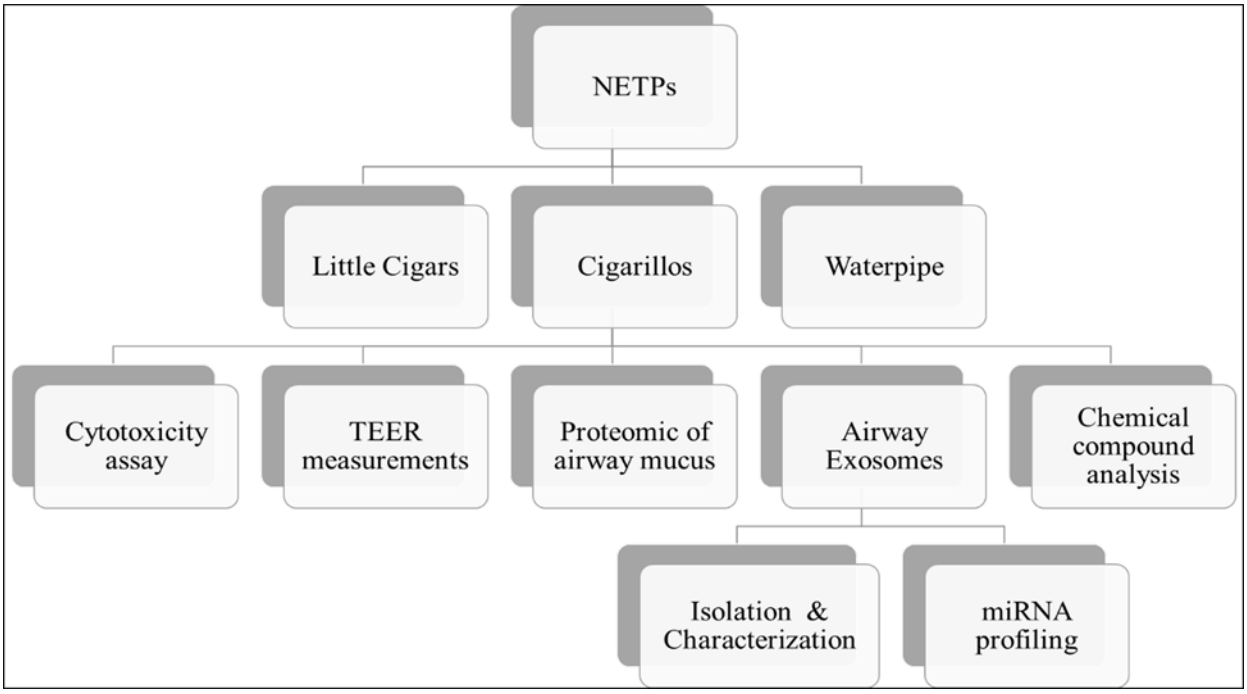


Figure 1: Flow Chart of proposed studies for characterizing the effect of New and Emerging Tobacco Products (NETPs) on airway innate mucosal defense. Human Trachea-Bronchial Epithelial (HTBE) cell culture was exposed to little cigar, cigarillo or waterpipe. Cellular viability and Trans-Epithelial Electrical Resistance (TEER) of smoke-exposed epithelia were evaluated. Apical secretions from NETP-exposed cultures were collected and subjected to label-free quantification mass spectrometric analysis. Chemical composition analysis of some different cigarillo brands was also performed. Part of the collected apical secretions from NETPs smoked HTBE were processed for isolation of the exosome-like vesicles in which airway miRNA profiling was performed.

Table 1*: Alternate forms of tobacco and nicotine delivery, which can be categorized broadly, into two types of tobacco products: combustible tobacco, which is intended to be smoked, and non-combustible, which is those that do not require the burning of the product for consumption.

Smoked tobacco	Non-combustible forms of nicotine
Cigar[6] includes large, cigarillo and little cigar	Electronic nicotine delivery devices (ENDs), including e-cigarettes
Hookah: also known as shisha, pipes, hubble-bubble, and narghile.	Dissolvable tobacco (strips, sticks, or orbs)
Kretek (Clove cigarette)	Snus
Bidi	Snuff (Pinch, dip)
Heat-not-burn tobacco such as IQOS[7]	Chewing tobacco (spit tobacco)

*Adopted from www-uptodate-com, as updated: Aug 24, 2018.

[6] Cigars are commonly categorized by their size and shape.
[7] I-Quit-Ordinary-Smoking (Philip Morris International).

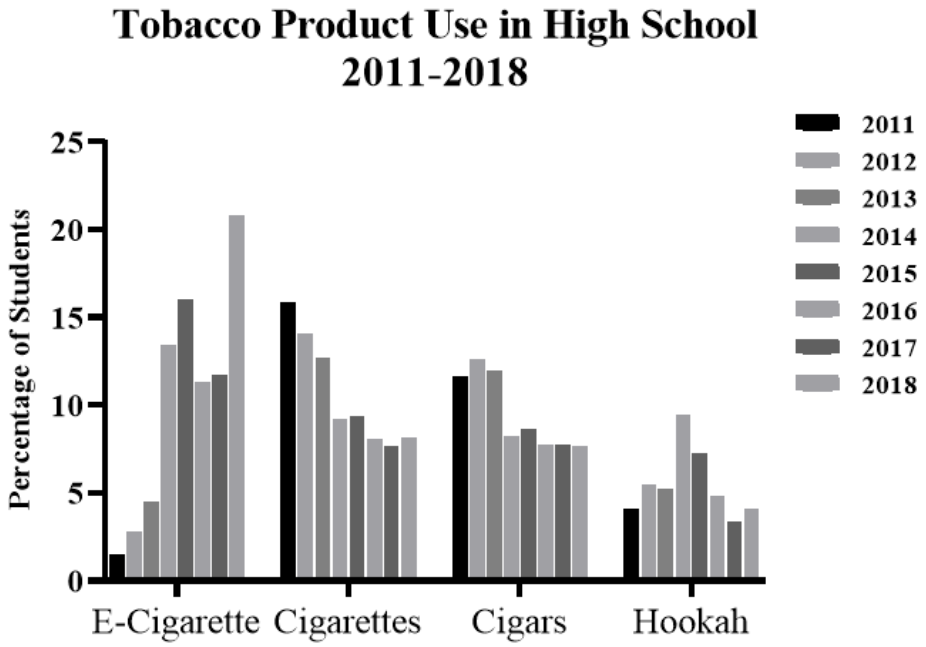


Figure 2: Recent trends in tobacco-product use by high school students. The bars represent the percent of students who said they had used each product in the past 30 days - adapted from T. Singh et al., April 15, 2016, MMWR and reproduced to update current data from 2016 -2018

Updated from CDC, Morbidity and Mortality Weekly Report (MMWR) [1]

[1] Jamal A, Gentzke A, Hu SS, et al. Tobacco Use Among Middle and High School Students — United States, 2011–2016. MMWR Morb Mortal Wkly Rep 2017; 66:597–603. DOI: http://dx.doi.org/10.15585/mmwr.mm6623a1 Gentzke AS, Creamer M, Cullen KA, et al. Vital Signs: Tobacco Product Use Among Middle and High School Students — United States, 2011–2018. MMWR Morb Mortal Wkly Rep 2019;68:157–164. DOI: http://dx.doi.org/10.15585/mmwr.mm6806e1

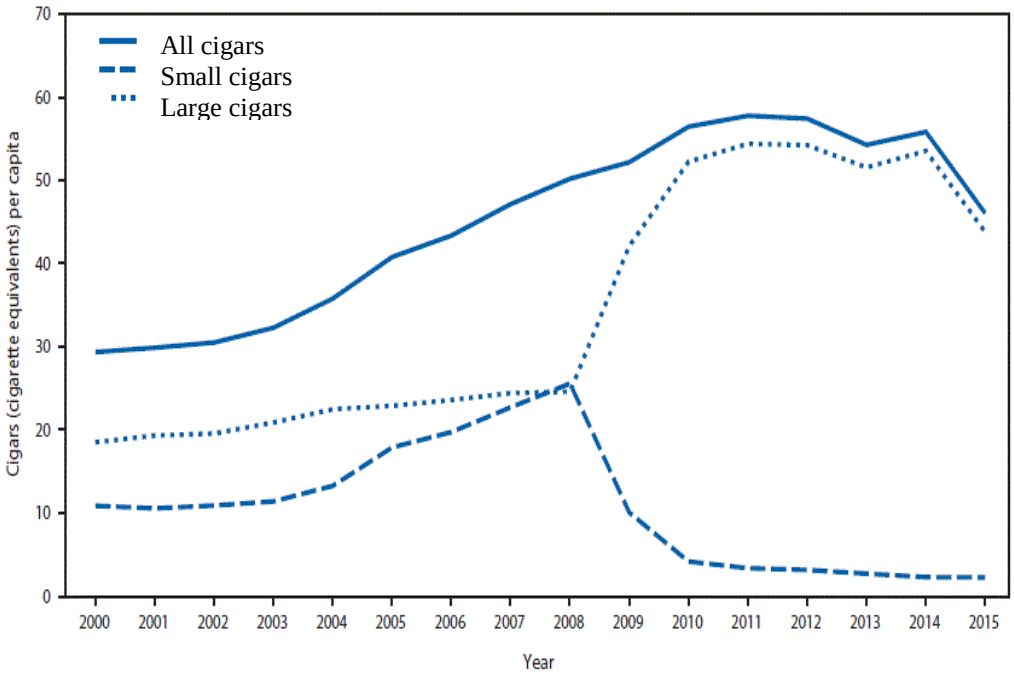


Figure 3: Consumption of cigars- United States, 2000 to 2015. Adopted form Wang TW et al., 2016, MMWR 2016.

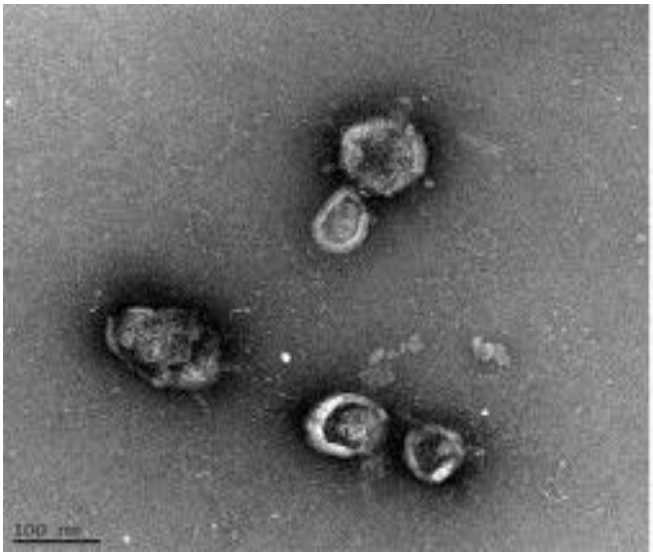


Figure 6: Electronic Micrograph of exosomes derived from human airway epithelial cell, which play an important role in airway biology, innate defense and epithelial remodeling. (Kesimer, et.al, 2009)

APPENDIX B: TABLES AND FIGURES FOR CHAPTER 2

Table 2[1,2] : Description of cigars[3] (cigarillo, little cigars as NETPs in comparison to conventional cigarettes)

Cigarillos	Little Cigars	Cigarette
Wrapped in tobacco leaves or brown tobacco-based paper	Wrapped in tobacco leaves or brown tobacco-based paper	Any roll of tobacco wrapped in paper or any substance not containing tobacco
Contain approximately 3 gram of tobacco	Contain about < 1gram of tobacco	Contain about < 1gram of tobacco
Come with different flavors	Come with different flavors	Banded from flavoring
Air-cured and fermented tobaccos	Air-cured and fermented tobaccos	Shredded or reconstituted tobacco
Length from 7-10 cm and diameter is 6-9 mm	Length is 8 cm and diameter is 8 mm	Length is 8 cm and diameter is 8 mm
Usually made without a filter	Usually made with filter	Made with filters
Sold individually or packs of 1-2	Sold in larger packs of 20	Sold in larger packs of 20
Less regulated and taxed at a lower rate than cigarettes	Less regulated and taxed at lower rate cigarettes	Relatively regulated and taxed at high rate than cigars

[1] King BA, Tynan MA, Dube SR, Arrazola R. Flavored-Little-Cigar and Flavored-Cigarette Use Among U.S. Middle and High School Students. Journal of Adolescent Health 2013; 54(1):40–6 [accessed 2015 Oct 19].
[2] Gammon DG, Loomis BR, Dench DL, King BA, Fulmer EB, Rogers T. Effect of Price Changes in Little Cigars and Cigarettes on Little Cigar Sales; USA, Q4 2011-Q4 2013. Tobacco Control 2016;25:538-44
[3] Cigars are measured as cigarette equivalents per capita. Small cigars are defined as cigars that weigh ≤3 lbs (1.36 kg) per 1000 cigars, and large cigars are defined as cigars that weigh >3 lbs per 1000 cigars.

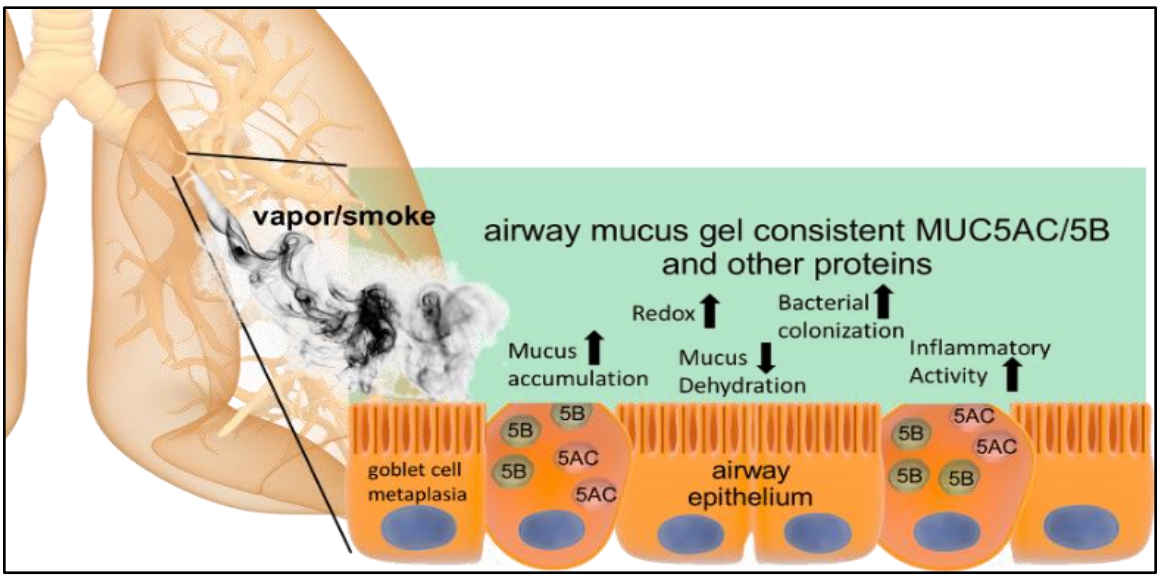


***Figure 7*:** Tobacco smoke exposure and the airway surface. The airway surface is the first point of contact with inhalants smoke and it is protecting the host by the secretion and continuous clearance of a mucus layer. Tobacco smoke exposure, the major cause of chronic obstructive pulmonary disease (COPD), instigates a dysfunctional clearance of thick obstructive mucus. Henceforth, tobacco smoke exposure leads to goblet cell metaplasia, mucus hypersecretion and dehydration, increased risk for infection and inflammation.

Table 3: *Demographic Characteristics of the airway primary epithelial cell primary cultures*.

Characteristic	**Studies**		
	Little cigars (n=2)[1]	**Cigarillos (n=6)**	**Waterpipes (n=6)**
Mean age, year	61 years	36 years	31.6 years
Gender	2 Male	4 Male, 2 Female	3 Male, 3 Female
Ethnicity	2 Caucasian	2 Black, 1 Caucasian, 2 Hispanic	4 Caucasian, 2 Hispanic

[1] Two donors = six cell cultures biological replicate samples

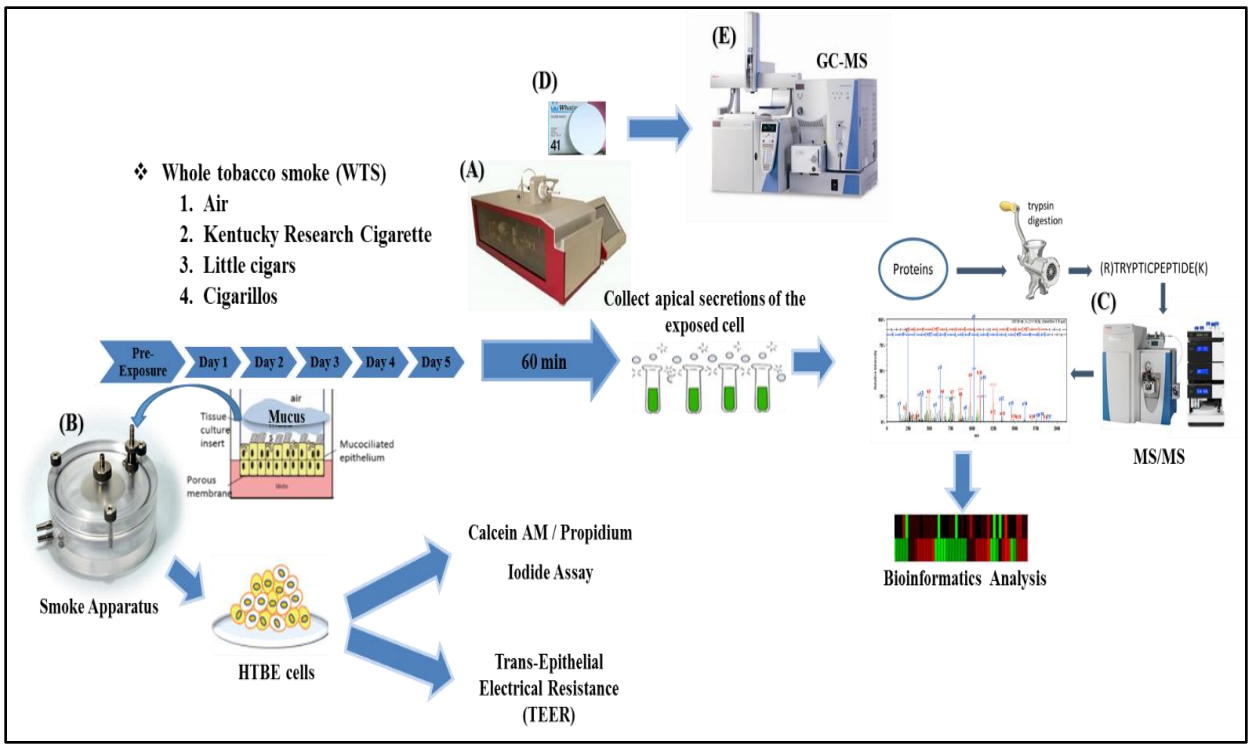


Figure 8: Experimental design set-up to investigate the effect of little cigar and cigarillo on airway epithelia and mucus barrier in vitro model. An LM1 smoke engine **(A)** was used to generate smoke from cigarettes, little cigars or cigarillos in which was applied to cultured human tracheal bronchial epithelial (HTBE) cells that were transferred to the smoke apparatus **(B)**. The cells were exposed to smoke or air once per day for five consecutive days. The apical surface of the culture was washed one-hour post exposure using phosphate-buffered saline. Apical secretions cultures of HTBE cells exposed to tobacco smoke were prepared and subjected to label-free quantitative proteomic analysis using mass spectrometer (MS/MS) **(C)** The proteome discoverer software was used to process the raw data and to identify proteins. Cytotoxicity assay (calcein AM/propidium Iodide assay) and Trans-Epithelial Electrical Resistance (TEER) measurements were performed on the smoked-HTBE cells. Smoke generated by the smoke engine was directed through Cambridge filter pads **(D)** to collect extracts from mainstream smoke to perform chemical compound analysis of cigarillo tobacco products using gas chromatography-mass spectrometry (GC-MS) **(E)**.

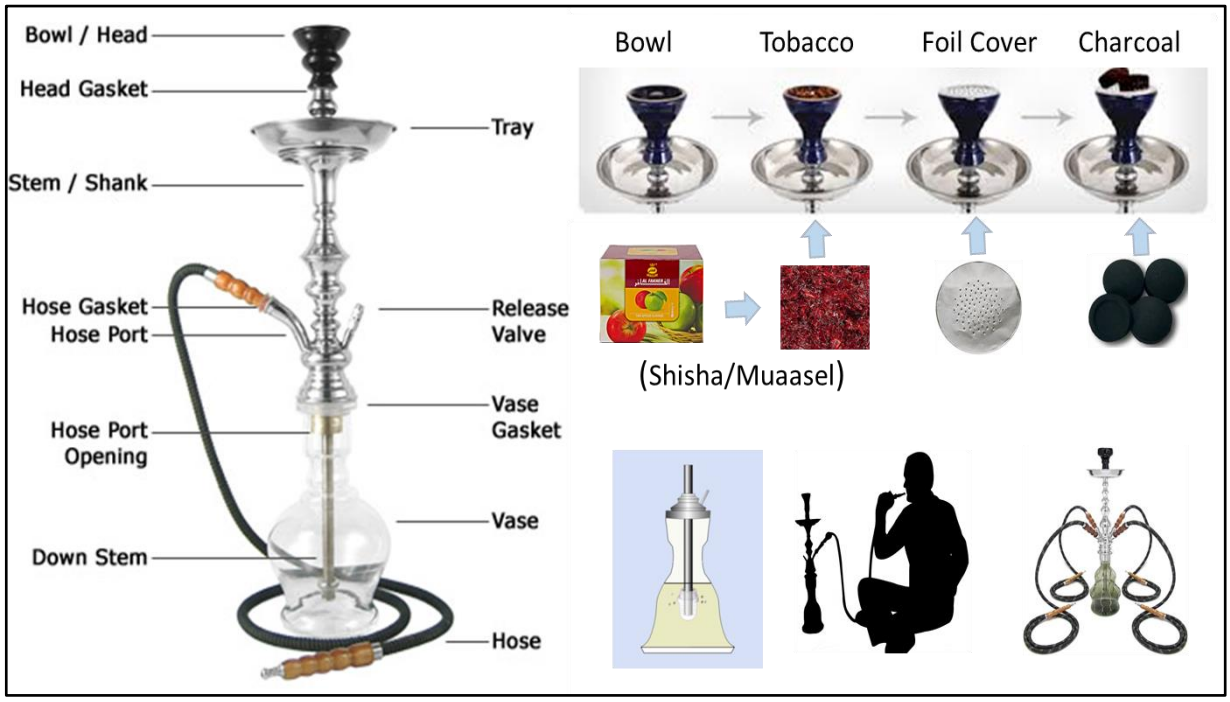


Figure 9: Waterpipe components and setup: The main components for waterpipe are a head, body, water bowl (vase), and hose(s) along with other accessories such as purge valve, grommets, and plate and vase gasket. A sticky mixture of tobacco, Muaasal is placed in the bowl at the top of the waterpipe. Then, the bowl covers by aluminum foil which heated by the burning charcoal on top. The charcoal heated the tobacco through the foil to produce smoke, then smoke traveled through the body of the waterpipe, and passed through a hose, which could be a single, or multiple to inhale the smoke as group simultaneously. The vase contain water which may cools the smoke, making many people think it is filtered out the smoke form chemical and harmful ingredients and that it is a healthier. Unfortunately, this is not true. Even after the smoke passing through the water, hookah smoke contains toxins and chemicals [1].

[1] Adopted and modified form http://dceg.cancer.gov/news-events/linkage-newsletter/2013-11/research

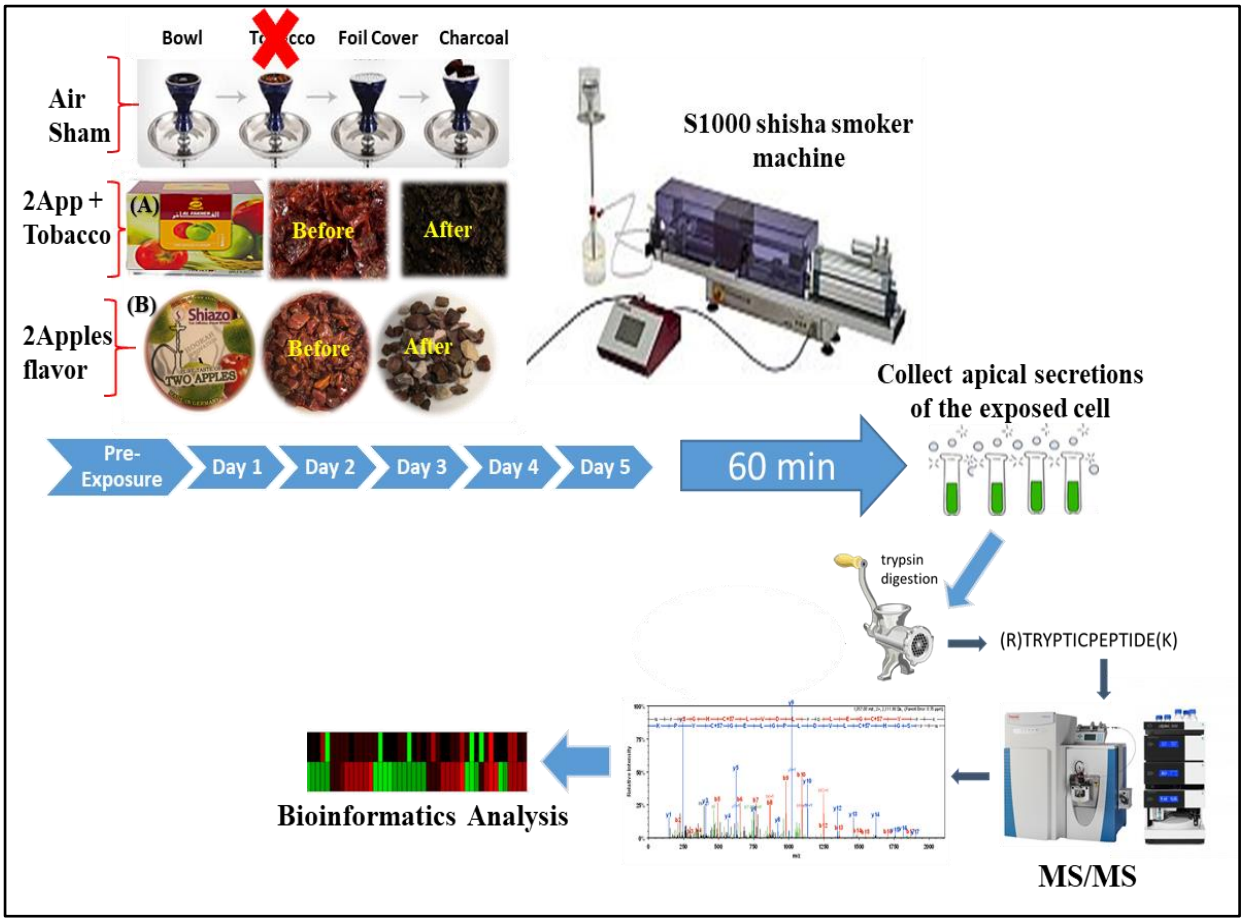


Figure 10: Experiment design to characterize shisha tobacco products: A S1000 shisha smoker machine generated waterpipe tobacco smoke. The bowl filled with 15 grams of tobacco products: shisha tobacco flavored with Two Apples (2App + Tobacco) and Shiazo steam stones Two Apples flavor only without tobacco (2App). The image rows showed 2App + Tobacco (A) and 2App (B) tobacco products before and after the smoking session. The same set up was prepared using the shisha smoker machine to generate air to expose HTBE cells, which represented as air-sham group. The HTBE cells subjected to one smoking or air session every day for 5 days, beginning with a warm-up wherein the cells received 20 puffs. The cells will receive 20 puffs at an interval of 60 seconds. One-hour post exposure PBS washes, apical secretions were collected and subjected to label-free quantitative proteomic and bioinformatics analysis.

Table 4: Physical characteristics of Kentucky research cigarette (KCS) and three different cigarillos tobacco products:

Tobacco Products	Weight/gram	Length/cm	Thickness/cm
Kentucky Research Cigarette (KCS)	1.02	8.5	0.7
Swisher-Sweets Cigarillo (SSW)	2.83	11	1.2 (first 1.5 cm length was 1)
Game-Black Cigarillo (GBK)	2.89	10.5	1
Hi-Fi Tropical Tango Cigarillo (HTT)	2.97	10.5	1

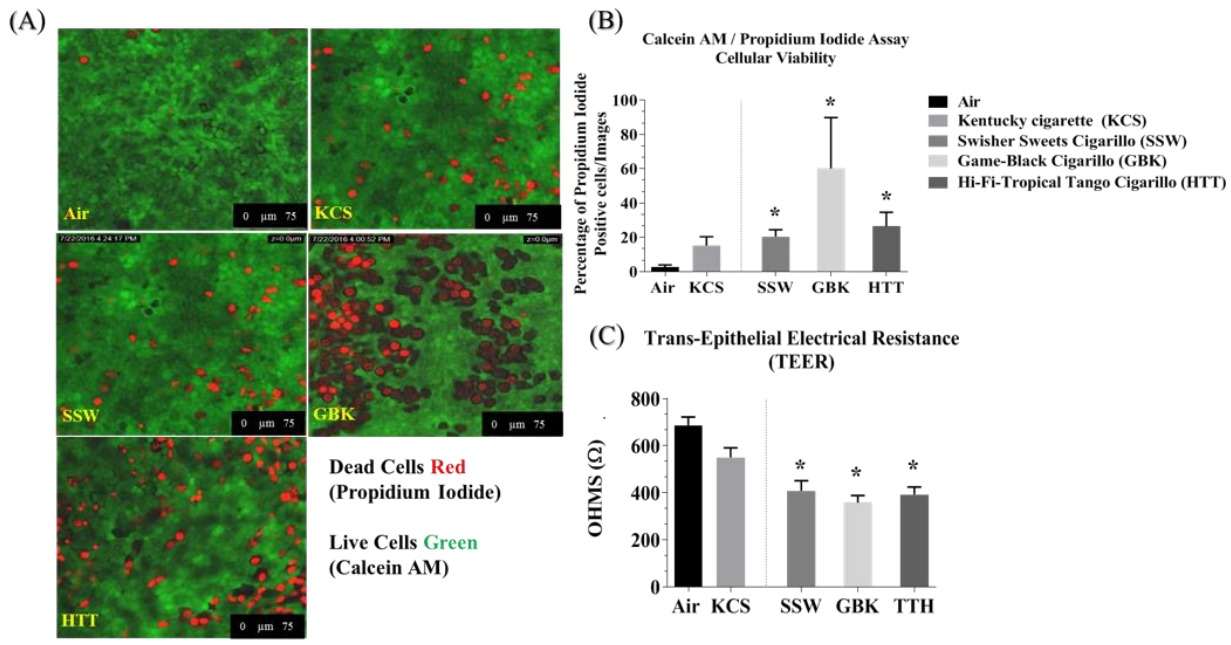


Figure 11: Effect of cigarillo tobacco products on HTBE cells. **(A)** Representative images of propidium iodide (red) uptake by chronic smoke-exposed HTBE cells showing calcein AM staining (green) for live cells after cigarillo smoke exposure. **(B)** Quantitation of the number of propidium iodide-positive cells per image (n=20 images). **(C)** Transepithelial electrical resistance (n=6) after cigarillo product (SSW, GBK, and HTT) and Kentucky research cigarette (KCS) smoke exposure. *Significantly different than epithelial cells exposed to air or KCS. Mean + SEM. One-way ANOVA, p-value < 0.05.

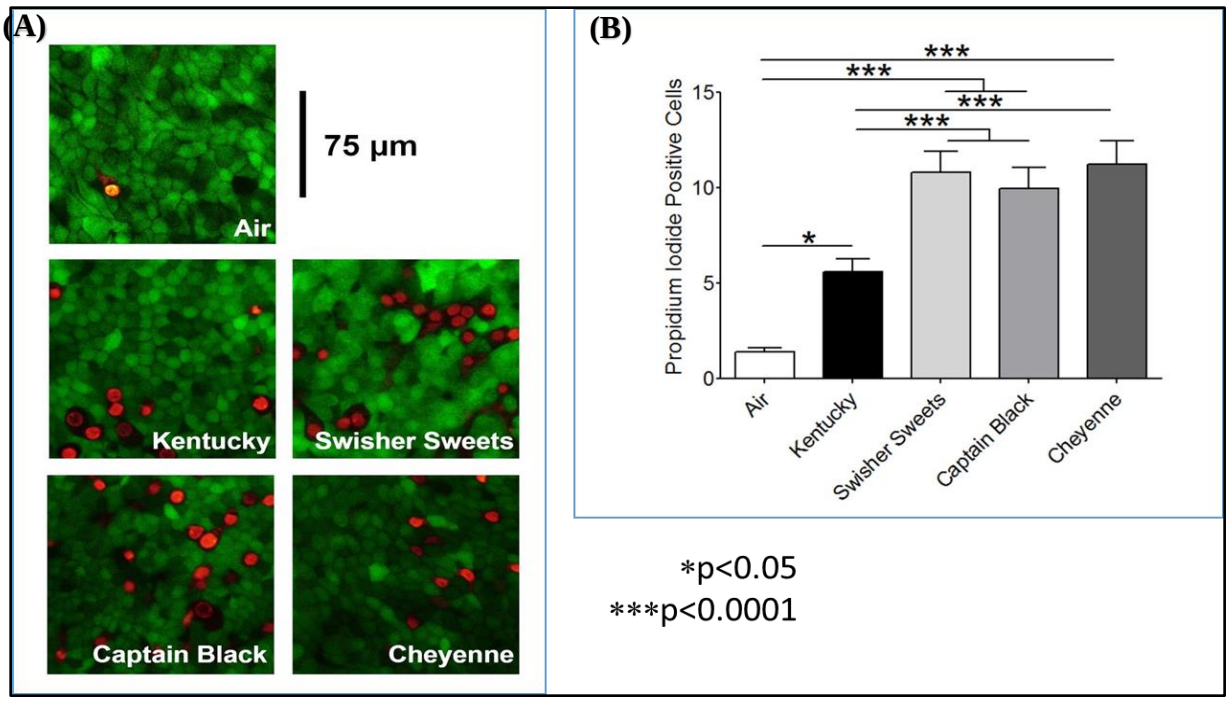


Figure 12: Chronic little cigar smoke exposure results in increased dead cells. **(A)** Representative images of propidium iodide (red) uptake by chronic smoke exposed HTBE cells with calcein-AM staining (green) for live cells after chronic smoke exposure. Each field had on average 220-230 cells. Scale Bar is 50 µm. **(B)** Bar graph of propidium iodide (PI) positive cells. n=6 biological replicates/group.

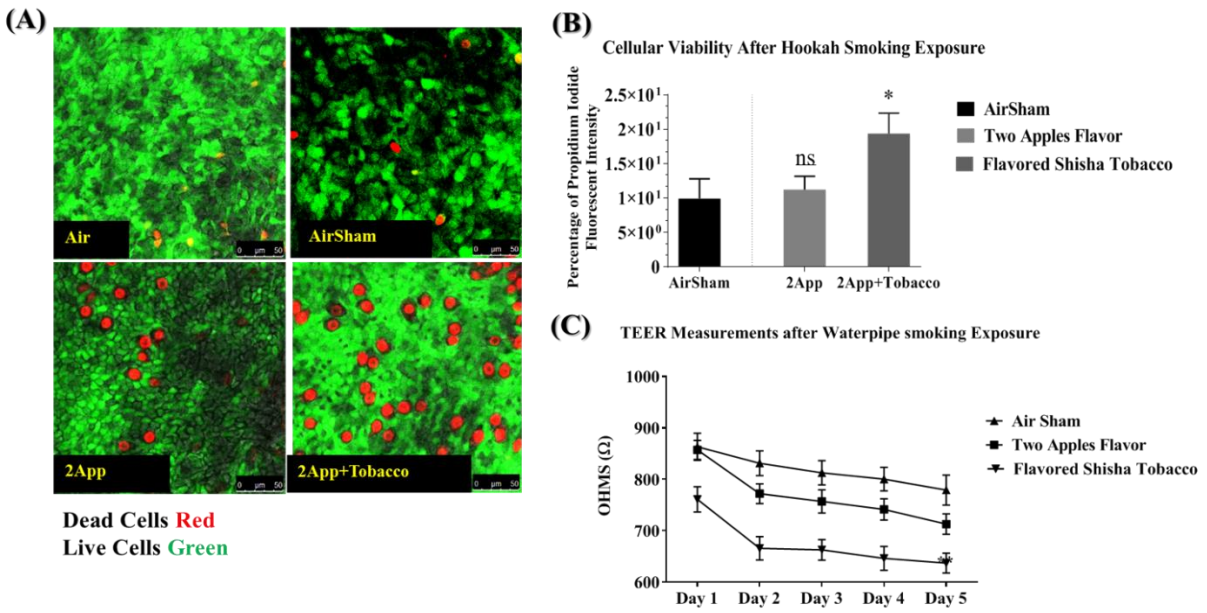


Figure 13: Waterpipe smoke exposure decreases cellular viability and Trans-Epithelial Electrical Resistance (TEER). **(A)** Representative images of HTBE cells exposed to air, airsham and waterpipe smoke includes Two Apples flavor (2App) and Two Apples flavor + Tobacco (2App+TOB) **(B)** Bar graph shows the percentage propidium iodide (PI) fluorescent intensity that measured by a microplate reader. **(C)** Trans-Epithelial Electrical Resistance (TEER) on smoked HTBE cells over five days exposure.*significantly different among the mean ± SEM measured by one-way ANOVA, **ANOVA with repeated measurement and p-value < 0.05. N=6/group.

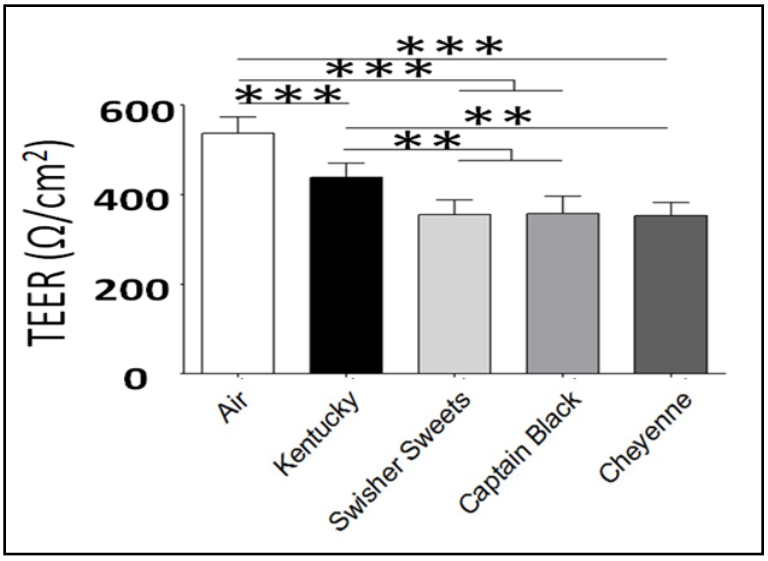


Figure 14: Chronic little cigar smoke exposure results in decreased Trans-Epithelial Electrical Resistance (TEER) on smoke exposed HTBE cells and disrupted of the epithelial cell layer. n=6 biological replicates/group.

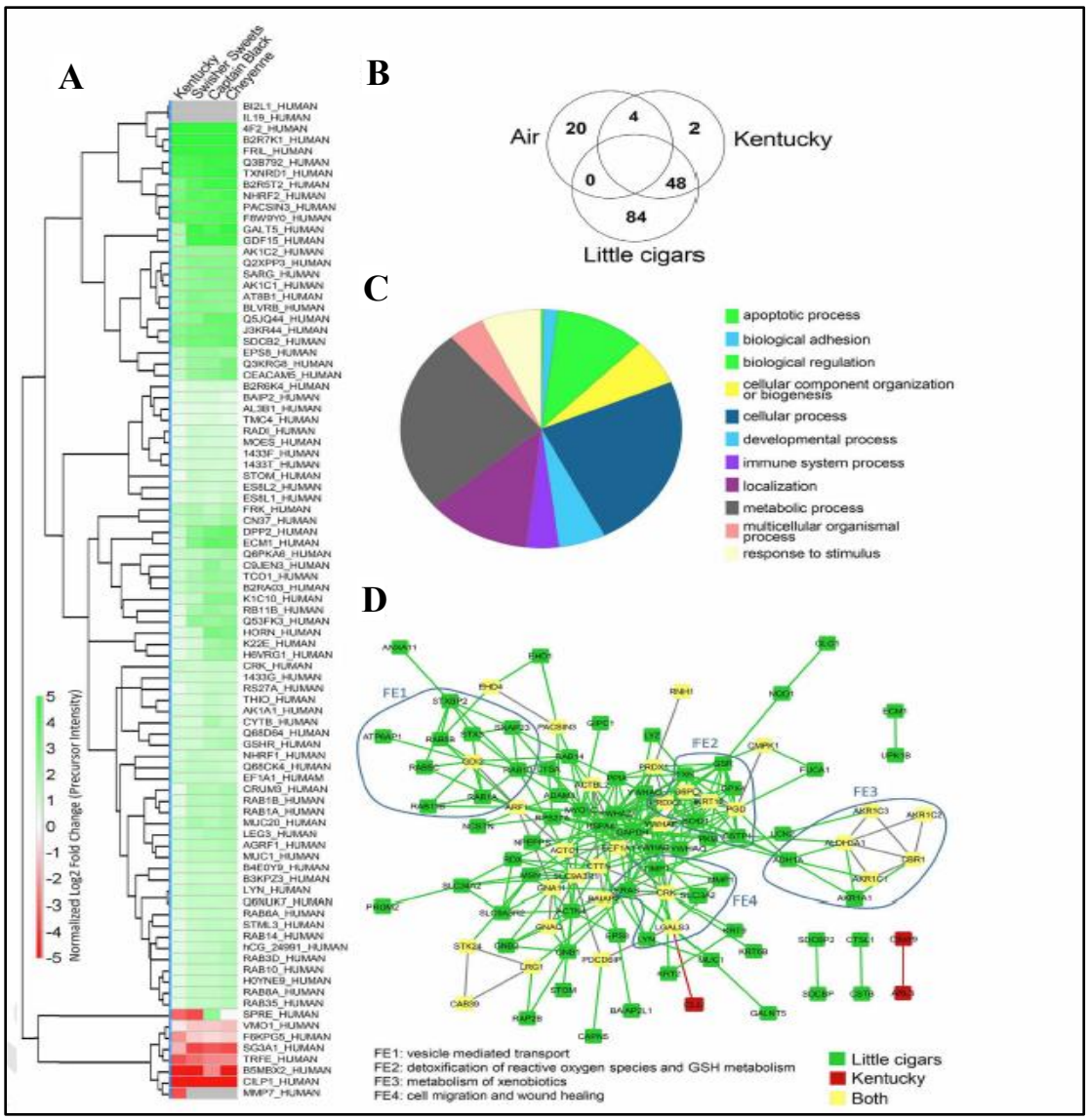


Figure 15 : Little cigar smoke exposure causes greater changes to the HTBE cell apical secretion's proteome than cigarette smoke exposure. **(A)** Heat map of significantly changed proteins relative to air controls. Significance was set at $p \leq 0.001$. **(B)** Venn diagram showing proteins that are upregulated in each group. **(C)** Pie chart representing the biological process classification for significantly changing proteins of all exposure groups. **(D)** Reactome map showing the functional enrichment **(FE)** of proteins with significant increases after Kentucky cigarette and LC exposure.[14]

[14] Ghosh A, Abdelwahab SH, Reeber SL, et al. Little Cigars are More Toxic than Cigarettes and Uniquely Change the Airway Gene and Protein Expression. *Sci Rep*. 2017; 7:46239. Published 2017 Apr 27. doi:10.1038/srep46239

Table 5: Partially list of differential proteins of HTBE cell apical secretions associated with little cigars exposure determined by mass spectrometry

Identified Protein	*Quantitative Value (Normalized Total Precursor Intensity)					**ANOVA Test P-Value
			Little cigars			
	Air	†KCS	†LCCN	†LCCB	†LCSS	
Proteins associated with immune system process						
BPI fold-containing family A member 1	5.15E+09	5.88E+09↑	3.07E+09↓	3.40E+09↓	3.94E+09↓	< 0.00010
Galectin-3	3.92E+08	7.89E+08↓	1.19E+09↑	1.06E+09↑	8.40E+08↑	< 0.00010
Complement C3	3.07E+10	2.50E+10↓	1.95E+10↓	2.16E+10↓	2.25E+10↓	< 0.00010
Spondin-2	1.44E+08	4.82E+07↓	2.05E+07↓	4.01E+07↓	3.48E+07↓	0.00043
Protein S100-A14	3.73E+07	7.09E+07↓	1.16E+08↑	1.03E+08↑	1.24E+08↑	0.011
Antioxidant activity associated proteins						
Protein S100-A9	7.65E+08	6.18E+08↓	1.12E+09↑	1.05E+09↑	7.99E+08↓	0.099
Peroxiredoxin-1	1.20E+09	2.14E+09↑	3.03E+09↑	2.91E+09↑	2.24E+09↑	0.013
Glutathione reductase	9.09E+07	1.55E+08↑	2.77E+08↑	2.29E+08↑	1.81E+08↑	< 0.00010
Peroxiredoxin-2	3.66E+08	5.74E+08↑	7.59E+08↑	7.20E+08↑	6.35E+08↑	0.0058
Glutathione peroxidase	2.57E+07	2.53E+07↓	4.05E+07↑	3.94E+07↑	4.25E+07↑	0.015
Secretory granule related proteins						
Uteroglobin	6.93E+10	4.42E+10↓	2.40E+10↓	3.05E+10↓	3.05E+10↓	0.00078
Alpha-actinin-4	9.19E+08	1.12E+09↑	1.60E+09↑	1.53E+09↑	1.28E+09↑	0.027
Ras-related protein Rab-3D	8.90E+07	1.18E+08↑	1.98E+08↑	1.86E+08↑	2.02E+08↑	< 0.00010
Ras-related protein Rab-10	2.19E+08	2.98E+08↑	4.75E+08↑	4.15E+08↑	4.83E+08↑	< 0.00010
Superoxide dismutase	1.36E+08	2.63E+08↑	4.23E+08↑	4.73E+08↑	3.33E+08↑	0.0020
Secretory fluid proteins process						
Aquaporin-5	5.17E+07	1.39E+08↑	2.52E+08↑	2.67E+08↑	2.51E+08↑	0.030
Ras-related protein Rab-14	8.67E+07	1.19E+08↑	2.00E+08↑	1.90E+08↑	1.98E+08↑	< 0.00010
Adhesion related proteins						
Calcium and integrin-binding protein 1	1.52E+09	1.89E+09↑	1.78E+09↑	2.01E+09↑	2.33E+09↑	0.0055
Moesin	4.36E+09	5.00E+09↑	6.86E+09↑	6.37E+09↑	7.36E+09↑	0.00016
Nck-associated protein 1	2.36E+06	8.41E+06↑	1.16E+07↑	2.12E+07↑	1.05E+07↑	0.032
Transforming growth factor-beta-induced protein ig-h3	2.75E+07	4.26E+06↓	9.90E+06↓	1.45E+07↓	9.68E+06↓	0.019

†Kentucky Research Cigarette (KCS), Little Cigar Cheyenne (LCCN), Little Cigar Captain Black (LCCB), Little Cigar Swisher Sweets (LCSS). *****Quantitative analysis was performed by Scaffold version 4.4.3 software. Precursor intensity peptides MS/MS based peptide and protein identifications. Peptide identifications were greater than 95.0% probability by the Scaffold Local FDR algorithm. Protein identifications were greater than 95.0% probability and contained at least two identified peptides. ******P-value ≤ 0.05: statistical significant of multiple groups. The values were regenerated by ANOVA test for the biological samples (N=2 donors cultures, 3 biological replications per cultures).

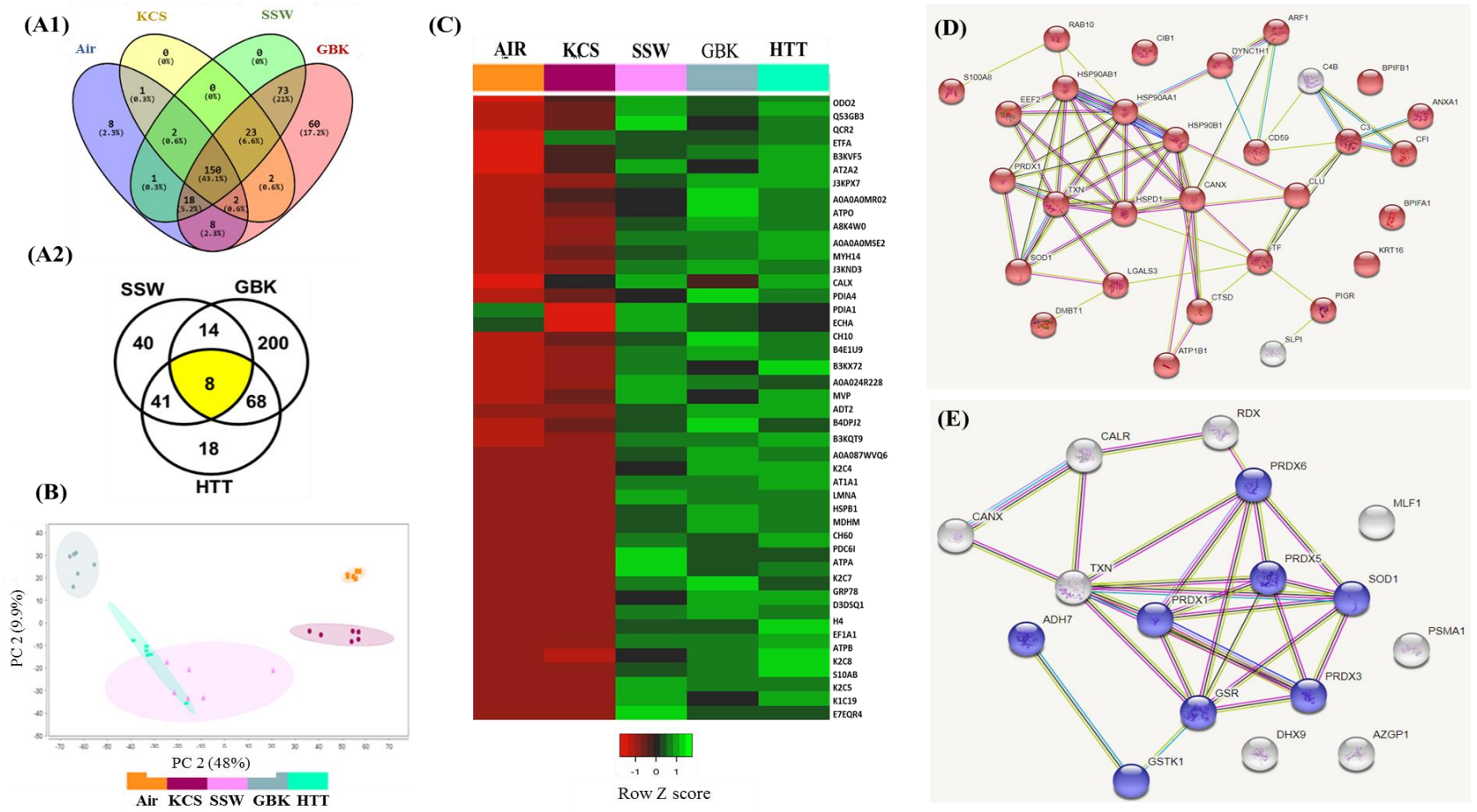


Figure 16: Proteomic analysis of the apical secretions of cigarillo smoke-exposed HTBE cells. (n=6 /group) Venn diagrams show significant differentially expressed proteins shared across and unique to each exposure: air, Kentucky research cigarettes (KCS) and cigarillos (SSW and GBK) **(A1).** Quantitative profile analysis of significant proteins across the cigarillo groups: Swisher-Sweets cigarillo (SSW)**,** Garcia y Vega Game black (GBK) and Hi-Fi Tropical Tango (HTT) cigarillos **(A2)**. Principal component analysis (PCA) of protein expression reveals a clustering of the cigarillo- (SSW, GBK, and HTT), KCS- and air-exposed groups. **(B)** A hierarchical heatmap displays the clustering analysis of protein expression after KCS and cigarillo smoke exposure. Cigarillos resulted in a clustering pattern different from that of cigarettes and air **(C)**. Functional enrichment pathway analysis of the differentially upregulated proteins after cigarillo exposure, demonstrating that the exposure induced changes in some proteins related to the innate immune (red) **(D)** and oxidative stress/oxidative stress-induced cell death pathways (blue) **(E)**

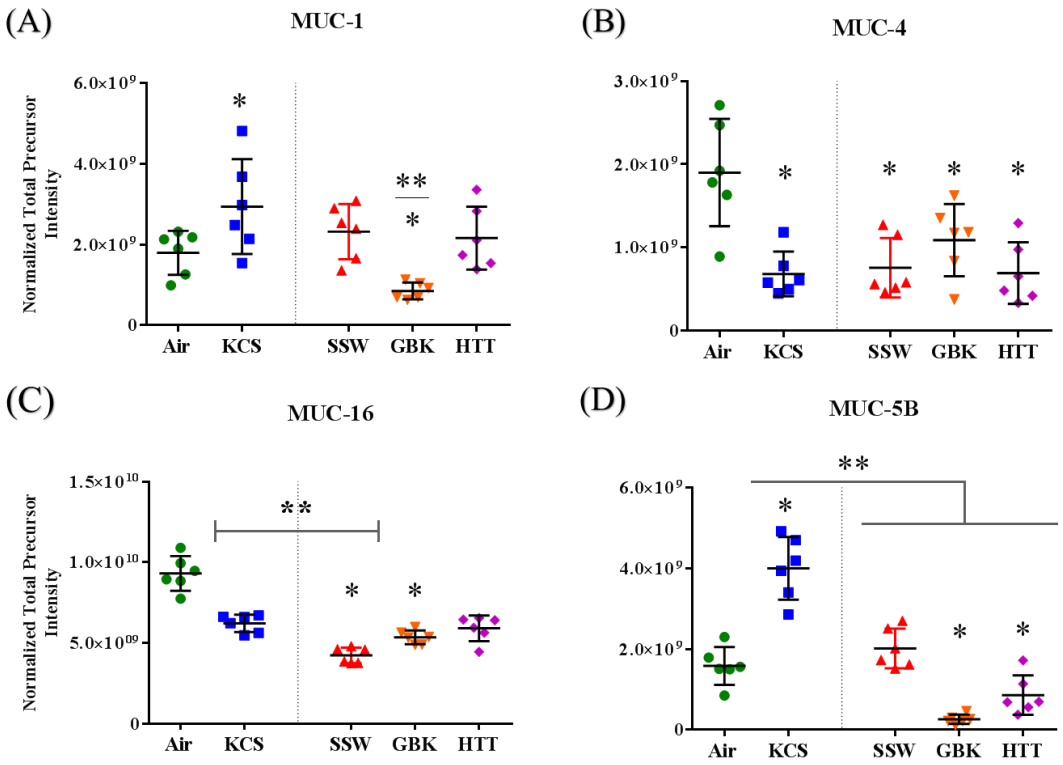


Figure 17: Cigarillo smoke exposure alters the expression of mucins: **(A)** MUC1, **(B)** MUC4, **(C)** MUC16 and **(D)** MUC5B in the apical secretions of smoke-exposed HTBE cells. Significantly different than epithelial cells exposed to *air or ** Kentucky research cigarettes (KCS) compared to Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black and Hi-Fi Tropical Tango (HTT). Mean ± SEM. One-way ANOVA, p value < 0.05.

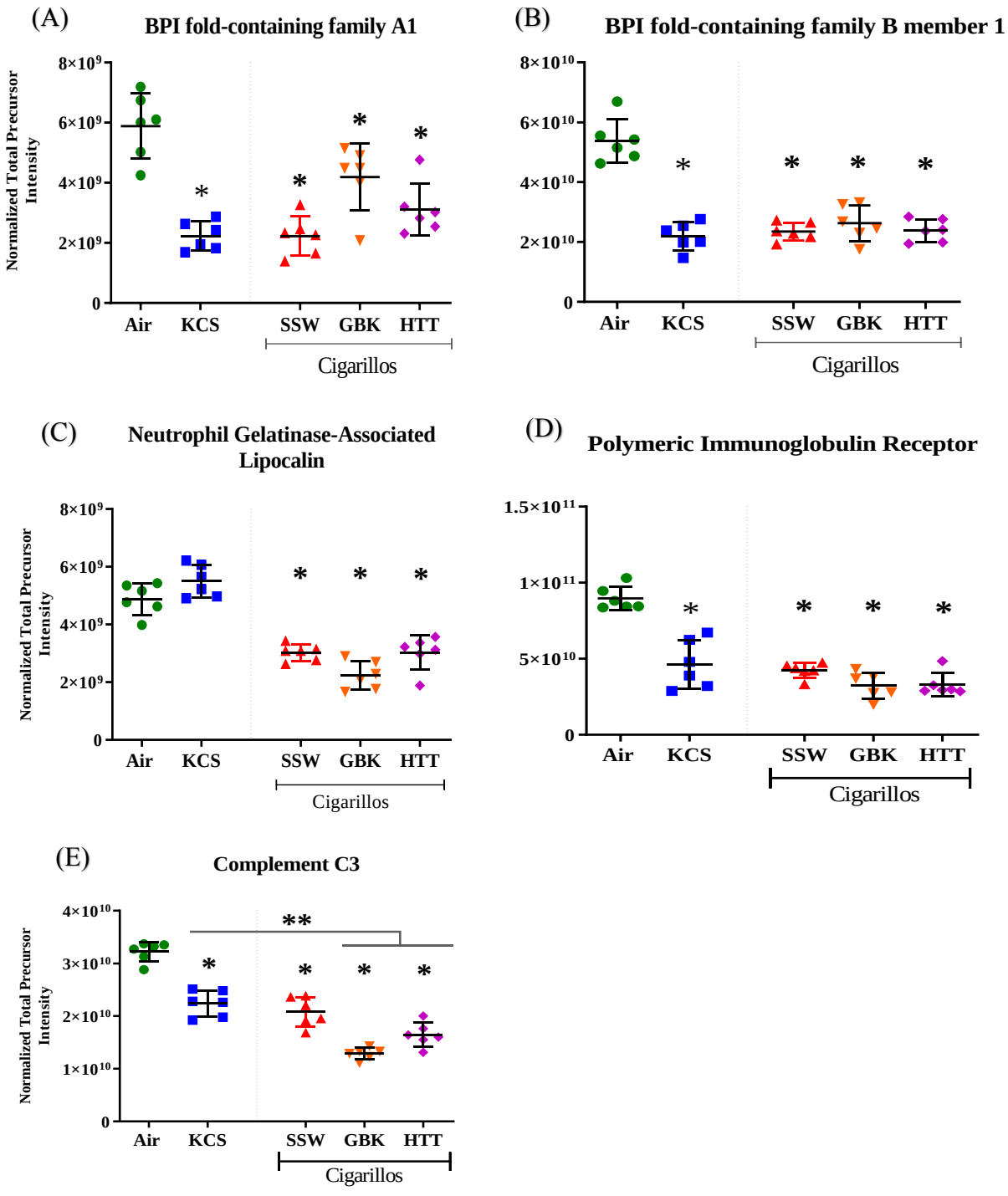


Figure 18: Cigarillo exposure changes protein expression related to the immune response. **(A)** BPI fold-containing family A1, **(B)** BPI fold-containing family B1, **(C)** neutrophil gelatinase-associated lipocalin, **(C)** complement C3 and **(D)** polyimmunoglobulin receptor. Significantly different than epithelial cells exposed to *air or ** Kentucky research cigarettes (KCS) compared to Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black and Hi-Fi Tropical Tango (HTT). Mean ± SEM. One-way ANOVA, p value < 0.05.

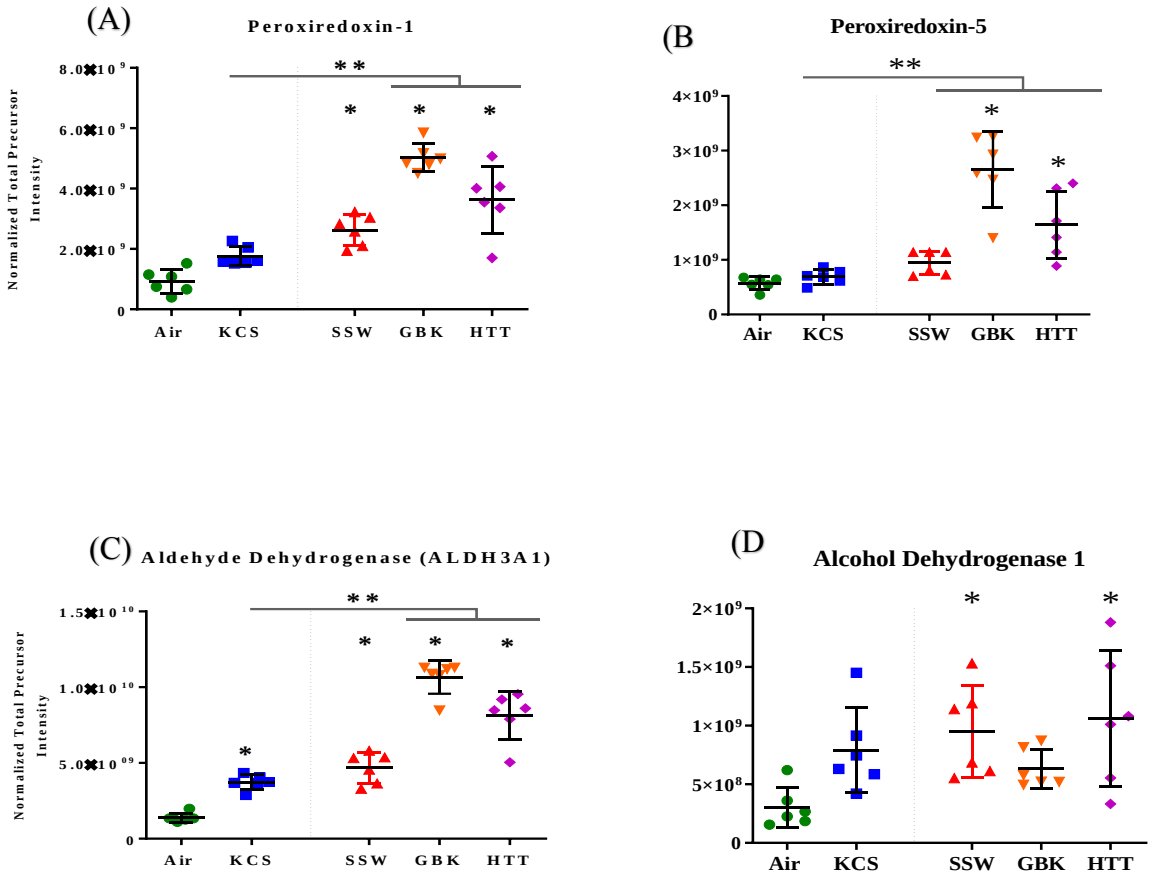


Figure 19: Cigarillos smoke exposure increase proteins expression in the oxidative stress pathway **(A)** Peroxiredoxin-1 and **(B)** Peroxiredoxin-5. It also upregulated **(C)** Aldehyde dehydrogenase- 3A1 and **(D)** Alcohol dehydrogenese-1 enzymes which involved in the detoxification process. Significantly different than epithelial cells exposed to *air or ** Kentucky research cigarettes (KCS) compared to Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black and Hi-Fi Tropical Tango (HTT). Mean ± SEM. One-way ANOVA, p value < 0.05.

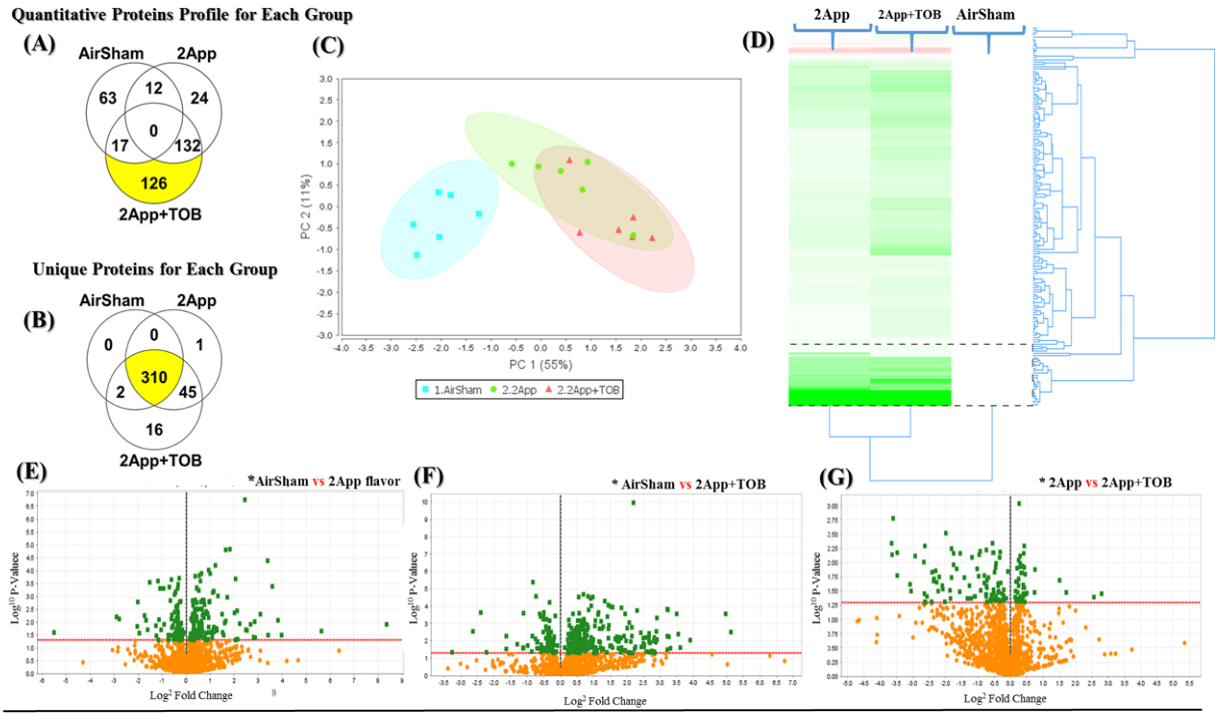


Figure 20: Proteomic quantitative analysis after waterpipe smoke exposure. **(A)** A Venn diagram shows the quantitative proteins significantly changed after airsham, Two Apples flavor (2App), Two Apples flavor+Tobacco (2App+TOB). **(B)** Quantitative profile of unique proteins identified for each group after the after exposure. **(C)** Principal component analysis (PCA) illustrated the clustering the air-sham differently from waterpipe smoked groups, 2App and 2App+TOB in conjunction with overlapping between them. **(D)** Clustering heatmap showed that pattern for protein expression for each exposed group. **Volcano** plots illustrated over all comparison of proteins expression in which the orange dots are insignificant and green dots are significant. Air-sham compared to **(E)** 2App and **(F)** 2App+TOB. **(G)** Compared 2App verses 2App+TOB. *t-test, $P<0.05$. N=6/group.

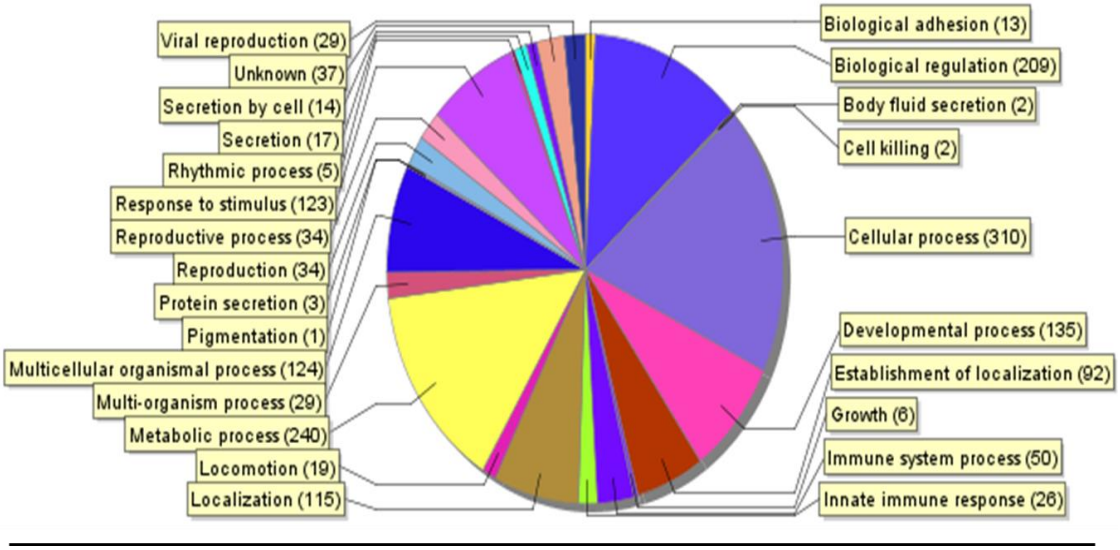


Figure 21: Pie chart representing the biological process classification for significantly changed proteins in the apical secretions of waterpipe smoked HTBE cells.

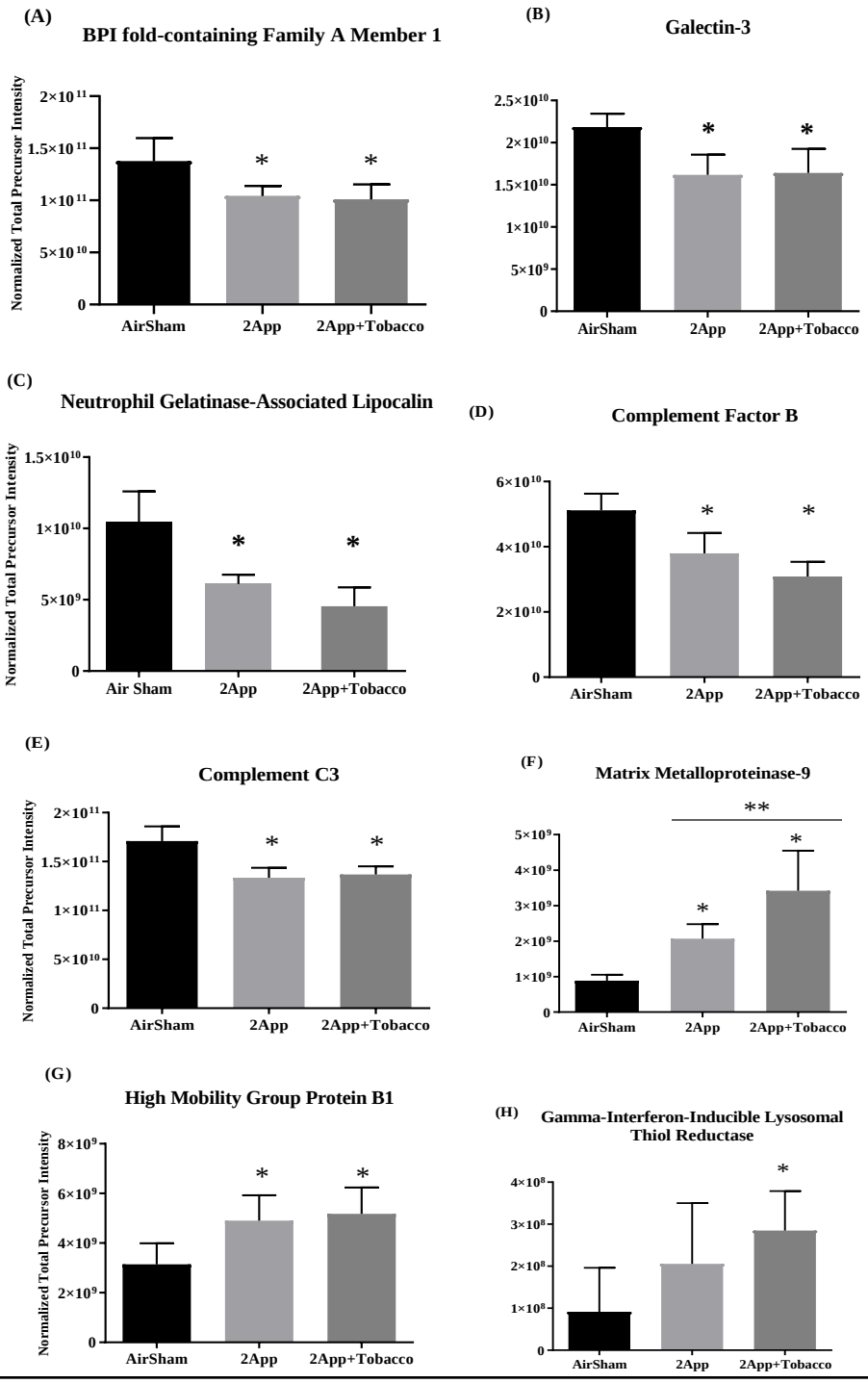


Figure 22: Waterpipe smoke exposure alters innate immune proteins: **(A)** BPI fold protein A1, **(B)** Galectin-3, **(C)** Neutrophil gelatinase-associated lipocalin (GILT), complement proteins include **(D)** factor B, and **(E)** C3, **(F)** Matrix metalloproteinase-9 (MMP9) High-mobility group protein-B1 and **(H)** Gamma-interferon-inducible lysosomal thiol reductase. *Mean ± SEM. One-way ANOVA, p value < 0.05. n=6/group.

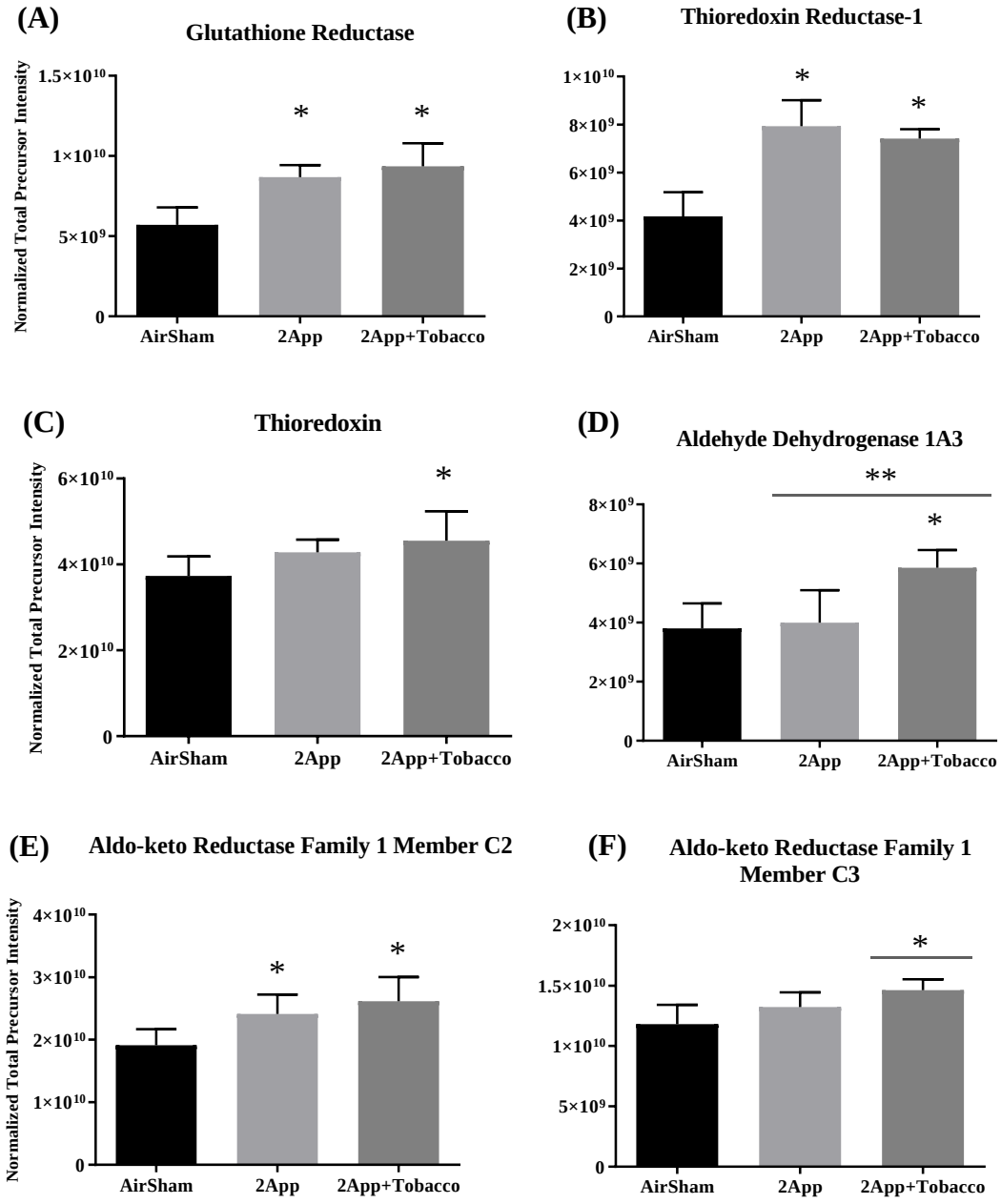


Figure 23: Waterpipe smoke exposure upregulates oxidative stress and detoxification enzymes protein **(A)** Glutathione reductase **(B)** Thioredoxin reductase-1, **(C)** Thioredoxin, **(D)** Aldehyde dehydrogenase 1A3, and Aldo-keto reductase family-1 which includes **(E)** member C2 and **(F)** member C2. *Mean ± SEM. One-way ANOVA, p value < 0.05. n=6/group.

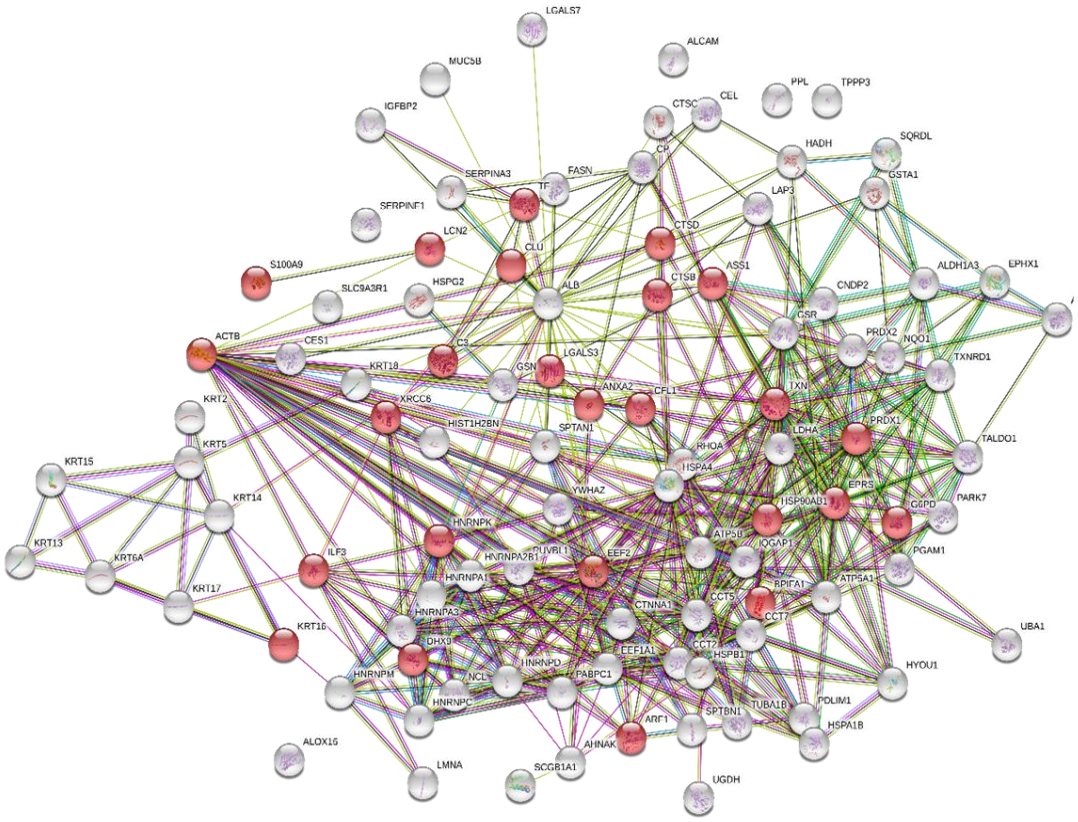


Figure 24: Enrichment pathway analysis of the significant differentially expressed proteins (red) changed after waterpipe smoke exposure shows pathways of the biological process involved such as activation of immune response and immune response-activating cell surface receptor signaling pathway.

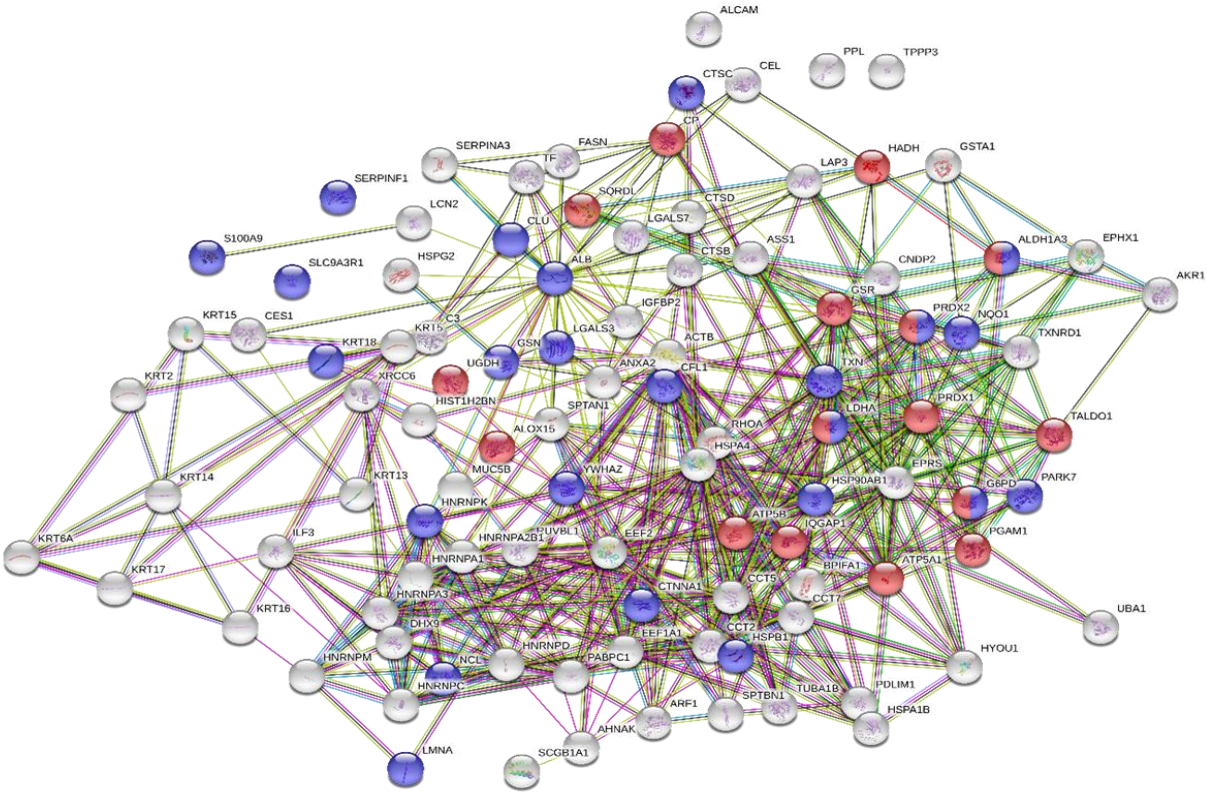


Figure 25: Enrichment pathway analysis of the significant differentially expressed proteins (red) changed after waterpipe smoke exposure shows pathways involved in oxidative stress (red) and cell death.

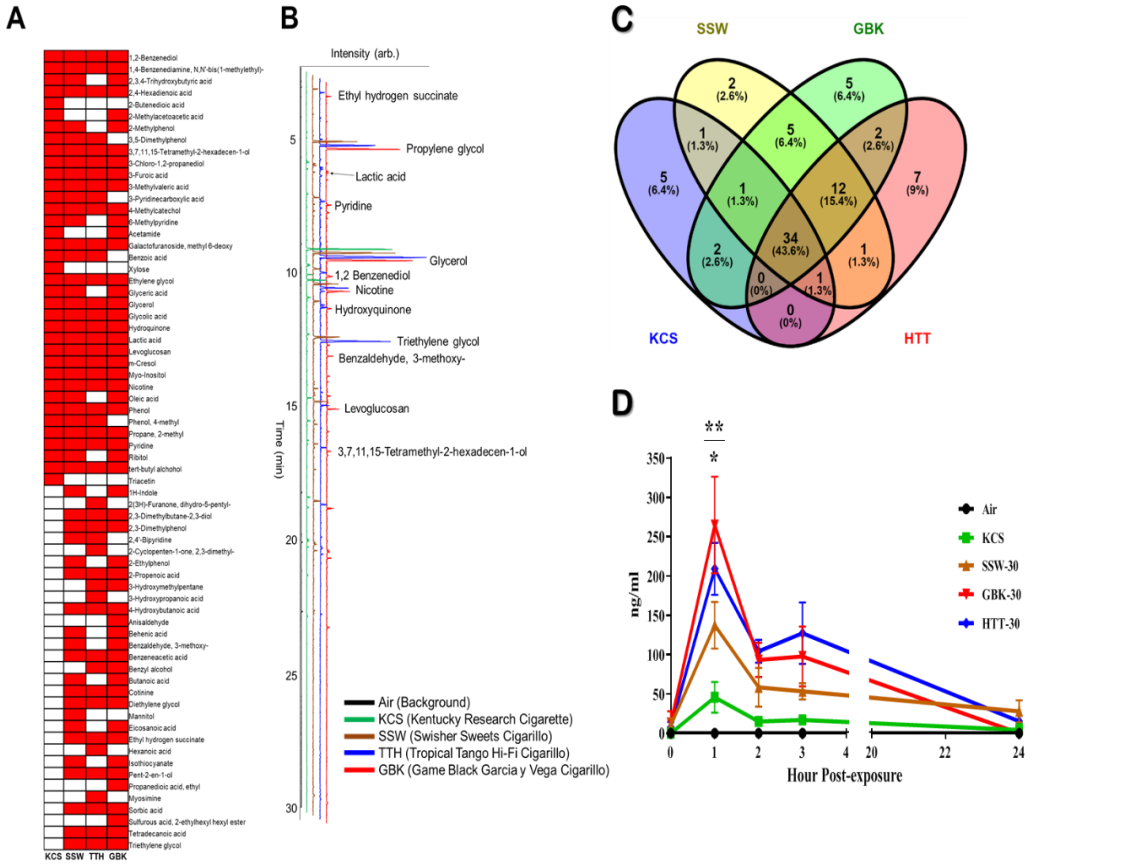
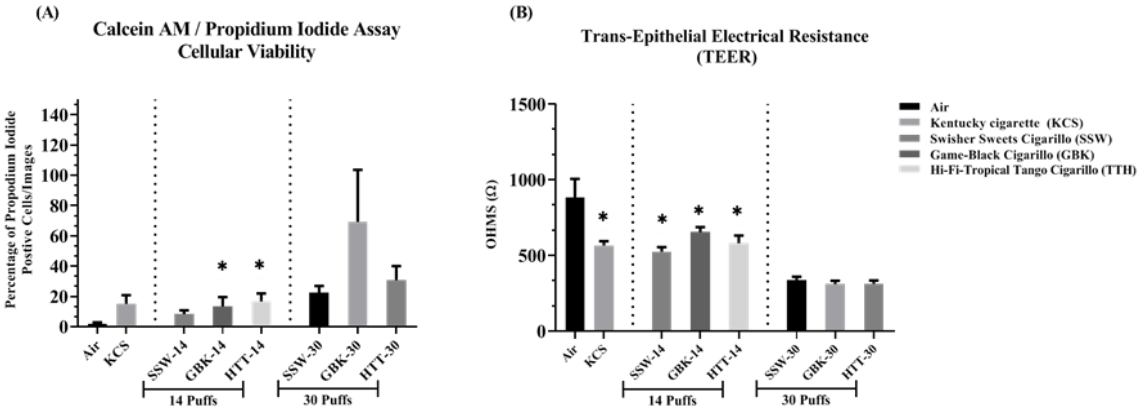
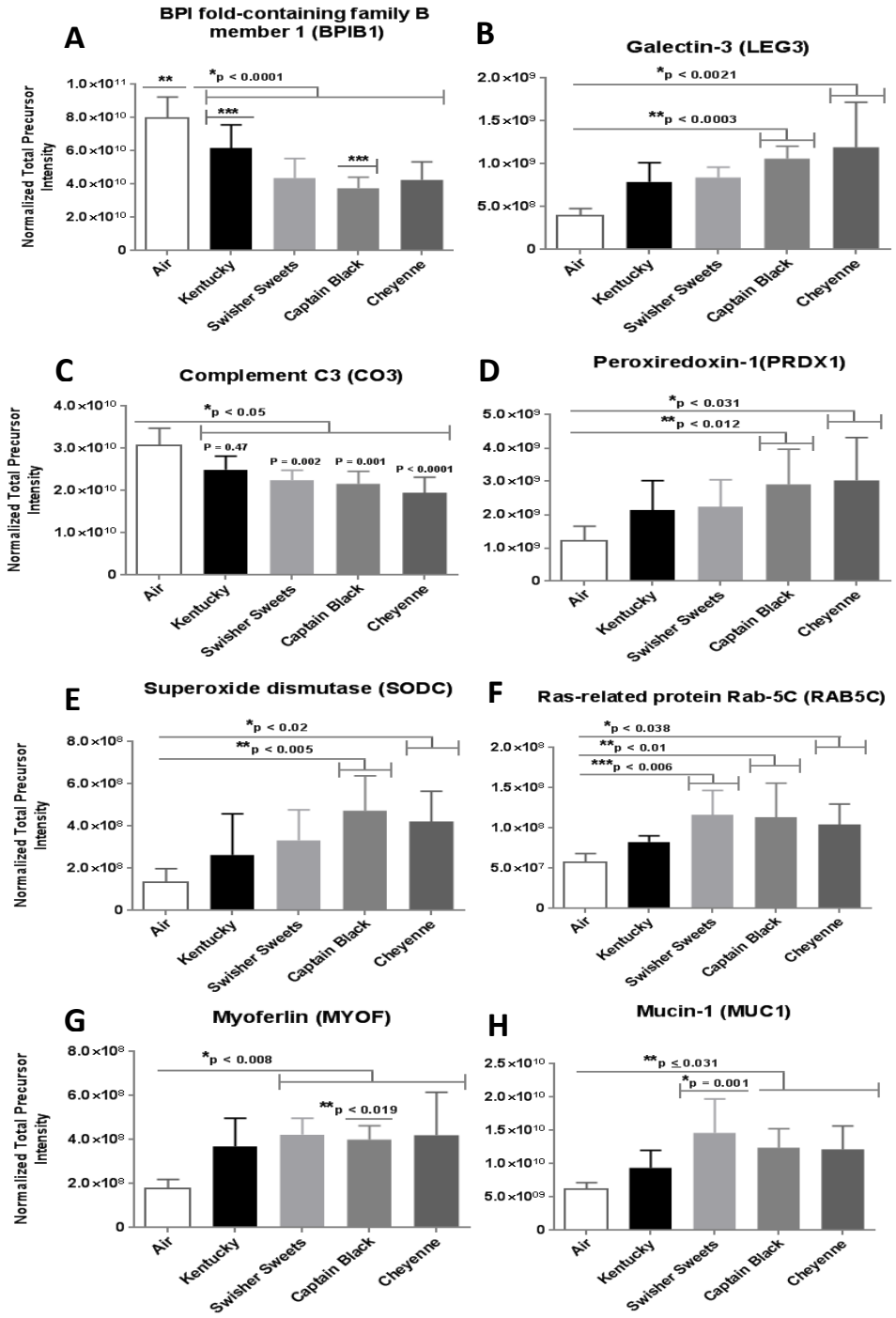


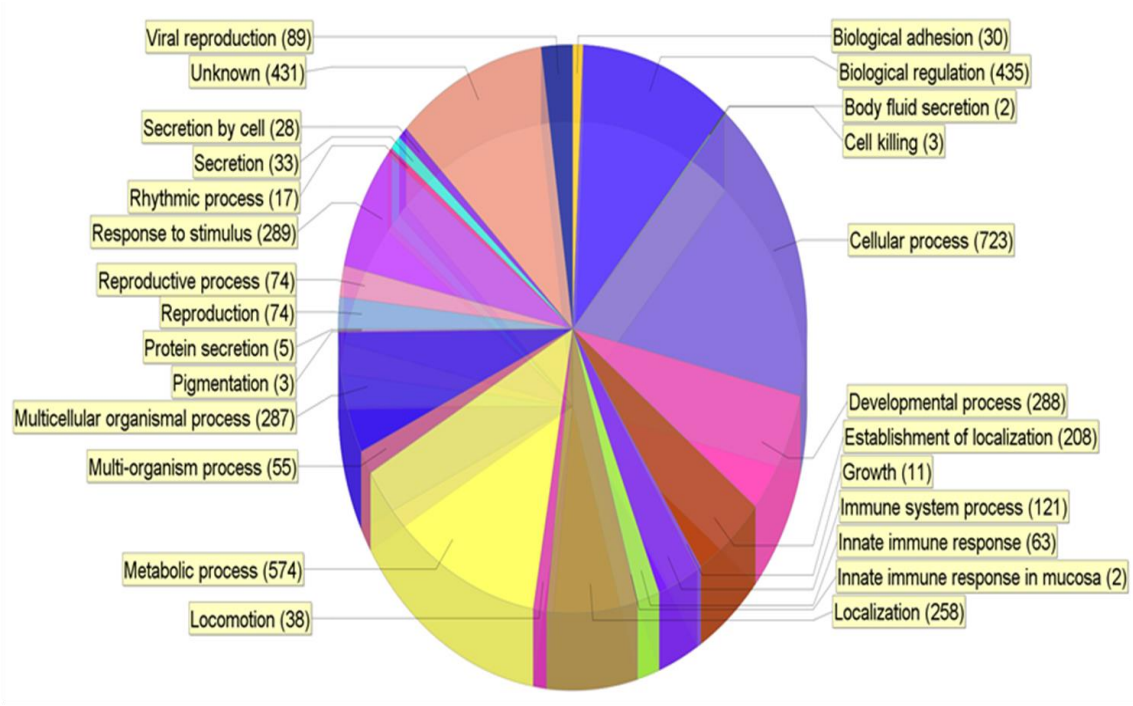
Figure 26: Analysis of chemical compounds. **(A)** Presence (red) and absence (white) of compounds identified in the particulate phase of smoke from tobacco products using gas chromatography-mass spectrometry (GC-MS). **(B)** Stacked chromatograms of trimethylsilyl (TMS)-derivatized filter extracts collected from mainstream smoke of Kentucky research cigarettes (KCS) and cigarillo products. **(C)** Unique and shared chemical entities were identified in smoking particles from different cigarillo products and KCS. **(D)** Time course of the nicotine concentrations in smoke-exposed HTBE cell apical secretions following exposure to tobacco products. Significantly different than mainstream smoke to *Air or ** Kentucky research cigarettes (KCS) compared to cigarillos (CLLO). Mean ± SEM. Two-way ANOVA with repeated measurement, sidak's multiple comparisons test, and p value < 0.05.



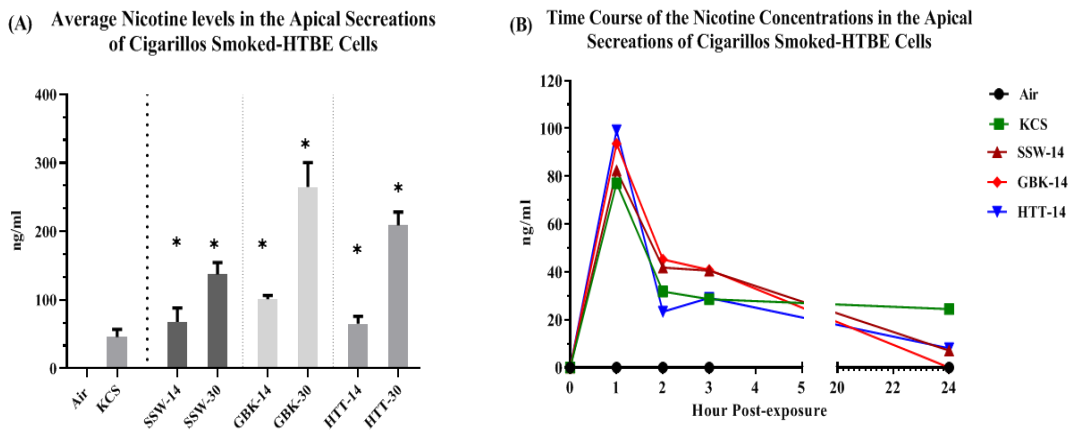
Supplement Figure 1: Effect of cigarillo tobacco smoke on HTBE cells. **(A)** Quantitation of the number of propidium iodide-positive cells per image (n=10-20 images). **(B)** Trans-epithelial electrical resistance (n=6) after cigarillo product (SSW, GBK, and HTT) and KCS smoke exposure. Among 14 puffs group, *significantly different than epithelial cells exposed to air. Mean + SEM. One-way ANOVA, p value < 0.05.



Supplement Figure 2 : Proteomic analysis of secretions from little cigar smoke exposed HBTE cells: Bar graphs showing quantitative analysis for selected proteins involved in immune system processes **(A, B, and C)**, proteins associated with antioxidant activity **(D)**, and secretory granule related proteins **(E and F)**, and proteins involved in repair mechanisms **(G)** and mucus **(H)**. Quantified protein hits were based on at least two identified peptides with a 2% FDR. Protein *Tukey's multiple comparisons test was performed on values generated by ANOVA test for the biological samples and analysis of variance statistical significance, p-value ≤ 0.05. (N= 2 donors cultures, 3 biological replications per cultures).



Supplement Figure 3: Proteomic analysis of the apical secretions of cigarillo smoke-exposed HTBE cells, (n= 6 /group): a pie chart summarizes the biological processes identified based on proteins that changed expression following exposure to each tobacco product.



Supplement Figure 4: (A) The average Nicotine level concentrations in smoke-exposed HTBE cell apical secretions following one-hour post exposure to cigarillos tobacco product, 14 and 30 puffs. **(B)** Time course of the nicotine concentrations in smoke-exposed HTBE cell apical secretions following exposure to 14 puffs of Kentucky research cigarettes (KCS) and cigarillo products. *significantly different than epithelial cells exposed to air. Mean + SEM. Ordinary One-way ANOVA, Uncorrected Fisher's LSD, p value < 0.05.

Supplement Table 1: ‡Level of the nicotine detected on the apical secretions of smoked-HTBE cells over 24-hours post exposure to air (control), Kentucky research cigarettes (KCS) or cigarillos which include Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black cigarillo (GBK) and Hi-Fi Tropical Tango cigarillo (HTT)

Group	Pre-exposure	1-hour	2-hour	3-hour	24-hour
Air	<LOQ[15]	<LOQ	<LOQ	<LOQ	<LOQ
KCS	<LOQ	46±20	15±6	17±5	<LOQ
SSW-14	<LOQ	68±35	38±5	37±8	<LOQ
SSW-30	<LOQ	137±30	58±25	53±11	28±14
GBK-14	<LOQ	101±10	46±10	40±3	<LOQ
GBK-30	<LOQ	265±61	93±22	98±38	<LOQ
HTT14	<LOQ	68±17	36±11	33±3	<LOQ
HTT-30	<LOQ	207±32	106±14	128±38	19±3

‡All units in ng/ml

[15] LOQ (Limit of Quantitation)

Supplement Table 2: List of proteins significantly altered in HBEC ASL after chronic (5-day) exposure to air (control), Kentucky research cigarettes or little cigars. The mean of the total precursor ion intensity with p-value<0.001, as determined by ANOVA, is shown.

Accession Number	P-Value	Air Mean	Kentucky Mean	Cheyenne Mean	Captain Black Mean	Swisher Sweets Mean
B2R5T2_HUMAN	1.00E-04	15942017	68366667	2.17E+08	2.31E+08	1.78E+08
Q59EP1_HUMAN	1.10E-04	1.8E+08	3.87E+08	4.75E+08	4.05E+08	4.48E+08
A8K8D9_HUMAN	1.10E-04	8108917	28750000	39983333	42083333	45228167
AK1C1_HUMAN	1.20E-04	1.46E+08	4.33E+08	6.91E+08	5.81E+08	6.86E+08
ECM1_HUMAN	1.20E-04	35800000	59633333	2.86E+08	2.64E+08	2.21E+08
AT8B1_HUMAN	1.20E-04	9738017	26966667	45600000	46233333	58133333
V9HW42_HUMAN	1.30E-04	9.73E+09	1.16E+10	1.51E+10	1.44E+10	1.61E+10
ES8L2_HUMAN	1.30E-04	5.6E+08	9.21E+08	1.36E+09	1.15E+09	1.43E+09
GSHR_HUMAN	1.50E-04	84250000	1.44E+08	2.6E+08	2.14E+08	1.69E+08
ES8L1_HUMAN	1.60E-04	2.7E+08	3.76E+08	6.33E+08	5.09E+08	6.37E+08
G3P_HUMAN	1.70E-04	5.17E+08	6.09E+08	7.79E+08	7.16E+08	7.51E+08
SG3A1_HUMAN	1.80E-04	3.85E+09	9.48E+08	2.14E+08	2.9E+08	3.8E+08
MMP7_HUMAN	1.80E-04	22057600	0	0	0	0
BAIP2_HUMAN	2.10E-04	5.34E+08	7.18E+08	7.9E+08	8.23E+08	9.76E+08
A0A0G2JPR0_HUMAN	2.10E-04	46900117	13038233	3216667	5645817	1726600
BLVRB_HUMAN	2.10E-04	2819833	16662133	28473233	30100000	27233333
EF1A1_HUMAN	2.20E-04	3.18E+08	5.76E+08	6.52E+08	5.59E+08	6.11E+08
LEG3_HUMAN	2.60E-04	3.63E+08	7.33E+08	1.12E+09	9.92E+08	7.85E+08
ARF1_HUMAN	2.80E-04	1.33E+08	2.43E+08	3.28E+08	2.8E+08	2.97E+08
A0A087WWM1_HUMAN	3.10E-04	5.72E+09	8.72E+09	1.14E+10	1.16E+10	1.37E+10
A0A0C4DGG1_HUMAN	3.10E-04	4927485	20883333	31103517	28783333	31883333
Q3KRG8_HUMAN	3.20E-04	1.63E+08	3.68E+08	1.03E+09	6.76E+08	8.72E+08
Q53HG7_HUMAN	3.30E-04	30000000	43650000	56283333	52150000	59450000
RAB5C_HUMAN	3.40E-04	53366667	77066667	97866667	1.06E+08	1.09E+08
I1SRC5_HUMAN	3.60E-04	40283333	53016667	83016667	87866667	79316667
MOES_HUMAN	3.90E-04	4.05E+09	4.66E+09	6.44E+09	5.96E+09	6.87E+09
ITLN1_HUMAN	3.90E-04	8823900	409683.3	0	0	0
RADI_HUMAN	4.30E-04	4.83E+09	5.57E+09	7.56E+09	7.1E+09	7.94E+09
PTPRS_HUMAN	4.40E-04	5913517	1589017	211533.3	982583.3	91543.33
NHRF1_HUMAN	4.90E-04	1.68E+09	2.54E+09	2.84E+09	2.89E+09	3.14E+09
MYOF_HUMAN	4.90E-04	1.65E+08	3.44E+08	3.94E+08	3.75E+08	3.96E+08
B4E0Y9_HUMAN	5.00E-04	56033333	1.03E+08	1.38E+08	1.18E+08	1.44E+08
RS27A_HUMAN	5.10E-04	1.99E+08	2.87E+08	4.72E+08	4.86E+08	4.33E+08
SPON2_HUMAN	5.10E-04	1.34E+08	45100000	19184600	37576000	32583333
B2RA03_HUMAN	5.20E-04	1.2E+08	4.57E+08	6.67E+08	6.54E+08	3.8E+08

SNP23_HUMAN	5.20E-04	16816667	30400000	45283333	51550000	58266667
B2R6S5_HUMAN	6.30E-04	6024523	30853183	57966667	59836933	46583333
A8K8Z4_HUMAN	6.30E-04	5388283	2180850	724333.3	1828867	889266.7
Q5JQ44_HUMAN	6.60E-04	2150617	17788650	38684150	34718667	30066667
CTL4_HUMAN	7.70E-04	2.88E+08	3.56E+08	4.23E+08	4.26E+08	4.8E+08
Q53FK3_HUMAN	7.80E-04	36533333	73150000	2E+08	1.52E+08	1.99E+08
CBR1_HUMAN	9.00E-04	25950000	55850000	56650000	47866667	53433333
F6KPG5_HUMAN	9.50E-04	4.64E+09	5.84E+08	1.94E+09	1.29E+09	1.19E+09
1433Z_HUMAN	1.00E-03	7.81E+08	9.95E+08	1.27E+09	1.26E+09	1.31E+09
FUCO_HUMAN	1.00E-03	1.06E+08	1.9E+08	3.32E+08	3.17E+08	3.24E+08
GNA11_HUMAN	1.10E-03	2.22E+08	3.43E+08	4.11E+08	4.25E+08	4.33E+08
KLK10_HUMAN	1.10E-03	5.87E+08	1.12E+09	6.41E+08	7.87E+08	8.19E+08
GSLG1_HUMAN	1.10E-03	17616667	22333333	50683333	43950000	37366667
A0A024RC87_HUMAN	1.10E-03	6853950	25283333	20466667	23166667	24150000
SARG_HUMAN	1.40E-03	20643200	63450000	1.63E+08	1.43E+08	1.43E+08
AL3A1_HUMAN	1.50E-03	1.17E+09	2.69E+09	4.33E+09	4.1E+09	3.21E+09
Q68CK4_HUMAN	1.80E-03	4.41E+08	7E+08	7.88E+08	9.55E+08	9.07E+08
SODC_HUMAN	1.80E-03	1.26E+08	2.46E+08	3.97E+08	4.42E+08	3.11E+08
A0A087WT12_HUMAN	1.90E-03	23800000	23633333	37816667	36800000	39633333
A0A024R462_HUMAN	2.30E-03	4184733	0	0	0	0
A0A024R872_HUMAN	2.40E-03	60183333	95466667	1.22E+08	1.18E+08	1.2E+08
PRDX2_HUMAN	2.60E-03	3.4E+08	5.35E+08	7.12E+08	6.74E+08	5.94E+08
CAB39_HUMAN	2.70E-03	1.34E+08	2.07E+08	2.25E+08	2.27E+08	2.42E+08
MA1C1_HUMAN	2.80E-03	13440367	3564350	0	2092300	3604983
RAB5B_HUMAN	2.80E-03	26400000	36316667	47266667	60650000	50150000
CLUS_HUMAN	2.90E-03	4.85E+09	4.13E+09	2.55E+09	2.97E+09	3.31E+09
THIO_HUMAN	2.90E-03	6.61E+08	8.84E+08	1.22E+09	1.28E+09	9.74E+08
B4E1P0_HUMAN	2.90E-03	6447733	21916667	37183333	37915517	27550000
BSSP4_HUMAN	3.00E-03	2.15E+08	2.08E+08	3.2E+08	3.58E+08	3.54E+08
TRFE_HUMAN	3.30E-03	1.63E+09	59500000	1.6E+08	1.63E+08	1E+08
BGH3_HUMAN	3.30E-03	25488050	3983400	9273667	13569783	9024017
STK24_HUMAN	3.40E-03	69333333	1.39E+08	1.66E+08	1.43E+08	1.86E+08
G9FP35_HUMAN	3.40E-03	1.46E+08	2.62E+08	3.4E+08	3.61E+08	3.56E+08
KPYM_HUMAN	3.50E-03	9.79E+08	1.1E+09	1.38E+09	1.26E+09	1.35E+09
STXB2_HUMAN	3.50E-03	1.62E+08	2.35E+08	3.17E+08	2.95E+08	3.52E+08
A8K2I7_HUMAN	3.50E-03	15082550	39550000	45766667	50866667	53583333
1433G_HUMAN	3.70E-03	2.07E+08	2.91E+08	4.79E+08	3.86E+08	4.27E+08
K22E_HUMAN	3.90E-03	4.36E+08	6.29E+08	2.22E+09	1.69E+09	9.21E+08
ARL3_HUMAN	3.90E-03	6070583	22000000	27266667	24590633	26643600
CN37_HUMAN	4.00E-03	21095750	44483333	76816667	62850000	62233333
B4E324_HUMAN	4.00E-03	45966667	63283333	1.28E+08	1.01E+08	1.21E+08

B2ZDQ1_HUMAN	4.10E-03	3.91E+09	5.14E+09	1.14E+10	9.38E+09	9.67E+09
K1C9_HUMAN	4.30E-03	2.93E+08	6.71E+08	1.31E+09	1.44E+09	7.99E+08
GALT5_HUMAN	4.30E-03	2800383	10825267	85050000	52317800	64050000
Q4W4Y1_HUMAN	4.40E-03	5.56E+08	7.97E+08	8.08E+08	8.29E+08	9.13E+08
CI009_HUMAN	4.60E-03	5791067	4001767	0	1192883	929033.3
STOM_HUMAN	4.70E-03	6.89E+08	8.56E+08	1.78E+09	1.51E+09	1.43E+09
A0A024RE18_HUMAN	4.80E-03	43250000	71716667	79150000	1.02E+08	99666667
HSP7C_HUMAN	4.90E-03	8E+08	9.89E+08	1.22E+09	1.18E+09	1.16E+09
A8KAH3_HUMAN	4.90E-03	855566.7	7133333	15500000	14660233	17147733
CLIC1_HUMAN	5.00E-03	1.23E+09	1.67E+09	2.19E+09	1.91E+09	2.26E+09

Supplement Table 3: List of proteins significantly altered in HTBE cell apical secretions after exposure to air (control), Kentucky research cigarettes (KCS) or cigarillos, which include Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black cigarillo (GBK) and Hi-Fi Tropical Tango cigarillo (HTT). The mean of the total precursor ion intensity with p-value<0.05, as determined by ANOVA, is shown.

Accession Number	ANOVA (p-value)	Air	KCS	SSW	GBK	HTT
PIGR_HUMAN	1.00E-04	8.97E+10	4.62E+10	4.23E+10	3.23E+10	3.29E+10
CO3_HUMAN	1.00E-04	3.22E+10	2.24E+10	2.08E+10	1.29E+10	1.64E+10
BPIB1_HUMAN	1.00E-04	5.38E+10	2.19E+10	2.35E+10	2.63E+10	2.38E+10
E7EQR4_HUMAN	1.50E-04	1.19E+10	6.00E+09	1.01E+10	1.10E+10	9.21E+09
GELS_HUMAN	3.10E-04	7.90E+09	6.00E+09	5.30E+09	4.17E+09	4.83E+09
Q53G99_HUMAN	2.80E-04	1.85E+10	2.77E+10	3.05E+10	1.92E+10	2.55E+10
MUC16_HUMAN	1.00E-04	9.30E+09	6.21E+09	4.22E+09	5.35E+09	5.91E+09
Q8IZ29_HUMAN	1.00E-04	1.08E+09	2.06E+09	2.90E+09	9.88E+09	5.57E+09
LG3BP_HUMAN	1.30E-03	2.09E+09	3.25E+09	3.24E+09	2.03E+09	2.32E+09
AL3A1_HUMAN	1.00E-04	1.40E+09	3.74E+09	4.66E+09	1.06E+10	8.13E+09
TBA1A_HUMAN	1.00E-04	9.60E+08	1.32E+09	1.96E+09	6.54E+09	3.27E+09
KPYM_HUMAN	1.00E-04	5.84E+08	1.08E+09	2.38E+09	4.49E+09	3.67E+09
V9HW65_HUMAN	1.00E-04	1.89E+09	8.51E+09	6.02E+09	3.14E+09	4.73E+09
ANXA1_HUMAN	1.00E-04	1.60E+09	6.04E+09	4.50E+09	3.73E+09	5.36E+09
K1C19_HUMAN	1.00E-04	9.77E+08	5.40E+09	1.33E+10	9.58E+09	1.26E+10
AL1A1_HUMAN	1.00E-04	4.63E+08	9.90E+08	1.19E+09	5.70E+09	3.29E+09
MUC5B_HUMAN	1.00E-04	1.59E+09	4.00E+09	2.02E+09	2.63E+08	8.63E+08
K2C5_HUMAN	1.00E-04	1.31E+09	3.80E+09	1.02E+10	6.13E+09	7.93E+09
BPIA1_HUMAN	1.00E-04	5.89E+09	2.23E+09	2.22E+09	4.19E+09	3.11E+09
B2ZDQ1_HUMAN	1.00E-04	4.89E+09	5.50E+09	3.02E+09	2.23E+09	3.02E+09
MUC4_HUMAN	1.80E-04	1.90E+09	6.79E+08	7.56E+08	1.09E+09	6.90E+08
B2R920_HUMAN	1.20E-03	8.96E+08	3.03E+09	1.13E+09	6.33E+09	3.92E+09
TRFE_HUMAN	2.40E-04	1.89E+09	3.42E+09	2.35E+09	2.64E+09	2.55E+09
SPB3_HUMAN	1.00E-04	1.37E+09	3.08E+09	3.91E+09	1.91E+09	6.29E+09
LOX15_HUMAN	1.00E-04	1.81E+08	1.30E+08	7.32E+08	1.49E+09	1.34E+09
H6VRF8_HUMAN	6.70E-04	1.74E+09	5.59E+09	3.95E+09	1.57E+09	3.00E+09
HSP7C_HUMAN	1.00E-04	6.31E+08	1.83E+09	1.78E+09	4.00E+09	2.94E+09
PRDX5_HUMAN	1.00E-04	5.69E+08	6.91E+08	9.45E+08	2.65E+09	1.64E+09
CATD_HUMAN	3.60E-03	1.61E+09	1.20E+09	1.01E+09	2.36E+09	2.14E+09
CLUS_HUMAN	1.00E-04	6.27E+09	4.46E+09	3.35E+09	2.72E+09	2.89E+09
B4E1Z4_HUMAN	1.00E-04	4.00E+09	4.96E+09	2.00E+09	1.13E+09	1.92E+09
HS90A_HUMAN	1.00E-04	2.65E+08	1.87E+09	1.40E+09	4.93E+09	2.89E+09
SLPI_HUMAN	1.00E-04	4.34E+10	5.70E+10	3.77E+10	1.43E+10	3.06E+10
S10AB_HUMAN	1.80E-02	1.66E+09	3.27E+09	3.02E+09	3.08E+09	3.34E+09
K2C8_HUMAN	1.00E-04	1.05E+09	3.09E+09	4.54E+09	4.74E+09	5.81E+09
A0A0A0N0M1_HUMAN	1.00E-04	1.16E+09	5.58E+08	9.12E+08	5.85E+08	6.21E+08
AT12A_HUMAN	1.10E-02	1.18E+09	6.80E+08	1.36E+09	8.81E+08	1.03E+09
B7Z5Q2_HUMAN	1.00E-04	1.37E+09	1.32E+09	9.35E+08	3.43E+08	6.42E+08
GSTP1_HUMAN	2.90E-03	3.70E+08	7.99E+08	7.84E+08	2.57E+09	1.62E+09
K1C10_HUMAN	1.00E-04	8.91E+08	4.60E+09	1.77E+09	7.05E+08	1.44E+09
A0A0G2JIW1_HUMAN	1.00E-04	5.68E+08	1.53E+09	1.81E+09	3.20E+09	2.93E+09

CEL_HUMAN	1.00E-04	2.64E+09	3.38E+08	5.84E+08	8.49E+08	8.47E+08
ENOA_HUMAN	1.00E-04	4.12E+08	9.95E+08	8.14E+08	1.72E+09	1.40E+09
ACTN4_HUMAN	4.10E-04	4.12E+08	5.63E+08	5.09E+08	1.18E+09	8.44E+08
B4DPP6_HUMAN	1.10E-02	2.90E+10	4.30E+10	4.14E+10	3.11E+10	3.95E+10
IBP2_HUMAN	1.00E-04	4.35E+09	3.35E+09	2.71E+09	2.07E+09	2.42E+09
PRDX1_HUMAN	1.00E-04	9.26E+08	1.76E+09	2.62E+09	5.03E+09	3.63E+09
CIB1_HUMAN	1.10E-02	9.69E+08	9.04E+08	7.59E+08	5.06E+08	3.87E+08
ATPB_HUMAN	1.00E-04	1.77E+07	2.64E+08	1.04E+09	1.18E+09	1.20E+09
EF1A1_HUMAN	1.00E-04	5.71E+08	9.25E+08	2.78E+09	3.84E+09	3.56E+09
1433Z_HUMAN	1.10E-02	6.51E+08	1.61E+09	1.16E+09	1.64E+09	1.71E+09
G3P_HUMAN	1.00E-04	3.78E+08	8.44E+08	1.15E+09	3.05E+09	2.14E+09
H4_HUMAN	1.00E-04	9.95E+08	5.83E+09	1.11E+10	9.16E+09	1.01E+10
SG3A1_HUMAN	1.00E-04	6.79E+09	2.71E+09	2.97E+09	1.94E+09	2.49E+09
K7EL21_HUMAN	1.00E-04	1.27E+09	1.99E+09	1.91E+09	7.26E+09	3.72E+09
D3DSQ1_HUMAN	1.00E-04	1.36E+08	3.09E+08	5.09E+08	8.66E+08	7.35E+08
A0A087WVJ0_HUMAN	1.10E-03	1.80E+09	2.94E+09	2.33E+09	8.54E+08	2.16E+09
TKT_HUMAN	1.00E-04	1.32E+08	6.46E+08	4.06E+08	1.55E+09	5.21E+08
TPIS_HUMAN	1.00E-04	7.73E+08	2.07E+09	1.32E+09	2.09E+09	2.37E+09
GRP78_HUMAN	1.00E-04	1.74E+07	6.25E+08	7.88E+08	1.53E+09	1.20E+09
K2C7_HUMAN	1.00E-04	8.53E+07	1.06E+09	2.22E+09	1.39E+09	2.21E+09
ATPA_HUMAN	1.00E-04	1.05E+07	2.73E+07	6.63E+08	8.55E+08	9.30E+08
PGK1_HUMAN	1.00E-04	1.56E+08	2.18E+08	3.39E+08	1.17E+09	6.24E+08
NHRF1_HUMAN	6.20E-03	2.28E+09	2.68E+09	2.32E+09	1.24E+09	1.83E+09
ALDOA_HUMAN	1.00E-04	6.72E+08	9.45E+08	1.46E+09	2.82E+09	2.22E+09
SBP1_HUMAN	1.00E-04	1.22E+08	1.31E+08	1.76E+08	9.63E+08	5.73E+08
PDC6I_HUMAN	1.40E-04	2.69E+08	7.87E+07	3.22E+08	2.80E+08	2.29E+08
STOM_HUMAN	1.50E-03	3.77E+08	4.35E+08	5.31E+08	1.69E+08	2.73E+08
K1C9_HUMAN	1.00E-04	3.37E+08	2.58E+09	1.12E+09	2.97E+08	5.57E+08
CH60_HUMAN	1.00E-04	0.00E+00	2.06E+08	9.00E+08	9.84E+08	8.91E+08
DMBT1_HUMAN	1.00E-04	2.45E+09	1.13E+09	6.90E+08	1.10E+09	8.25E+08
B4DRR0_HUMAN	1.00E-04	1.54E+09	3.25E+09	3.79E+09	2.36E+09	3.54E+09
WFDC2_HUMAN	1.00E-04	3.50E+09	1.22E+10	3.78E+09	1.53E+09	4.15E+09
KCRU_HUMAN	1.00E-04	1.91E+06	4.92E+07	1.09E+08	6.46E+08	2.45E+08
MDHM_HUMAN	1.00E-04	0.00E+00	1.42E+08	3.31E+08	6.19E+08	4.63E+08
HSPB1_HUMAN	1.00E-04	3.76E+07	2.82E+08	9.16E+08	1.26E+09	1.12E+09
EF2_HUMAN	1.00E-04	4.08E+07	6.91E+07	1.50E+08	6.61E+08	3.45E+08
CALM_HUMAN	3.10E-02	4.42E+08	7.54E+08	5.95E+08	8.37E+08	7.63E+08
BASP1_HUMAN	1.00E-04	7.27E+09	8.15E+09	6.27E+09	3.95E+09	5.91E+09
IBP7_HUMAN	1.00E-04	2.16E+09	1.22E+09	8.79E+08	7.55E+08	7.46E+08
AK1C1_HUMAN	1.00E-04	1.37E+08	1.20E+08	5.58E+08	1.10E+09	8.93E+08
LMNA_HUMAN	1.00E-04	4.14E+07	2.56E+08	4.68E+08	5.54E+08	6.84E+08
PEDF_HUMAN	1.00E-04	8.29E+08	2.32E+08	9.46E+07	3.97E+08	2.76E+08
IDHC_HUMAN	1.00E-04	3.23E+08	6.49E+08	9.62E+08	2.22E+09	1.86E+09
HS90B_HUMAN	1.00E-04	1.75E+07	6.48E+08	7.07E+08	3.34E+09	1.78E+09
GBB2_HUMAN	1.40E-03	1.00E+09	7.23E+08	1.21E+09	3.15E+08	1.39E+09
ANXA5_HUMAN	2.70E-02	6.75E+07	5.47E+08	3.41E+08	3.07E+08	3.35E+08
K1C17_HUMAN	1.00E-04	4.82E+08	2.45E+09	1.20E+09	1.06E+09	1.34E+09
A0A024R962_HUMAN	1.00E-04	4.02E+08	2.25E+08	1.75E+08	6.66E+07	1.07E+08

TAGL2_HUMAN	1.90E-03	4.46E+07	6.58E+07	9.89E+07	1.62E+08	8.01E+07
ANXA4_HUMAN	1.00E-04	4.94E+07	4.33E+08	4.24E+08	2.19E+08	2.51E+08
TERA_HUMAN	1.00E-04	5.75E+06	4.58E+07	5.54E+07	3.19E+08	9.63E+07
K22E_HUMAN	1.00E-04	8.35E+08	3.52E+09	2.58E+09	1.11E+09	1.84E+09
6PGD_HUMAN	1.00E-04	2.95E+07	7.38E+07	1.45E+08	4.92E+08	4.35E+08
ADH1_YEAST	1.30E-02	3.02E+08	7.91E+08	9.52E+08	6.34E+08	1.06E+09
AT1A1_HUMAN	3.10E-04	0.00E+00	0.00E+00	8.74E+08	5.23E+08	5.04E+08
K2C4_HUMAN	1.00E-04	0.00E+00	7.17E+07	5.31E+08	3.58E+08	6.48E+08
G9K388_HUMAN (+1)	6.30E-03	2.04E+08	3.68E+08	2.47E+08	6.46E+08	5.19E+08
CD59_HUMAN (+2)	2.50E-03	2.81E+09	2.68E+09	2.80E+09	1.41E+09	2.16E+09
A0A087WVQ6_HUMAN	1.00E-04	0.00E+00	5.68E+06	2.61E+08	4.39E+08	3.72E+08
B3KQT9_HUMAN (+1)	1.00E-04	0.00E+00	7.53E+07	3.96E+08	4.83E+08	4.52E+08
MYOF_HUMAN	1.00E-04	1.14E+08	1.47E+07	3.13E+08	1.16E+08	1.90E+08
B4E0X1_HUMAN	1.00E-04	1.69E+09	2.62E+09	1.12E+09	7.27E+08	8.86E+08
U3KQK0_HUMAN	1.30E-03	1.47E+08	3.36E+09	5.39E+09	2.35E+09	3.59E+09
A8K486_HUMAN	1.00E-04	1.16E+08	6.13E+07	2.67E+08	8.41E+08	6.79E+08
C9JIZ6_HUMAN (+3)	2.00E-02	2.68E+08	1.75E+08	1.73E+08	4.05E+08	2.10E+08
MMP9_HUMAN	2.10E-02	1.52E+08	6.99E+07	8.30E+07	3.74E+07	7.56E+07
FLNB_HUMAN	1.00E-04	2.99E+07	1.41E+08	1.46E+08	3.20E+08	2.35E+08
LDHB_HUMAN	9.00E-03	6.26E+07	4.26E+08	1.60E+08	7.71E+08	6.67E+08
TPPP3_HUMAN	2.20E-04	1.97E+08	2.95E+08	4.26E+08	9.21E+08	5.02E+08
A8KAJ3_HUMAN (+1)	8.80E-04	5.56E+08	3.64E+08	2.77E+08	2.58E+08	2.26E+08
IQGA1_HUMAN	4.50E-02	3.42E+07	1.36E+07	2.69E+07	7.98E+07	4.44E+07
B4DPJ2_HUMAN	1.20E-04	2.78E+07	1.57E+08	2.47E+08	1.44E+08	2.32E+08
GNA11_HUMAN (+1)	1.00E-04	4.03E+08	7.38E+07	2.25E+08	1.73E+08	1.48E+08
PROF1_HUMAN	1.00E-04	2.34E+08	2.13E+08	3.09E+08	7.25E+08	5.97E+08
TIG1_HUMAN	1.20E-02	2.56E+08	1.73E+08	1.41E+08	2.25E+08	6.73E+07
ADH7_HUMAN	1.00E-04	5.90E+07	0.00E+00	1.09E+08	7.53E+08	2.71E+08
PARK7_HUMAN	1.00E-04	2.01E+08	2.62E+08	4.73E+08	6.51E+08	8.22E+08
RADI_HUMAN	1.10E-03	4.65E+09	2.66E+09	3.65E+09	4.11E+09	3.14E+09
ADT2_HUMAN	2.90E-03	0.00E+00	0.00E+00	6.66E+08	4.33E+08	7.76E+08
A0A087WVI4_HUMAN	4.30E-02	1.28E+08	2.01E+07	1.13E+08	6.73E+07	5.16E+07
TMC5_HUMAN	4.70E-04	3.14E+08	8.80E+07	2.16E+08	1.33E+08	1.89E+08
MVP_HUMAN	1.00E-04	0.00E+00	7.64E+07	1.66E+08	2.75E+08	2.15E+08
AT1B1_HUMAN (+1)	2.80E-03	2.42E+08	1.35E+08	3.97E+08	2.48E+08	3.39E+08
EFTU_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.35E+08	5.19E+08	1.76E+08
MDHC_HUMAN	1.00E-04	5.64E+07	1.59E+08	1.03E+08	5.86E+08	2.51E+08
FAS_HUMAN	1.20E-04	0.00E+00	0.00E+00	6.10E+07	1.27E+08	9.33E+07
PLEC_HUMAN	1.20E-03	0.00E+00	0.00E+00	5.15E+07	5.23E+07	1.23E+08
B4DL49_HUMAN (+1)	1.50E-03	1.37E+08	2.61E+08	2.42E+08	6.61E+08	4.62E+08
UBA1_HUMAN	1.00E-04	0.00E+00	0.00E+00	5.16E+07	2.30E+08	8.98E+07
A0A087WUA5_HUMAN	1.00E-04	1.36E+08	0.00E+00	1.73E+08	8.85E+07	7.08E+07
B3KQF4_HUMAN (+2)	1.00E-04	4.99E+08	2.48E+08	1.77E+08	1.88E+08	1.14E+08
A0A087WSV8_HUMAN	7.20E-03	3.71E+08	9.58E+07	1.83E+08	3.89E+08	1.73E+08
Q59ER5_HUMAN	1.00E-04	7.80E+07	8.53E+06	8.15E+07	3.29E+08	1.51E+08
CO4B_HUMAN	1.00E-04	3.51E+08	0.00E+00	0.00E+00	1.75E+07	0.00E+00
A0A024R228_HUMAN	1.00E-04	0.00E+00	3.96E+06	1.93E+08	3.45E+08	3.09E+08
THIO_HUMAN	3.00E-03	4.04E+08	9.52E+08	7.40E+08	3.01E+08	8.22E+08

SODC_HUMAN	1.00E-04	3.67E+07	1.67E+07	1.32E+08	4.68E+08	1.31E+08
LEG3_HUMAN (+2)	1.40E-03	3.32E+07	2.31E+08	1.89E+08	6.40E+08	1.36E+08
CILP1_HUMAN	1.00E-04	3.44E+08	0.00E+00	0.00E+00	1.13E+08	4.45E+07
DYHC1_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	4.49E+07	0.00E+00
PLSI_HUMAN	2.10E-03	4.11E+07	5.48E+06	1.90E+07	8.15E+07	2.13E+07
B3KX72_HUMAN (+1)	1.60E-04	0.00E+00	1.04E+07	1.24E+08	1.37E+08	1.50E+08
MYO1D_HUMAN	1.00E-04	1.13E+08	0.00E+00	3.50E+07	3.51E+07	9.49E+06
ENPL_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	2.18E+08	5.98E+08	4.73E+08
GRP75_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	1.23E+08	2.89E+08	2.24E+08
ROA2_HUMAN	1.00E-04	1.78E+07	2.23E+08	5.00E+08	1.14E+09	7.86E+08
CFAI_HUMAN (+2)	1.00E-04	3.86E+08	2.73E+07	3.73E+07	4.01E+07	4.34E+07
B4DV28_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	2.05E+07	2.95E+08	1.45E+08
H13_HUMAN	3.90E-03	0.00E+00	4.00E+07	6.30E+08	8.73E+07	3.38E+08
A0A024R884_HUMAN	3.20E-02	1.57E+08	1.46E+08	1.19E+08	4.70E+07	4.91E+07
B4E3A8_HUMAN (+1)	1.00E-04	2.33E+07	3.04E+07	2.25E+07	1.99E+08	1.19E+08
HYEP_HUMAN	1.00E-04	0.00E+00	0.00E+00	4.77E+07	2.88E+08	8.33E+07
Q59EF6_HUMAN	1.00E-04	8.63E+06	3.67E+07	1.37E+08	4.38E+08	2.91E+08
FUCO_HUMAN	1.00E-04	4.99E+07	0.00E+00	1.09E+08	1.67E+08	3.52E+08
B4E1U9_HUMAN (+1)	6.70E-03	4.11E+08	1.25E+08	4.47E+08	3.57E+08	3.31E+08
CYTC_HUMAN	2.70E-03	5.02E+08	6.56E+06	3.03E+08	2.36E+08	6.18E+07
TALDO_HUMAN	1.00E-04	0.00E+00	3.22E+07	1.81E+07	2.87E+08	1.77E+08
A2A274_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	8.61E+07	3.63E+08	2.61E+08
CTL4_HUMAN	6.30E-04	8.20E+08	7.74E+08	6.61E+08	4.58E+08	5.53E+08
CH10_HUMAN	1.50E-04	0.00E+00	6.31E+07	4.15E+08	3.51E+08	2.48E+08
S10A8_HUMAN	1.00E-04	1.53E+08	5.71E+08	6.36E+07	7.99E+07	4.94E+07
PEBP1_HUMAN	1.00E-04	3.81E+07	6.83E+07	1.04E+08	2.90E+08	2.04E+08
CD9_HUMAN (+1)	1.00E-04	1.58E+08	1.11E+08	6.36E+08	5.69E+07	3.62E+08
A6XND0_HUMAN	2.40E-04	1.58E+08	2.75E+08	1.74E+08	5.38E+07	1.29E+08
ECHA_HUMAN	7.10E-03	0.00E+00	0.00E+00	9.30E+07	6.91E+07	7.55E+07
AK1A1_HUMAN	1.00E-04	1.31E+07	0.00E+00	3.99E+07	2.16E+08	9.08E+07
A0A0A0MTS2_HUMAN	1.00E-04	0.00E+00	2.04E+07	3.38E+07	2.37E+08	1.95E+07
B5ME49_HUMAN	1.00E-04	7.44E+09	7.97E+08	2.30E+09	3.77E+09	4.53E+09
PDIA1_HUMAN	5.70E-03	0.00E+00	1.67E+07	4.69E+07	6.66E+07	4.31E+07
K1C16_HUMAN	1.60E-04	3.58E+08	2.45E+09	6.16E+08	7.19E+08	5.24E+08
Q32Q12_HUMAN	7.90E-03	5.29E+07	8.81E+07	1.66E+08	3.06E+08	3.47E+08
IDHP_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.38E+08	7.22E+08	2.89E+08
Q53EY8_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.81E+07	2.94E+08	8.42E+07
HNRPM_HUMAN	1.10E-02	0.00E+00	0.00E+00	5.65E+07	1.17E+08	6.88E+07
A4D2P0_HUMAN (+1)	1.50E-02	1.97E+08	0.00E+00	8.18E+07	1.29E+08	3.81E+07
PRDX6_HUMAN	2.90E-04	2.79E+07	1.00E+07	2.86E+07	2.08E+08	7.91E+07
Q5M8T4_HUMAN (+1)	1.20E-03	1.76E+08	0.00E+00	6.18E+07	6.07E+07	6.04E+07
ECHM_HUMAN	6.50E-04	0.00E+00	0.00E+00	1.27E+07	4.30E+07	1.55E+07
MMP10_HUMAN	1.00E-04	1.35E+08	1.42E+08	6.91E+07	4.32E+07	6.20E+07
STEA4_HUMAN	7.30E-03	8.12E+07	1.41E+07	4.86E+07	4.26E+07	0.00E+00
AATM_HUMAN	1.00E-04	0.00E+00	4.75E+07	9.01E+07	2.33E+08	1.89E+08
A0A024RB53_HUMAN	1.00E-04	0.00E+00	0.00E+00	2.26E+08	1.01E+09	5.22E+08
A0A087X208_HUMAN	9.20E-03	6.86E+07	1.63E+07	1.23E+07	2.36E+07	7.08E+06
HEXB_HUMAN	2.00E-04	1.81E+07	9.84E+06	0.00E+00	1.76E+08	3.31E+07

PRDX3_HUMAN	1.70E-02	0.00E+00	1.12E+07	3.01E+07	1.06E+08	4.67E+07
C9J0K6_HUMAN (+1)	2.50E-02	2.63E+06	0.00E+00	4.28E+07	8.76E+07	5.27E+07
A0A024RDF4_HUMAN	1.00E-04	3.98E+06	0.00E+00	7.23E+07	3.98E+08	1.91E+08
CBR1_HUMAN	1.00E-04	1.09E+07	0.00E+00	1.52E+07	1.76E+08	3.71E+07
PDIA4_HUMAN	1.20E-02	0.00E+00	3.03E+07	7.87E+07	4.54E+07	8.93E+07
B2RDI5_HUMAN	4.50E-03	0.00E+00	3.81E+07	2.55E+07	1.76E+08	6.20E+07
AGR2_HUMAN (+3)	3.40E-04	0.00E+00	8.83E+06	1.31E+08	3.24E+08	2.66E+08
D3DPU2_HUMAN	1.00E-04	1.30E+07	6.29E+07	3.57E+07	2.14E+08	1.55E+08
1433G_HUMAN	3.00E-04	8.72E+07	1.49E+08	9.64E+07	3.25E+08	1.81E+08
DEST_HUMAN	7.60E-03	2.01E+08	3.12E+07	5.95E+07	2.72E+08	2.41E+08
KLK11_HUMAN	8.60E-04	2.64E+08	1.96E+08	5.00E+07	4.20E+07	4.31E+07
RUVB2_HUMAN	1.00E-04	0.00E+00	0.00E+00	3.84E+07	3.23E+08	0.00E+00
PP1B_HUMAN	1.80E-04	8.51E+06	5.76E+05	1.81E+07	5.68E+07	3.70E+05
CALX_HUMAN	7.50E-03	0.00E+00	0.00E+00	6.34E+07	7.76E+07	1.08E+08
RAB10_HUMAN (+1)	1.20E-04	1.32E+08	5.00E+07	4.36E+08	2.00E+08	2.24E+08
ARF1_HUMAN	4.30E-03	8.47E+06	0.00E+00	1.14E+07	7.04E+07	2.25E+07
J3KTA4_HUMAN	1.00E-04	0.00E+00	0.00E+00	3.50E+07	9.04E+07	5.51E+07
B4DJ30_HUMAN (+1)	6.50E-03	0.00E+00	0.00E+00	5.19E+07	1.19E+08	8.21E+07
B5BUB1_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.56E+07	1.67E+08	3.47E+07
EF1G_HUMAN	1.00E-04	0.00E+00	0.00E+00	4.46E+07	2.29E+08	8.73E+07
J3KND3_HUMAN (+1)	4.70E-03	1.45E+07	0.00E+00	7.48E+07	1.09E+08	1.32E+08
MOES_HUMAN	9.20E-04	4.34E+09	4.40E+08	1.18E+09	1.21E+09	0.00E+00
MYH14_HUMAN	1.30E-02	0.00E+00	0.00E+00	3.80E+07	3.35E+07	4.21E+07
DHE3_HUMAN	1.00E-04	0.00E+00	0.00E+00	6.68E+07	1.57E+08	6.74E+07
A0A087X0D5_HUMAN	5.20E-03	2.93E+08	8.02E+07	9.28E+07	1.29E+08	1.90E+08
ASSY_HUMAN	3.90E-04	0.00E+00	0.00E+00	4.05E+07	7.03E+07	1.15E+08
A0A0A0MSE2_HUMAN	5.30E-03	0.00E+00	0.00E+00	3.20E+07	3.56E+07	5.66E+07
A0A024RC87_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.46E+07	1.39E+08	5.49E+07
ADH1G_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	3.67E+08	7.10E+07
S10A6_HUMAN	2.10E-02	0.00E+00	1.31E+07	0.00E+00	1.17E+08	0.00E+00
GBG12_HUMAN	3.50E-04	5.87E+07	2.77E+08	2.18E+08	2.66E+07	9.12E+07
1433B_HUMAN	1.00E-04	0.00E+00	4.75E+08	2.85E+08	1.27E+09	6.43E+08
GSHR_HUMAN	2.10E-04	2.27E+07	1.53E+07	1.04E+08	2.06E+08	1.23E+08
E9PCY7_HUMAN (+2)	9.30E-03	0.00E+00	0.00E+00	3.00E+07	4.96E+07	7.87E+07
PGAM1_HUMAN	1.00E-04	2.11E+06	1.81E+07	1.56E+07	1.57E+08	1.08E+08
RS2_HUMAN	6.70E-04	0.00E+00	0.00E+00	1.48E+08	3.00E+08	1.62E+08
Q59H77_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	5.51E+07	1.66E+08	1.21E+07
GNAS1_HUMAN	2.80E-03	1.89E+08	3.51E+07	1.10E+08	4.14E+06	1.23E+08
CALR_HUMAN (+1)	1.00E-04	0.00E+00	5.84E+07	1.41E+08	1.68E+08	8.15E+07
S6A14_HUMAN	1.60E-02	1.53E+08	4.26E+08	3.36E+08	3.82E+07	3.06E+08
ELAF_HUMAN	9.40E-04	2.06E+08	3.15E+08	5.85E+07	4.24E+07	4.22E+07
TCPB_HUMAN	1.00E-04	0.00E+00	1.01E+07	9.64E+07	2.59E+08	2.03E+08
A8K7F6_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	2.69E+07	1.96E+08	6.71E+07
A8K8D9_HUMAN (+3)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	7.41E+07	3.21E+07
CYTB_HUMAN	1.00E-04	3.22E+07	2.17E+07	9.65E+06	3.74E+08	5.65E+07
FOLR1_HUMAN	4.70E-04	1.01E+08	5.07E+06	6.29E+06	4.02E+07	0.00E+00
A8K4W0_HUMAN (+2)	1.00E-04	0.00E+00	0.00E+00	1.13E+08	1.43E+08	1.25E+08
ATRN_HUMAN	4.10E-03	7.42E+07	1.29E+07	1.15E+07	1.17E+07	1.63E+07

LDHA_HUMAN	1.20E-02	0.00E+00	0.00E+00	0.00E+00	2.05E+08	0.00E+00
SPTB2_HUMAN	3.60E-02	0.00E+00	0.00E+00	0.00E+00	3.07E+07	5.95E+06
B4DJV2_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	5.76E+07	2.15E+08	8.21E+07
RS3_HUMAN	1.00E-04	0.00E+00	0.00E+00	4.07E+07	1.25E+08	5.44E+07
ATPO_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.31E+08	2.49E+08	2.30E+08
AMPL_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	2.35E+08	7.90E+07
VDAC1_HUMAN	1.00E-04	0.00E+00	0.00E+00	6.33E+07	2.05E+08	1.73E+08
G5EA09_HUMAN (+1)	2.40E-02	1.20E+08	0.00E+00	1.03E+08	3.95E+07	2.96E+07
CROCC_HUMAN	1.60E-02	0.00E+00	0.00E+00	5.24E+06	5.60E+08	1.78E+08
Q53HU0_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	1.91E+07	1.09E+08	2.80E+07
Q6FIG4_HUMAN (+1)	1.00E-04	2.77E+07	0.00E+00	1.96E+08	2.41E+08	0.00E+00
B7Z6Q5_HUMAN (+3)	1.00E-04	0.00E+00	0.00E+00	2.61E+06	8.93E+07	1.24E+07
MLF1_HUMAN	4.30E-04	1.40E+07	1.41E+07	1.97E+07	3.02E+08	2.19E+08
A0A0A0MR02_HUMAN	1.90E-02	0.00E+00	0.00E+00	1.52E+08	2.10E+08	1.11E+08
RS8_HUMAN	1.00E-04	0.00E+00	0.00E+00	4.62E+07	1.83E+08	3.58E+07
B7Z899_HUMAN (+3)	1.00E-04	0.00E+00	0.00E+00	3.89E+06	4.19E+07	5.63E+07
A0A0C4DGQ5_HUMAN	2.30E-02	0.00E+00	1.91E+07	4.66E+07	1.33E+08	7.70E+07
J3KPX7_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	1.68E+08	1.06E+08	1.14E+08
PHB_HUMAN (+1)	5.70E-04	0.00E+00	0.00E+00	2.99E+07	1.34E+08	4.13E+07
CD166_HUMAN	1.00E-04	0.00E+00	1.03E+07	1.47E+05	1.33E+08	2.06E+07
KCRB_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	2.98E+08	0.00E+00
A8K6V6_HUMAN (+3)	1.00E-04	1.09E+07	0.00E+00	5.89E+06	6.59E+07	1.78E+07
AT2A2_HUMAN	2.60E-02	0.00E+00	0.00E+00	5.93E+07	3.29E+07	3.52E+07
Q53EP4_HUMAN (+3)	1.20E-03	0.00E+00	0.00E+00	2.66E+07	9.42E+07	1.92E+07
DHSO_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	7.23E+06	9.27E+07	5.81E+07
Q59GF8_HUMAN	7.30E-03	0.00E+00	0.00E+00	0.00E+00	3.31E+07	3.92E+06
RL15_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.13E+08	3.94E+08	1.66E+08
NUCB1_HUMAN	1.00E-04	5.29E+07	8.47E+06	0.00E+00	1.61E+06	0.00E+00
GSLG1_HUMAN	2.20E-03	9.86E+07	1.12E+07	1.69E+07	5.67E+07	2.38E+07
ECHB_HUMAN	6.50E-04	0.00E+00	0.00E+00	0.00E+00	6.23E+07	3.35E+06
B1AK87_HUMAN (+2)	5.40E-04	1.24E+07	0.00E+00	1.10E+07	6.54E+07	4.09E+06
UGDH_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.17E+07	1.33E+08	4.05E+07
B3KVF5_HUMAN	4.30E-03	0.00E+00	0.00E+00	1.34E+09	1.22E+09	1.68E+09
SFPQ_HUMAN	2.20E-02	0.00E+00	0.00E+00	2.51E+07	8.72E+07	2.25E+07
AMY2B_HUMAN	1.00E-04	1.32E+08	0.00E+00	1.17E+07	2.50E+07	0.00E+00
Q53HV2_HUMAN (+2)	6.20E-04	0.00E+00	0.00E+00	1.12E+06	3.07E+07	6.95E+06
EST1_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	8.36E+07	0.00E+00
PIP_HUMAN	1.00E-04	9.06E+07	1.78E+07	1.54E+07	1.09E+07	0.00E+00
CEAM5_HUMAN	6.00E-03	4.86E+07	4.59E+08	3.38E+08	0.00E+00	2.14E+08
ETFA_HUMAN	7.20E-03	0.00E+00	0.00E+00	5.86E+07	8.89E+07	6.89E+07
A0A0C4DFZ2_HUMAN	1.00E-04	0.00E+00	0.00E+00	5.13E+06	5.49E+07	9.58E+06
A0A087WTT1_HUMAN	1.00E-04	0.00E+00	0.00E+00	4.76E+06	4.34E+07	4.34E+06
QCR2_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.36E+08	1.86E+08	1.48E+08
A0A024R8Q1_HUMAN	1.20E-03	1.28E+06	0.00E+00	0.00E+00	5.07E+07	2.73E+07
J3QQ67_HUMAN (+1)	1.00E-04	0.00E+00	6.56E+06	1.31E+08	4.00E+08	2.33E+08
B3KPS3_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	5.68E+09	0.00E+00
E7EQB2_HUMAN (+1)	1.80E-03	5.61E+07	0.00E+00	0.00E+00	2.23E+07	0.00E+00
Q6IPH7_HUMAN	3.30E-02	0.00E+00	0.00E+00	9.25E+07	1.09E+08	6.10E+07

SAMH1_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	3.63E+07	0.00E+00
A0A0C4DFU2_HUMAN	1.60E-03	0.00E+00	0.00E+00	5.39E+06	4.14E+07	0.00E+00
FUMH_HUMAN	1.00E-04	0.00E+00	0.00E+00	2.11E+07	2.02E+08	1.75E+07
DPP2_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.62E+08	0.00E+00
3HIDH_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.49E+07	1.06E+08	1.58E+07
AL1A3_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	8.82E+07	1.03E+08
B4DEA8_HUMAN	3.30E-02	0.00E+00	0.00E+00	0.00E+00	7.17E+07	6.65E+07
ALDH2_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	6.16E+06	6.72E+07	2.78E+06
B4E0U6_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.39E+08	0.00E+00
A0A0C4DG17_HUMAN	1.00E-04	0.00E+00	0.00E+00	6.90E+07	1.40E+08	1.14E+08
Q53G25_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.26E+08	4.86E+07
Q53GB3_HUMAN	6.30E-03	0.00E+00	0.00E+00	6.95E+07	5.80E+07	5.90E+07
LAMC2_HUMAN	7.80E-03	2.29E+07	1.16E+08	1.50E+07	1.72E+07	1.27E+07
Q8TBK5_HUMAN	3.10E-03	0.00E+00	0.00E+00	2.30E+07	1.86E+08	3.57E+07
A0A0G2JM65_HUMAN	2.90E-03	1.30E+09	0.00E+00	5.10E+08	1.39E+08	7.43E+07
A0A024RBK9_HUMAN	1.00E-04	0.00E+00	1.90E+07	0.00E+00	7.42E+07	5.61E+06
Q9BU08_HUMAN	5.60E-03	0.00E+00	0.00E+00	0.00E+00	4.65E+07	2.16E+07
RL10_HUMAN	4.30E-02	0.00E+00	0.00E+00	0.00E+00	8.49E+07	7.55E+07
TCO1_HUMAN	1.40E-04	2.84E+06	1.19E+07	4.71E+06	2.41E+05	7.67E+05
A0A0A0MTI5_HUMAN	1.80E-03	0.00E+00	0.00E+00	6.18E+06	1.01E+08	9.05E+06
A0A0C4DFY5_HUMAN	4.70E-04	9.09E+07	1.48E+07	3.17E+07	7.70E+06	2.12E+07
Q5HYG7_HUMAN	6.00E-03	0.00E+00	0.00E+00	3.05E+07	8.63E+07	3.85E+07
B7Z1Y2_HUMAN	5.80E-03	2.01E+06	8.72E+06	1.47E+07	2.81E+07	3.28E+07
B3GN7_HUMAN	4.10E-04	1.02E+08	5.57E+06	0.00E+00	0.00E+00	0.00E+00
Q59ET3_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	9.14E+07	1.31E+07
H15_HUMAN	1.90E-03	0.00E+00	0.00E+00	1.41E+08	2.16E+07	0.00E+00
SERA_HUMAN	1.00E-04	0.00E+00	0.00E+00	5.61E+06	4.23E+07	6.55E+06
A0A087WXI2_HUMAN	1.00E-04	1.14E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00
B2R983_HUMAN (+1)	1.70E-03	0.00E+00	0.00E+00	0.00E+00	8.00E+07	2.48E+07
J3KQE5_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.42E+08	3.88E+07
A8K3B4_HUMAN	2.30E-04	0.00E+00	0.00E+00	0.00E+00	4.69E+07	2.21E+06
B4DIT7_HUMAN (+1)	3.60E-02	0.00E+00	0.00E+00	3.33E+06	7.87E+06	2.04E+07
GBLP_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.10E+07	1.00E+08	4.46E+07
SYEP_HUMAN	1.20E-02	0.00E+00	0.00E+00	0.00E+00	2.93E+07	0.00E+00
A8K335_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	5.71E+07	0.00E+00
AL9A1_HUMAN (+1)	2.70E-04	0.00E+00	0.00E+00	0.00E+00	3.98E+07	5.81E+06
B4E2I4_HUMAN (+1)	3.10E-03	0.00E+00	0.00E+00	3.12E+06	7.38E+07	1.97E+07
CO6_HUMAN	1.00E-04	1.11E+08	0.00E+00	0.00E+00	0.00E+00	0.00E+00
SPHM_HUMAN	1.00E-04	0.00E+00	4.95E+06	6.33E+06	1.16E+08	2.22E+07
A8K8U1_HUMAN (+1)	2.60E-03	0.00E+00	0.00E+00	0.00E+00	8.28E+06	0.00E+00
A0A087X1X7_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.94E+08	7.40E+07
A8K690_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	7.02E+07	1.47E+06
SYDC_HUMAN	1.40E-02	0.00E+00	0.00E+00	0.00E+00	1.47E+07	0.00E+00
RAB7A_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	4.36E+07	1.60E+06
PDIA6_HUMAN	2.40E-03	0.00E+00	0.00E+00	4.46E+06	1.79E+07	2.67E+06
A0A087WXI5_HUMAN	1.00E-04	0.00E+00	0.00E+00	3.41E+06	2.63E+07	0.00E+00
J3KTL2_HUMAN (+1)	2.10E-03	0.00E+00	0.00E+00	8.90E+07	4.12E+07	5.73E+07
TBB5_HUMAN	1.70E-03	0.00E+00	0.00E+00	1.33E+09	5.98E+09	2.82E+09

B4DVA7_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	7.03E+07	0.00E+00
G3V295_HUMAN	1.30E-02	0.00E+00	3.09E+07	2.16E+07	9.79E+07	3.81E+07
AK1C2_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.02E+09	2.15E+08
APT_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.14E+06	2.99E+07	0.00E+00
VTM2L_HUMAN	1.00E-04	6.46E+07	6.22E+06	1.68E+07	0.00E+00	7.02E+06
A8KA83_HUMAN	2.00E-04	0.00E+00	0.00E+00	1.78E+07	1.04E+08	1.42E+07
ZA2G_HUMAN	1.00E-04	6.18E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
ROA3_HUMAN	1.00E-04	0.00E+00	0.00E+00	3.64E+07	1.89E+08	1.38E+08
Q5W0H4_HUMAN (+2)	4.80E-02	0.00E+00	0.00E+00	0.00E+00	3.76E+07	0.00E+00
Q53TD0_HUMAN (+1)	1.60E-04	0.00E+00	0.00E+00	0.00E+00	1.11E+08	0.00E+00
PCBP1_HUMAN	2.00E-02	0.00E+00	0.00E+00	2.43E+07	9.17E+07	4.33E+07
UGPA_HUMAN	4.50E-02	0.00E+00	0.00E+00	0.00E+00	2.54E+07	8.48E+06
Q5HYB6_HUMAN	3.90E-04	8.31E+06	0.00E+00	7.09E+07	1.57E+07	1.19E+08
PSA1_HUMAN	2.10E-03	0.00E+00	0.00E+00	0.00E+00	7.56E+07	1.42E+07
ODPA_HUMAN	1.10E-02	0.00E+00	0.00E+00	0.00E+00	4.12E+07	2.76E+07
MSLN_HUMAN	3.20E-02	1.89E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
SYNC_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	2.65E+07	0.00E+00
B3KXC3_HUMAN (+2)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	2.51E+08	0.00E+00
A0A024QZX5_HUMAN	4.90E-04	1.52E+06	0.00E+00	0.00E+00	2.20E+07	1.46E+06
A8K686_HUMAN	4.00E-02	1.09E+07	2.42E+07	7.24E+06	8.06E+07	4.52E+07
AMPB_HUMAN	4.20E-02	0.00E+00	0.00E+00	0.00E+00	1.58E+07	0.00E+00
A8K3C3_HUMAN (+1)	1.30E-04	0.00E+00	0.00E+00	0.00E+00	9.75E+07	0.00E+00
Q6NZ55_HUMAN (+1)	4.00E-04	0.00E+00	0.00E+00	2.58E+07	1.01E+08	1.76E+07
AIFM1_HUMAN	2.60E-04	0.00E+00	0.00E+00	0.00E+00	6.09E+07	0.00E+00
NAGAB_HUMAN	1.80E-02	0.00E+00	0.00E+00	0.00E+00	5.26E+07	3.82E+07
CYB5_HUMAN (+1)	4.60E-04	0.00E+00	0.00E+00	0.00E+00	7.74E+07	7.75E+06
THIL_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	8.29E+07	3.14E+05
GPC1_HUMAN	4.50E-02	1.01E+07	0.00E+00	0.00E+00	3.22E+07	0.00E+00
F8W1A4_HUMAN (+1)	4.50E-03	0.00E+00	0.00E+00	0.00E+00	2.65E+07	0.00E+00
H0YI09_HUMAN (+2)	7.80E-03	0.00E+00	0.00E+00	1.44E+07	4.37E+07	0.00E+00
ECI1_HUMAN	1.00E-04	0.00E+00	0.00E+00	1.45E+06	4.43E+07	0.00E+00
COX41_HUMAN	3.50E-02	0.00E+00	0.00E+00	0.00E+00	6.99E+07	2.28E+07
SYWC_HUMAN	2.30E-04	0.00E+00	0.00E+00	0.00E+00	1.10E+08	0.00E+00
CATZ_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.27E+08	2.78E+07
ODO2_HUMAN	6.90E-04	0.00E+00	0.00E+00	8.93E+07	9.43E+07	1.07E+08
GNA14_HUMAN	1.00E-04	2.86E+08	0.00E+00	0.00E+00	1.40E+07	0.00E+00
DPP3_HUMAN (+3)	1.70E-02	0.00E+00	0.00E+00	0.00E+00	4.00E+07	4.50E+07
B4E2G8_HUMAN	1.80E-02	0.00E+00	0.00E+00	0.00E+00	4.11E+06	7.61E+05
SRSF3_HUMAN	3.90E-02	0.00E+00	0.00E+00	5.30E+06	4.37E+07	3.05E+07
GDIA_HUMAN	1.90E-04	0.00E+00	0.00E+00	0.00E+00	3.34E+07	0.00E+00
HS105_HUMAN	6.10E-04	0.00E+00	0.00E+00	0.00E+00	2.03E+07	0.00E+00
RS23_HUMAN	1.10E-02	0.00E+00	0.00E+00	0.00E+00	1.03E+08	0.00E+00
DX39B_HUMAN	5.50E-04	0.00E+00	0.00E+00	0.00E+00	4.87E+07	0.00E+00
HNRPL_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	7.13E+07	0.00E+00
B4DJB4_HUMAN	1.40E-02	0.00E+00	0.00E+00	0.00E+00	3.31E+07	6.11E+06
A1L1A8_HUMAN	7.70E-04	0.00E+00	0.00E+00	0.00E+00	7.34E+07	0.00E+00
E9PGN7_HUMAN	1.20E-02	1.90E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
A0A0A0MRV0_HUMAN	2.30E-02	0.00E+00	0.00E+00	0.00E+00	2.70E+07	0.00E+00

2AAA_HUMAN	3.50E-04	0.00E+00	0.00E+00	0.00E+00	6.75E+07	0.00E+00
ODPB_HUMAN	3.70E-02	0.00E+00	0.00E+00	4.29E+06	1.72E+07	0.00E+00
E7ET40_HUMAN	1.80E-03	6.12E+07	9.39E+06	2.25E+07	6.15E+06	0.00E+00
DECR_HUMAN	1.30E-02	0.00E+00	0.00E+00	1.02E+07	2.72E+07	5.83E+07
O95036_HUMAN	1.60E-02	0.00E+00	0.00E+00	0.00E+00	3.88E+07	0.00E+00
SYAC_HUMAN	5.40E-03	0.00E+00	0.00E+00	0.00E+00	7.61E+06	0.00E+00
PDXK_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.19E+08	5.73E+06
B1AHL2_HUMAN	1.00E-04	6.16E+07	0.00E+00	7.43E+06	0.00E+00	1.72E+07
ARK72_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.77E+07	0.00E+00
RS7_HUMAN	4.50E-03	0.00E+00	0.00E+00	0.00E+00	4.74E+07	0.00E+00
RL35_HUMAN	6.10E-03	0.00E+00	0.00E+00	0.00E+00	3.17E+07	0.00E+00
B2RDX5_HUMAN	2.10E-02	0.00E+00	0.00E+00	0.00E+00	2.79E+07	0.00E+00
RALB_HUMAN	1.00E-04	1.24E+08	0.00E+00	5.35E+06	8.40E+06	0.00E+00
B2R944_HUMAN	3.80E-02	2.40E+07	0.00E+00	7.48E+06	0.00E+00	0.00E+00
ELAV1_HUMAN	2.10E-03	0.00E+00	0.00E+00	0.00E+00	6.42E+07	1.42E+07
SPON2_HUMAN	4.70E-03	3.65E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
FSTL1_HUMAN	1.40E-02	4.17E+07	0.00E+00	0.00E+00	1.24E+07	0.00E+00
FRIL_HUMAN	5.70E-03	0.00E+00	0.00E+00	0.00E+00	2.65E+07	0.00E+00
H1X_HUMAN	2.30E-02	0.00E+00	0.00E+00	1.24E+07	4.67E+07	0.00E+00
SAHH_HUMAN	4.70E-03	0.00E+00	0.00E+00	0.00E+00	4.17E+07	0.00E+00
A0A024QZB4_HUMAN	2.20E-02	0.00E+00	0.00E+00	0.00E+00	2.83E+07	0.00E+00
B4E324_HUMAN	1.20E-04	0.00E+00	0.00E+00	0.00E+00	4.11E+07	0.00E+00
PSB6_HUMAN	8.40E-03	0.00E+00	0.00E+00	0.00E+00	4.91E+07	2.52E+07
SIAS_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	6.71E+07	0.00E+00
BLVRB_HUMAN	1.00E-02	0.00E+00	0.00E+00	0.00E+00	3.74E+07	0.00E+00
TEBP_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	1.73E+08	5.75E+06
A4D1W8_HUMAN	5.60E-03	0.00E+00	0.00E+00	0.00E+00	4.98E+07	0.00E+00
Q59GX9_HUMAN	1.00E-04	0.00E+00	0.00E+00	0.00E+00	6.10E+07	0.00E+00
GSTK1_HUMAN	1.20E-04	0.00E+00	0.00E+00	0.00E+00	3.61E+07	0.00E+00
A0A087X1J9_HUMAN	5.60E-03	2.22E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00
B0YIW6_HUMAN	9.00E-03	0.00E+00	0.00E+00	0.00E+00	3.38E+07	0.00E+00
Q5IWS5_HUMAN	6.00E-03	2.18E+07	0.00E+00	0.00E+00	0.00E+00	0.00E+00

Supplement Table 4: List of proteins significantly altered in HTBE cell apical secretions after exposure to air-sham (control), waterpipe Two Apples shisha flavor (2App) or Two Apples with shisha tobacco (2App+TOB). The mean of the total precursor ion intensity with p-value<0.05, as determined by ANOVA, is shown.

Accession Number	ANOVA (p-value)	Air-sham	2App	2App+TOB
CO3_HUMAN	1.00E-04	1.32E+10	9.46E+09	8.87E+09
BPIB1_HUMAN	1.40E-02	1.17E+10	1.04E+10	1.31E+10
K2C5_HUMAN	1.70E-03	4.29E+10	3.70E+10	3.19E+10
PIGR_HUMAN	8.70E-04	2.26E+10	1.64E+10	2.88E+10
Q53HR5_HUMAN	2.60E-02	1.00E+10	7.68E+09	6.78E+09
GELS_HUMAN	1.00E-04	2.43E+09	3.28E+09	4.83E+09
ANXA1_HUMAN	2.30E-02	1.22E+10	1.05E+10	1.12E+10
ANXA2_HUMAN (+1)	1.00E-04	1.17E+10	9.58E+09	7.61E+09
SPB3_HUMAN	1.00E-04	4.24E+09	7.37E+09	4.54E+09
AL1A1_HUMAN	3.40E-03	4.50E+09	6.82E+09	5.20E+09
PRDX5_HUMAN	2.30E-02	5.76E+09	4.72E+09	3.75E+09
LG3BP_HUMAN	2.80E-02	8.50E+08	1.07E+09	1.38E+09
G3P_HUMAN	6.40E-04	5.99E+09	7.21E+09	4.85E+09
ACTN4_HUMAN	4.00E-04	3.77E+09	3.78E+09	2.89E+09
A8K2I0_HUMAN	3.50E-04	3.71E+10	2.77E+10	2.46E+10
MUC5B_HUMAN	1.30E-03	2.20E+09	7.08E+08	1.51E+09
B2ZDQ1_HUMAN (+1)	1.00E-04	1.04E+10	6.11E+09	4.55E+09
MUC16_HUMAN	2.30E-03	1.61E+09	1.42E+09	2.08E+09
AT12A_HUMAN	1.20E-03	1.69E+09	1.31E+09	1.51E+09
S10A6_HUMAN	1.00E-04	2.75E+07	8.58E+07	1.89E+08
B4E022_HUMAN (+1)	5.50E-04	2.05E+09	3.66E+09	1.98E+09
TRFE_HUMAN	1.00E-04	9.74E+08	7.12E+09	4.73E+09
CH60_HUMAN	2.00E-03	1.10E+09	1.16E+09	1.72E+09
ALDOA_HUMAN	3.60E-02	6.52E+09	7.24E+09	5.82E+09
LMNA_HUMAN	2.10E-02	2.38E+09	2.17E+09	3.11E+09
ATPA_HUMAN	1.20E-03	2.90E+09	2.39E+09	1.58E+09
PRDX1_HUMAN	1.60E-02	7.02E+09	1.02E+10	8.37E+09
B4E1Z4_HUMAN	3.70E-04	4.41E+09	2.73E+09	2.42E+09
AHNK_HUMAN	6.90E-04	9.42E+07	4.27E+08	3.96E+08
MVP_HUMAN	3.00E-02	7.75E+08	9.51E+08	8.67E+08
GRP78_HUMAN	7.70E-04	2.61E+09	2.55E+09	2.22E+09
ROA2_HUMAN	5.20E-04	1.57E+09	1.23E+09	3.16E+09
A0A0G2JNM3_HUMAN	2.60E-02	7.87E+08	6.12E+08	9.64E+08
CERU_HUMAN	6.80E-03	1.38E+09	7.52E+08	8.26E+08
1433Z_HUMAN	4.10E-03	5.28E+09	4.65E+09	3.30E+09

A1A4E9_HUMAN	1.80E-02	1.19E+10	7.86E+09	8.76E+09
B4E3A8_HUMAN (+1)SERPINB1	3.60E-02	1.06E+09	1.35E+09	7.55E+08
KCRU_HUMAN	3.00E-02	6.37E+08	9.35E+08	8.32E+08
A0A024RB53_HUMAN (+1)	2.00E-02	4.41E+09	4.62E+09	3.47E+09
HSPB1_HUMAN	1.00E-04	3.78E+09	3.57E+09	2.08E+09
ADH7_HUMAN	3.00E-02	1.13E+09	1.25E+09	1.40E+09
Q53FJ5_HUMAN (+1)	2.70E-02	1.09E+09	9.77E+08	1.18E+09
B3KX72_HUMAN (+1)HNRNPU	1.00E-04	2.82E+08	6.18E+08	6.60E+08
A4QPB0_HUMAN	2.10E-02	4.37E+07	1.38E+08	2.24E+08
H0YA55_HUMAN	1.00E-04	1.74E+10	6.29E+10	5.27E+10
SAMH1_HUMAN	9.50E-03	3.68E+08	4.51E+08	4.81E+08
S10A9_HUMAN	1.00E-04	5.51E+09	4.07E+09	3.33E+09
AT1A1_HUMAN	4.80E-02	7.07E+08	6.70E+08	8.50E+08
SPTB2_HUMAN	1.00E-04	1.17E+08	2.02E+08	2.87E+08
IDHC_HUMAN	1.00E-04	5.73E+09	3.99E+09	1.40E+09
K1C14_HUMAN	4.50E-03	1.35E+10	9.64E+09	7.35E+09
A0A0D9SGF6_HUMAN (+1)	1.10E-03	6.26E+07	1.12E+08	2.26E+08
ASSY_HUMAN	2.90E-03	1.20E+08	4.15E+08	2.61E+08
A0A0A0MTS2_HUMAN	6.70E-03	3.81E+08	4.48E+08	4.30E+08
1433S_HUMAN	3.10E-02	1.92E+09	2.15E+09	1.54E+09
IBP2_HUMAN	7.00E-04	9.58E+08	1.06E+09	1.90E+09
A2A274_HUMAN (+1)	2.80E-02	2.11E+08	2.64E+08	4.43E+08
LEG7_HUMAN	1.50E-02	1.78E+09	1.32E+09	1.12E+09
A0A024RDF4_HUMAN (+1)	1.00E-04	8.74E+08	1.34E+09	1.41E+09
1433G_HUMAN	5.00E-04	1.30E+09	8.85E+08	9.26E+08
PROF1_HUMAN	1.00E-04	2.64E+09	3.42E+09	1.61E+09
K22E_HUMAN	3.60E-04	1.08E+10	7.65E+09	6.87E+09
KCY_HUMAN	3.50E-03	1.84E+08	3.41E+08	4.99E+08
B4DR52_HUMAN (+2)	4.20E-02	3.56E+09	1.24E+09	8.06E+09
LDHA_HUMAN	8.30E-03	1.29E+09	1.38E+09	6.86E+08
QCR2_HUMAN	5.40E-03	8.71E+08	9.38E+08	5.95E+08
B3KNF4_HUMAN	1.60E-02	2.77E+08	2.57E+08	3.18E+08
LEG3_HUMAN (+3)	3.20E-02	1.65E+09	1.22E+09	1.10E+09
NHRF1_HUMAN	1.10E-03	1.27E+09	7.23E+08	1.43E+09
V9HW38_HUMAN	1.20E-03	4.95E+08	3.44E+08	8.55E+08
SLPI_HUMAN	3.40E-03	6.12E+09	5.03E+09	7.61E+09
MDHC_HUMAN	2.50E-02	7.90E+08	1.07E+09	1.03E+09
CIB1_HUMAN	1.50E-02	4.88E+08	2.70E+08	5.96E+08
B4DPJ2_HUMAN	4.40E-02	2.83E+08	3.36E+08	4.34E+08
RUVB2_HUMAN	2.80E-02	3.53E+08	4.09E+08	5.77E+08
Q59EF6_HUMAN	3.90E-02	6.47E+08	6.94E+08	4.76E+08

1433B_HUMAN	2.20E-03	3.78E+09	3.17E+09	2.16E+09
SPB5_HUMAN	4.10E-04	1.04E+08	2.93E+08	9.31E+07
SFPQ_HUMAN	1.60E-02	5.90E+08	8.44E+08	6.00E+08
KAD1_HUMAN (+1)	4.30E-02	2.96E+08	2.79E+08	4.79E+08
A8K9G0_HUMAN (+1)	2.70E-02	1.08E+09	7.64E+08	1.23E+09
H2AY_HUMAN	3.10E-03	2.22E+08	3.46E+08	3.89E+08
PRKDC_HUMAN	3.50E-02	2.38E+07	0.00E+00	6.61E+07
PDC6I_HUMAN	4.00E-02	1.34E+08	2.26E+08	1.52E+08
1433T_HUMAN	3.40E-03	2.89E+09	2.42E+09	1.53E+09
K7EKI8_HUMAN (+1)	1.00E-04	3.36E+07	3.50E+07	1.99E+08
J3KPX7_HUMAN (+1)	3.20E-02	8.66E+08	6.09E+08	5.89E+08
S10A2_HUMAN	7.40E-04	8.25E+09	7.58E+09	4.71E+09
GBB2_HUMAN	3.10E-03	2.24E+08	2.19E+08	5.46E+08
PRDX6_HUMAN	3.60E-03	1.15E+08	3.50E+08	1.58E+08
K1C16_HUMAN	1.60E-02	1.07E+10	7.96E+09	5.34E+09
B4E2I4_HUMAN (+1)	3.00E-02	2.19E+07	1.21E+08	1.16E+08
B4DFL1_HUMAN	1.30E-03	1.28E+08	4.08E+08	2.14E+08
TALDO_HUMAN	2.10E-02	4.91E+08	8.88E+08	9.12E+08
ARF1_HUMAN (+1)	7.00E-03	1.50E+07	5.68E+07	1.49E+08
Q6ZR44_HUMAN	1.00E-02	1.06E+08	2.60E+08	2.17E+08
SODC_HUMAN	3.60E-03	8.26E+08	1.54E+09	1.34E+09
B1AHC9_HUMAN (+1)	7.40E-04	3.06E+07	1.65E+08	1.42E+08
A0A024R6W0_HUMAN (+1)	4.60E-03	2.90E+08	5.15E+08	3.75E+08
BASP1_HUMAN	4.60E-03	3.17E+09	1.73E+09	4.85E+09
DX39B_HUMAN	1.00E-04	7.01E+07	1.38E+08	1.01E+08
QSOX1_HUMAN	2.20E-02	2.44E+07	8.84E+07	8.83E+07
A8K8U1_HUMAN (+1)	2.80E-02	2.04E+06	4.60E+07	1.61E+07
PSME1_HUMAN	3.20E-02	1.10E+08	1.47E+08	2.04E+08
AL1A3_HUMAN (+1)	6.80E-03	3.72E+07	1.59E+08	2.38E+08
HMGB1_HUMAN	1.00E-04	1.56E+08	4.06E+08	6.36E+08
A8K3C3_HUMAN (+1)	1.60E-02	1.71E+07	1.06E+08	1.40E+08
UGDH_HUMAN	2.00E-03	9.24E+07	7.66E+07	1.95E+08
HNRPL_HUMAN (+1)	8.70E-03	8.18E+07	1.62E+08	3.28E+08
Q53HV2_HUMAN (+1)	1.00E-02	1.11E+07	4.14E+07	1.26E+08
IBP3_HUMAN	1.00E-04	9.40E+08	6.01E+08	2.93E+08
GNA11_HUMAN (+1)	2.00E-02	2.44E+08	1.54E+08	1.45E+08
PDIA6_HUMAN	1.90E-02	9.73E+07	8.89E+07	5.94E+07
IBP7_HUMAN	3.70E-04	2.52E+08	3.27E+08	4.67E+08
STML2_HUMAN	1.40E-02	1.25E+07	1.75E+07	6.02E+07
PDLI1_HUMAN	1.00E-04	3.11E+07	1.91E+08	2.70E+08
A0A087X0D5_HUMAN (+2)	7.10E-03	1.35E+08	1.11E+08	3.39E+08

ETFA_HUMAN	4.10E-03	8.03E+07	5.78E+07	2.02E+08
B4DKR1_HUMAN	2.40E-02	1.10E+08	2.68E+08	1.79E+08
ODF3B_HUMAN	1.20E-02	1.37E+08	2.49E+08	2.41E+08
A0A087X1Z3_HUMAN (+2)	2.10E-03	2.93E+06	5.73E+07	5.34E+07
B4DPJ8_HUMAN	6.00E-03	2.07E+07	1.26E+08	5.31E+07
ECH1_HUMAN	3.20E-02	5.01E+07	1.36E+08	1.74E+08
B4E2G8_HUMAN	1.60E-03	8.92E+05	7.84E+06	3.44E+07
B2R7T8_HUMAN	2.20E-02	1.08E+08	1.78E+08	2.60E+08
B2RDE1_HUMAN	2.30E-02	1.64E+08	2.40E+07	2.30E+08
RBMX_HUMAN	1.30E-03	1.30E+07	2.82E+08	2.11E+08
LKHA4_HUMAN	1.00E-04	5.02E+06	2.93E+07	3.26E+07
NQO1_HUMAN	3.70E-03	3.34E+08	1.80E+08	4.21E+08
E9PCY7_HUMAN (+2)	1.10E-02	3.53E+07	1.42E+08	1.18E+08
DYL1_HUMAN	4.30E-02	1.20E+08	1.87E+08	5.30E+08
DHSO_HUMAN (+1)	2.80E-02	1.55E+08	1.85E+08	2.95E+08
K7ELC2_HUMAN (+2)	1.00E-04	2.43E+07	0.00E+00	1.60E+08
A0A087WUA5_HUMAN (+2)	6.10E-03	3.12E+07	2.47E+07	7.07E+07
B4E1U9_HUMAN	1.10E-03	2.62E+08	2.43E+08	7.29E+08
H14_HUMAN	1.60E-02	8.83E+07	5.28E+08	4.60E+08
FABP5_HUMAN	4.10E-02	2.30E+08	2.54E+08	1.61E+08
CEL_HUMAN (+2)	6.10E-04	0.00E+00	5.23E+07	2.57E+08
CD59_HUMAN (+2)	4.70E-02	1.35E+09	8.58E+08	1.54E+09
B3KWE0_HUMAN (+1)	3.60E-02	1.38E+08	4.36E+07	1.20E+08
TCO1_HUMAN	1.40E-02	1.91E+07	3.78E+07	1.04E+08
S10AG_HUMAN	2.60E-02	9.54E+07	7.31E+07	2.03E+08
SPR1B_HUMAN	1.30E-02	2.40E+08	1.28E+08	3.39E+08
GROA_HUMAN	4.40E-02	4.04E+08	2.82E+08	2.95E+08
ELAF_HUMAN	2.10E-02	1.05E+08	4.66E+08	3.37E+08
ATIF1_HUMAN	7.80E-03	4.79E+07	5.00E+07	2.62E+08
HNRPR_HUMAN	4.90E-02	3.23E+07	7.36E+07	1.46E+08
B9EKV4_HUMAN	4.60E-02	0.00E+00	1.11E+07	3.78E+07
B2RAR6_HUMAN (+2)	2.90E-02	1.82E+08	3.52E+08	3.19E+08
A0A0C4DFU2_HUMAN	4.30E-02	2.12E+07	1.38E+08	7.04E+07
GDIR1_HUMAN	3.10E-03	1.63E+07	1.22E+08	1.31E+08
H0YI09_HUMAN (+1)	1.60E-02	1.04E+08	7.97E+07	1.74E+08
CATS_HUMAN	1.00E-04	5.53E+07	8.88E+07	1.46E+08
Q53G25_HUMAN	1.00E-04	4.02E+07	0.00E+00	2.48E+08
J3KTL2_HUMAN (+1)	1.70E-02	2.54E+08	1.27E+08	2.30E+08
GSLG1_HUMAN	1.00E-02	0.00E+00	0.00E+00	7.71E+07
B4E0U6_HUMAN (+1)	1.00E-02	4.01E+07	1.20E+08	1.89E+08
J3QLI9_HUMAN (+2)	5.00E-03	6.61E+07	1.99E+07	8.69E+07

AK1BA_HUMAN	1.70E-03	0.00E+00	8.59E+06	4.76E+07
ODO2_HUMAN	7.30E-04	1.69E+08	6.28E+07	2.44E+08
Q6IPH7_HUMAN	4.40E-02	3.15E+07	1.59E+08	4.04E+07
E9PGC8_HUMAN (+1)	4.90E-02	1.99E+06	1.30E+07	1.73E+07
SERA_HUMAN	7.90E-03	6.33E+06	1.48E+07	6.21E+07
STIP1_HUMAN	7.10E-03	0.00E+00	3.80E+07	9.18E+07
G3V295_HUMAN (+2)	2.40E-04	2.62E+07	2.29E+08	1.75E+08
DPP3_HUMAN (+3)	1.00E-04	0.00E+00	8.68E+07	8.33E+07
RS12_HUMAN	1.00E-04	2.40E+07	2.93E+08	1.78E+08
MGST1_HUMAN (+1)	5.10E-04	4.79E+07	3.85E+07	1.55E+08
A0A0C4DGB5_HUMAN	1.50E-02	3.79E+07	5.90E+07	2.13E+08
A2GL_HUMAN	9.10E-03	0.00E+00	1.53E+06	2.29E+07
H1X_HUMAN	1.00E-04	0.00E+00	2.56E+08	1.66E+08
PLIN3_HUMAN	1.50E-02	0.00E+00	5.71E+07	1.09E+08
Q4LE33_HUMAN	2.20E-03	0.00E+00	7.08E+06	1.00E+08
MIEAP_HUMAN	5.50E-03	0.00E+00	5.44E+06	6.27E+07
B7Z3K9_HUMAN	3.30E-02	1.24E+07	2.64E+07	6.66E+07
A0A024QZN4_HUMAN (+1)	2.50E-02	0.00E+00	2.24E+07	6.50E+06
A0A024R5M3_HUMAN (+2)	1.00E-04	0.00E+00	9.34E+06	2.46E+08
CAZA1_HUMAN	1.10E-02	0.00E+00	0.00E+00	7.70E+07
RAB5C_HUMAN	2.10E-02	3.23E+07	4.57E+07	6.35E+07
PIP_HUMAN	1.00E-04	1.14E+07	2.38E+07	7.53E+07
Q59F44_HUMAN	3.00E-03	5.81E+07	2.72E+08	7.59E+07
CY1_HUMAN	9.80E-04	0.00E+00	6.51E+06	7.41E+07
CTL4_HUMAN	5.00E-02	1.30E+08	6.62E+07	2.96E+08
MNS1_HUMAN	6.50E-03	0.00E+00	0.00E+00	3.76E+07
B4DJI2_HUMAN (+1)	1.00E-04	3.77E+07	3.38E+07	0.00E+00
ACPH_HUMAN (+1)	9.00E-03	0.00E+00	2.14E+07	6.61E+06
ARPC2_HUMAN	6.10E-03	0.00E+00	0.00E+00	4.43E+07
PPOX_HUMAN	1.00E-02	0.00E+00	3.54E+07	2.84E+07
P5CS_HUMAN	3.00E-02	0.00E+00	0.00E+00	2.07E+07
6PGL_HUMAN	4.30E-04	0.00E+00	0.00E+00	4.10E+07
IPYR_HUMAN	1.60E-03	0.00E+00	4.82E+07	3.16E+07
F6TLX2_HUMAN (+1)	4.80E-02	2.22E+07	0.00E+00	2.98E+07
A0A087WXI5_HUMAN	3.90E-02	0.00E+00	7.01E+07	7.17E+07
B5BUB5_HUMAN	1.60E-03	0.00E+00	2.26E+07	6.56E+07
CPNE3_HUMAN	1.40E-02	5.44E+06	4.64E+07	1.17E+07
4F2_HUMAN (+2)	1.00E-04	0.00E+00	5.06E+06	4.87E+07
PEDF_HUMAN	1.60E-02	8.02E+06	0.00E+00	4.49E+07
LAP2B_HUMAN	1.00E-04	0.00E+00	8.57E+07	3.09E+07
Q7Z4Y4_HUMAN	5.00E-02	0.00E+00	1.77E+06	1.92E+07

E7EMS2_HUMAN	4.90E-02	0.00E+00	0.00E+00	2.67E+07
ERO1A_HUMAN	1.30E-03	7.05E+06	3.23E+06	2.29E+07
1433F_HUMAN	3.00E-03	0.00E+00	2.60E+07	1.45E+08
ACADM_HUMAN (+4)	3.70E-02	0.00E+00	0.00E+00	4.56E+07
B4DZ22_HUMAN	1.80E-02	1.15E+06	7.28E+05	2.08E+07
A0A087WTP3_HUMAN (+1)	3.30E-02	0.00E+00	4.34E+07	3.30E+07
Q9BTQ7_HUMAN (+1)	8.80E-03	1.11E+08	2.36E+07	0.00E+00
A8KAJ3_HUMAN (+1)	1.00E-04	0.00E+00	2.32E+07	8.61E+07
LASP1_HUMAN	4.80E-02	0.00E+00	6.79E+07	1.28E+08
Q2XPP3_HUMAN	6.20E-03	0.00E+00	2.40E+08	8.38E+08
B5BU25_HUMAN (+1)	2.40E-02	0.00E+00	2.32E+07	4.24E+07
E5RIW3_HUMAN	1.10E-02	1.31E+07	1.05E+08	2.96E+07
D3DRP5_HUMAN	1.20E-02	0.00E+00	2.88E+07	7.30E+07
PSA5_HUMAN (+1)	1.00E-04	0.00E+00	0.00E+00	9.09E+07
RALB_HUMAN	8.20E-03	0.00E+00	0.00E+00	5.37E+07
PGM2_HUMAN	2.30E-02	0.00E+00	5.53E+07	3.23E+07
IF6_HUMAN	3.40E-02	0.00E+00	7.34E+07	6.42E+07
A0A024RBF6_HUMAN	1.00E-04	3.41E+07	0.00E+00	3.09E+08
RL23A_HUMAN	4.30E-02	0.00E+00	0.00E+00	7.05E+07
ABRAL_HUMAN	2.90E-02	0.00E+00	2.33E+06	9.80E+06
B3GN7_HUMAN	3.10E-02	0.00E+00	0.00E+00	1.63E+07
B2R6K4_HUMAN (+1)	2.00E-02	0.00E+00	3.85E+07	2.82E+08
RS14_HUMAN	4.80E-04	0.00E+00	0.00E+00	1.66E+08
PPGB_HUMAN	1.00E-02	0.00E+00	0.00E+00	1.93E+07
NUDC_HUMAN	1.30E-03	0.00E+00	0.00E+00	5.35E+07
Q5QPL9_HUMAN	1.80E-02	0.00E+00	0.00E+00	4.66E+07
HNRH3_HUMAN (+1)	3.00E-02	0.00E+00	0.00E+00	4.11E+07
Q3SXP2_HUMAN (+1)	1.10E-03	0.00E+00	0.00E+00	6.30E+07
B0YIW6_HUMAN (+2)	4.50E-02	0.00E+00	0.00E+00	4.45E+07
A0A087WV23_HUMAN (+2)	1.60E-04	0.00E+00	0.00E+00	0.00E+00
B2R4C0_HUMAN (+2)	4.20E-02	0.00E+00	0.00E+00	2.39E+07
DDX17_HUMAN (+2)	4.60E-02	0.00E+00	0.00E+00	3.83E+07
SMD3_HUMAN	4.90E-02	0.00E+00	2.38E+07	8.32E+07
B3KQS9_HUMAN (+1)	9.90E-03	0.00E+00	0.00E+00	0.00E+00
B4DR80_HUMAN (+4)	3.40E-03	0.00E+00	0.00E+00	7.65E+06
Q13344_HUMAN	2.00E-02	0.00E+00	0.00E+00	3.01E+07
H7C579_HUMAN	1.00E-02	0.00E+00	0.00E+00	3.59E+07
VTM2L_HUMAN	1.20E-02	0.00E+00	0.00E+00	0.00E+00

Supplement Table 5: List of chemical compounds identified from gas chromatography-mass spectrometry (GC-MS) analysis in Kentucky research cigarettes (KCS) or cigarillos, which include Swisher-Sweets cigarillo (SSW), Garcia y Vega Game black cigarillo (GBK) and Hi-Fi Tropical Tango cigarillo (HTT).

KCS	SSW	GBK	HTT
1,2-Benzenediol	1,2-Benzenediol	1,2-Benzenediol	1,2-Benzenediol
1,4-Benzenediamine, N,N'-bis(1-methylethyl)-	1,4-Benzenediamine, N,N'-bis(1-methylethyl)-	1,4-Benzenediamine, N,N'-bis(1-methylethyl)-	1,4-Benzenediamine, N,N'-bis(1-methylethyl)-
2,3,4-Trihydroxybutyric acid	2,3,4-Trihydroxybutyric acid	2,3,4-Trihydroxybutyric acid	2,4-Hexadienoic acid
2,4-Hexadienoic acid	2,4-Hexadienoic acid	2,4-Hexadienoic acid	3,5-Dimethylphenol
2-Butenedioic acid	2-Methylphenol	2-Methylacetoacetic acid	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
2-Methylacetoacetic acid	3,5-Dimethylphenol	2-Methylphenol	3-Chloro-1,2-propanediol
2-Methylphenol	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3-Furoic acid
3,5-Dimethylphenol	3-Chloro-1,2-propanediol	3-Chloro-1,2-propanediol	3-Methylvaleric acid
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3-Furoic acid	3-Furoic acid	3-Pyridinecarboxylic acid
3-Chloro-1,2-propanediol	3-Methylvaleric acid	3-Methylvaleric acid	4-Methylcatechol
3-Furoic acid	3-Pyridinecarboxylic acid	4-Methylcatechol	Galactofuranoside, methyl 6-deoxy
3-Methylvaleric acid	4-Methylcatechol	6-Methylpyridine	Benzoic acid
3-Pyridinecarboxylic acid	6-Methylpyridine	Acetamide	Ethylene glycol
4-Methylcatechol	Galactofuranoside, methyl 6-deoxy	Galactofuranoside, methyl 6-deoxy	Glycerol
6-Methylpyridine	Benzoic acid	Ethylene glycol	Glycolic acid
Acetamide	Ethylene glycol	Glyceric acid	Hydroquinone
Galactofuranoside, methyl 6-deoxy	Glyceric acid	Glycerol	Lactic acid
Benzoic acid	Glycerol	Glycolic acid	Levoglucosan
Xylose	Glycolic acid	Hydroquinone	m-Cresol
Ethylene glycol	Hydroquinone	Lactic acid	Myo-Inositol
Glyceric acid	Lactic acid	Levoglucosan	Nicotine
Glycerol	Levoglucosan	m-Cresol	Phenol
Glycolic acid	m-Cresol	Myo-Inositol	Phenol, 4-methyl
Hydroquinone	Myo-Inositol	Nicotine	Propane, 2-methyl
Lactic acid	Nicotine	Oleic acid	Pyridine
Levoglucosan	Oleic acid	Phenol	tert-butyl alchohol
m-Cresol	Phenol	Propane, 2-methyl	2(3H)-Furanone, 5-butyldihydro-
Myo-Inositol	Phenol, 4-methyl	Pyridine	2(3H)-Furanone, dihydro-5-pentyl-
Nicotine	Propane, 2-methyl	Ribitol	2,3-Dimethylbutane-2,3-diol
Oleic acid	Pyridine	tert-butyl alchohol	2,3-Dimethylphenol
Phenol	Ribitol	1H-Indole	2,4'-Bipyridine
Phenol, 4-methyl	tert-butyl alchohol	2,2-Dimethyl-2-sila-1,3-dioxacyclohexane	2-Cyclopenten-1-one, 2,3-dimethyl-

Propane, 2-methyl	1H-Indole	2,3-Dimethylbutane-2,3-diol	2-Propenoic acid
Pyridine	2-(2-(2-Ethoxyethoxy)ethoxy)ethanol	2,3-Dimethylphenol	3-Ethoxybenzaldehyde
Ribitol	2,3-Dimethylbutane-2,3-diol	2-Ethylphenol	3-Hydroxymethylpentane
tert-butyl alchohol	2,3-Dimethylphenol	2-Propenoic acid	3-Hydroxypropanoic acid
Triacetin	2,4'-Bipyridine	3-Ethoxy-4-hydroxybenzaldehyde	4-Hydroxybutanoic acid
	2-Ethylphenol	3-Hydroxymethylpentane	Benzeneacetic acid
	2-Propenoic acid	4-Hydroxybutanoic acid	Benzyl alcohol
	4-Hydroxybutanoic acid	4-Methoxybenzaldehyde	Cotinine
	Behenic acid	Behenic acid	Diethylene glycol
	Benzaldehyde, 3-methoxy-	Benzaldehyde, 3-methoxy-	Ethyl hydrogen succinate
	Benzeneacetic acid	Benzeneacetic acid	Hexanoic acid
	Butanoic acid	Benzyl alcohol	Pent-2-en-1-ol
	Cotinine	Butanoic acid	Myosmine
	Diethylene glycol	Cotinine	Sorbic acid
	Mannitol	Diethylene glycol	Tetradecanoic acid
	Eicosanoic acid	Eicosanoic acid	Triethylene glycol
	Ethyl hydrogen succinate	Ethyl hydrogen succinate	
	Isopropanol	Isopropanol	
	Isothiocyanate	Isothiocyanate	
	Pent-2-en-1-ol	Pent-2-en-1-ol	
	Sorbic acid	Propanedioic acid, ethyl	
	Tetradecanoic acid	Sorbic acid	
	Triethylene glycol	Sulfurous acid, 2-ethylhexyl hexyl ester	
		Tetradecanoic acid	
		Triethylene glycol	

Appendix 3: TABLES AND FIGURES FOR CHAPTER 3

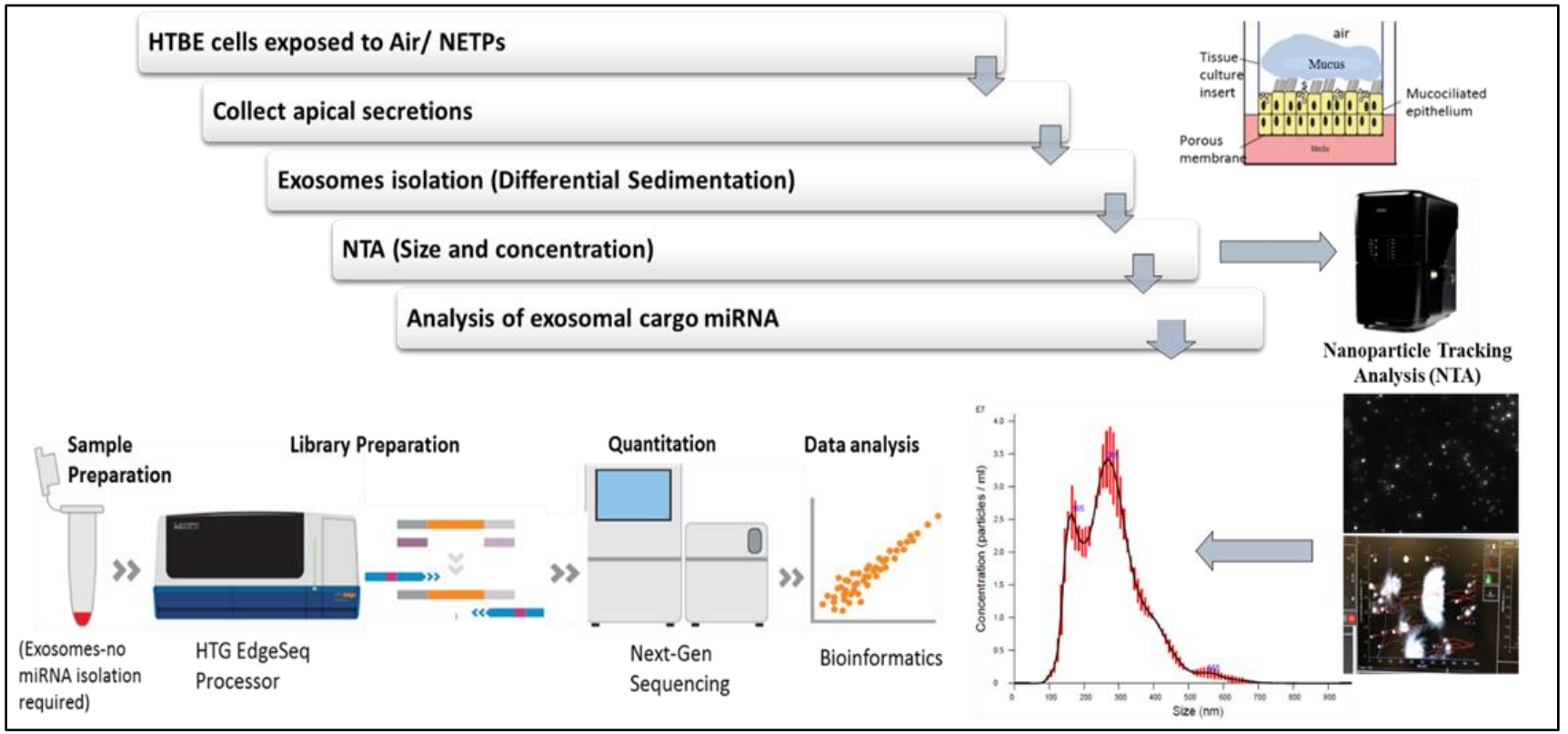


Figure 27: Methods for exosome isolation & miRNA analysis. NETPs smoked -HTBE cell apical secretions were collected and subjected to sequential differential sedimentation to purify airway exosome. Isolated exosome-like vesicles were characterized by Nanoparticle Tracking Analysis (NTA) instrument to provide the size and concentration. HTG EdgeSeq automated technology system was utilized to identify and purify exosomal miRNA in which library preparation was made for next-generation sequencing platforms. The differential expression analysis was performed by using a bioinformatics tool.

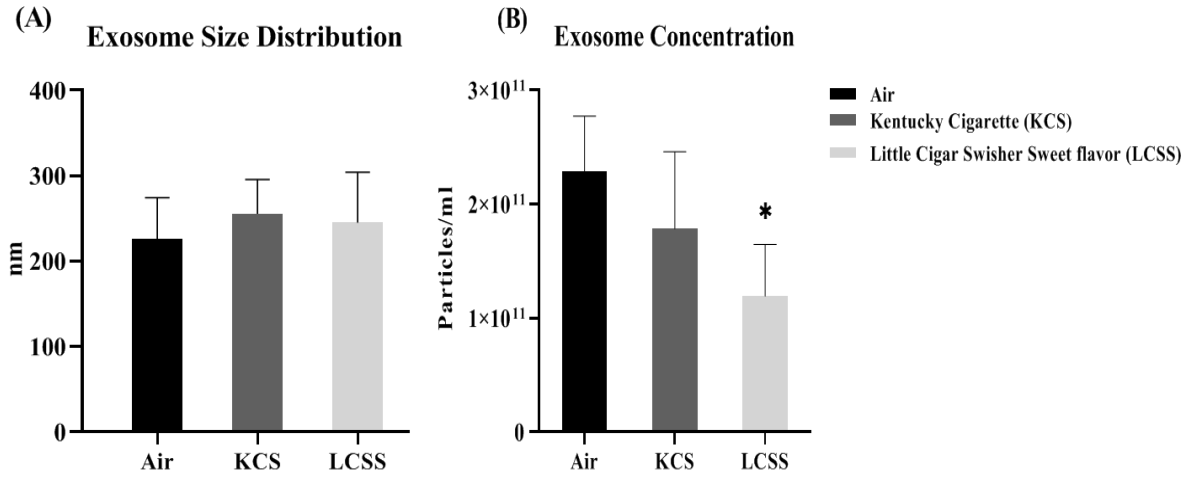


Figure 28: Characterization of exosome-like vesicles derived from apical secretion of HTBE cells exposed air, Kentucky cigarette (KCS) or little cigar swisher-sweets (LCSS). **(A)** Exosome size distribution and **(B)** concentration were measured by Nanoparticle Tracking Analysis (NTA) method. Significantly different than epithelial cells exposed to *air, mean ± SD. One-way ANOVA, p value <

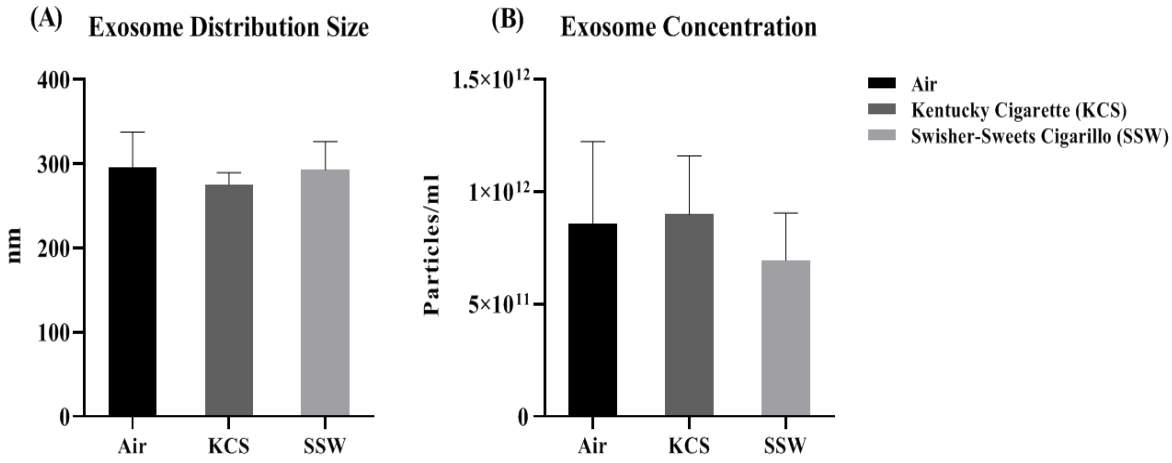


Figure 29: Characterization of exosome-like vesicles derived from apical secretion of HTBE cells exposed air, Kentucky cigarette or different cigarillo (SSW). **(A)** Exosome size distribution and **(B)** concentration were measured by Nanoparticle Tracking Analysis (NTA) method. N=6/group.

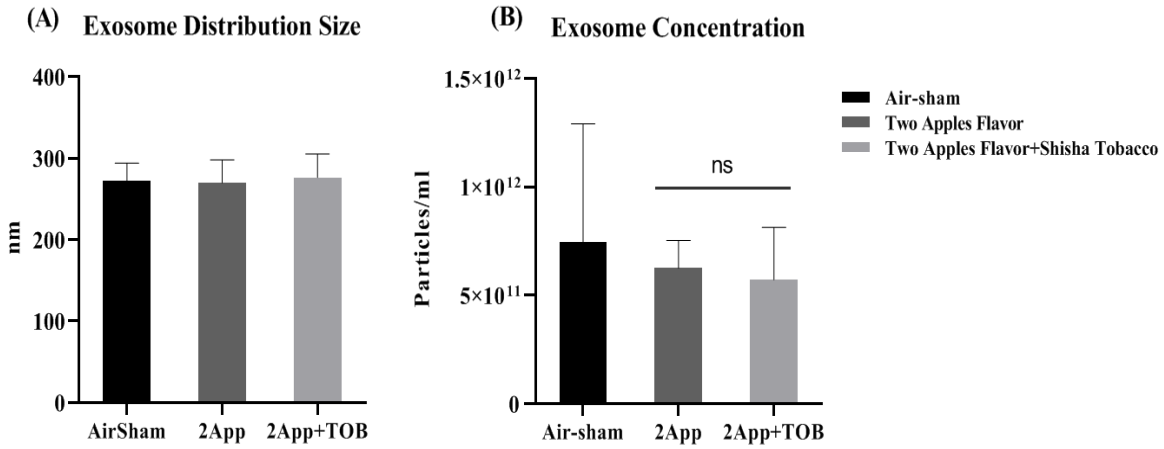


Figure 30: Characterization of exosome-like vesicles derived from apical secretion of HTBE cells exposed air, Two Apples flavor or shisha tobacco flavored Two Apples. **(A)** Exosome size distribution and **(B)** concentration were measured by Nanoparticle Tracking Analysis (NTA) method. N=6/group

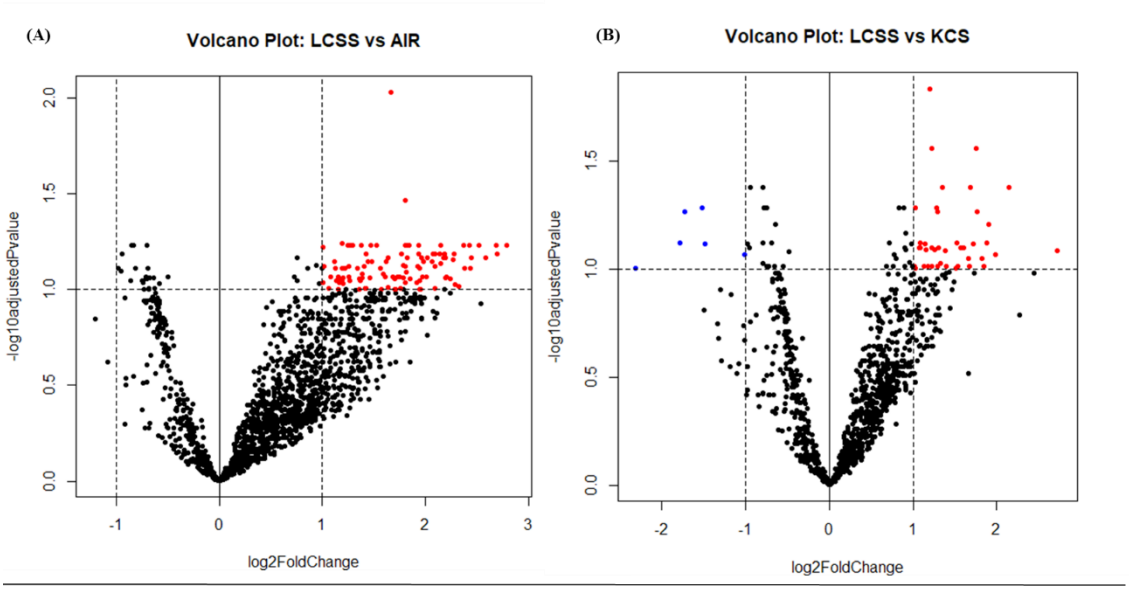


Figure 31: Differentially expressed exosomal miRNA after little cigar smoke exposure. Volcano plot showing exosomal miRNA differential expression in HTBE cells exposed to SSW vs air **(A)** and cigarette (KCS) **(B)** in which approximately 98 and 42 miRNAs were significantly differentially expressed in each comparison respectively. *P value < 0.05 and fold change > 2.

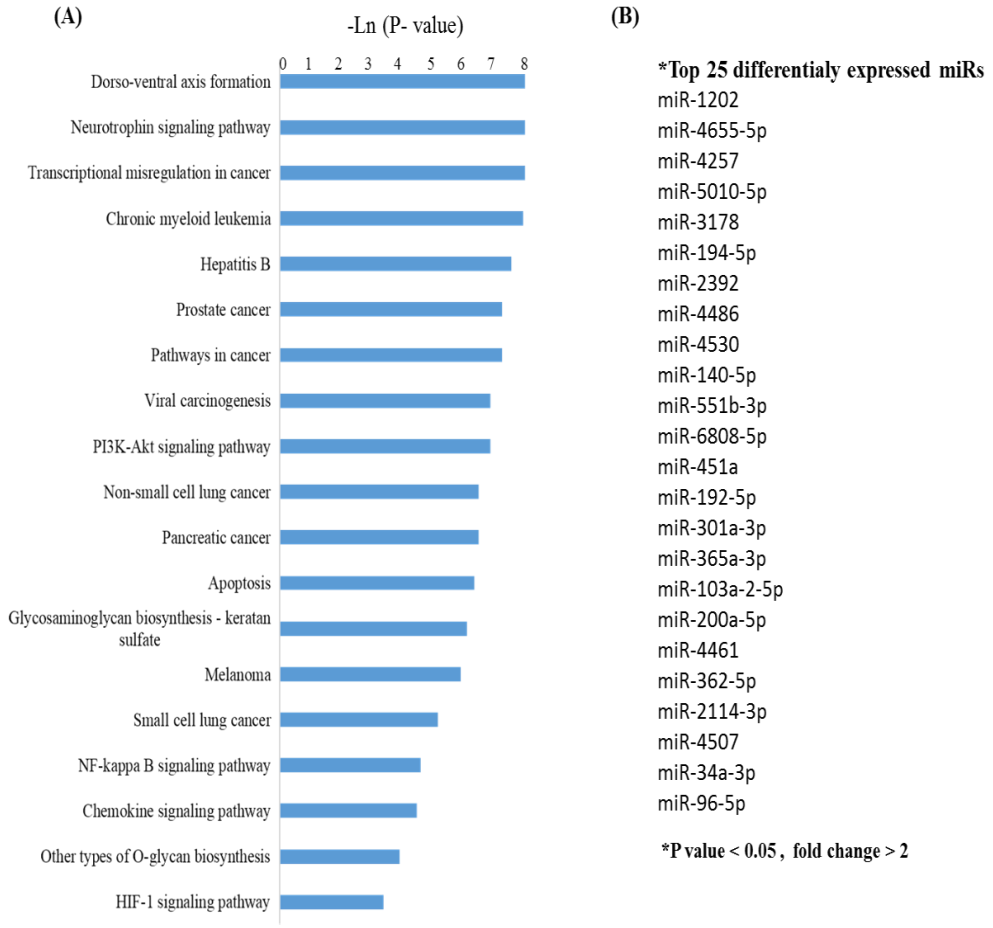


Figure 32: Pathway Analysis of miRNAs predict biological processes and functional pathways were affected by little cigar smoking exposure **(A)** and the list of top 25 signifcantly differentialy expressed miRNAs in smoke vs air groups **(B)**.

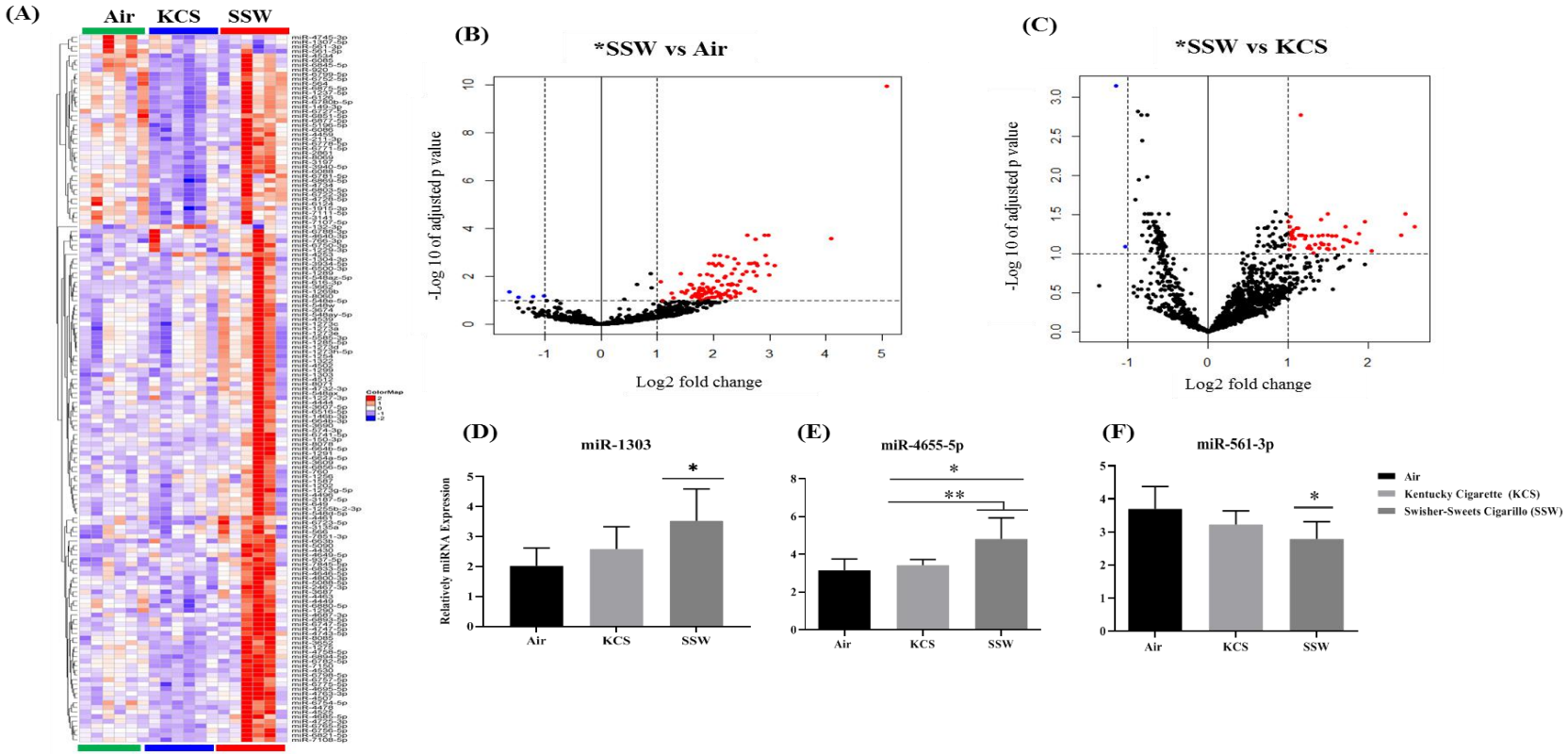


Figure 33: Exosomal miRNA analysis of apical secretion of HTBE cells exposed to swisher-Sweet cigarillo (SSW): **(A)** Heat map, a graphical representation displays the analysis result of a cluster of exosomal miRNA expression for air, cigarette and cigarillo exposure groups. Volcano plot showing exosomal miRNA differential expression in HTBE cells exposed to SSW vs Air **(B)** and KCS **(C)** were significantly differentially expressed in each comparison respectively. * Vs Air and ** vs KCS, P value < 0.05 and fold change > 2. cigarillo upregulated miRNAs that involved in NF-kappa B signaling pathway and Mucin-O-Glycan biosynthesis such as **(D)** miR-1303 and TGF-β signaling and MyD88-independent toll-like receptor signaling pathways like **(E)** miR-4566-5p while downregulated **(F)** miR-561-3p that involved in the genes regulated membrane organization, response to stress, regulated cell death and regulation of catalytic activity.

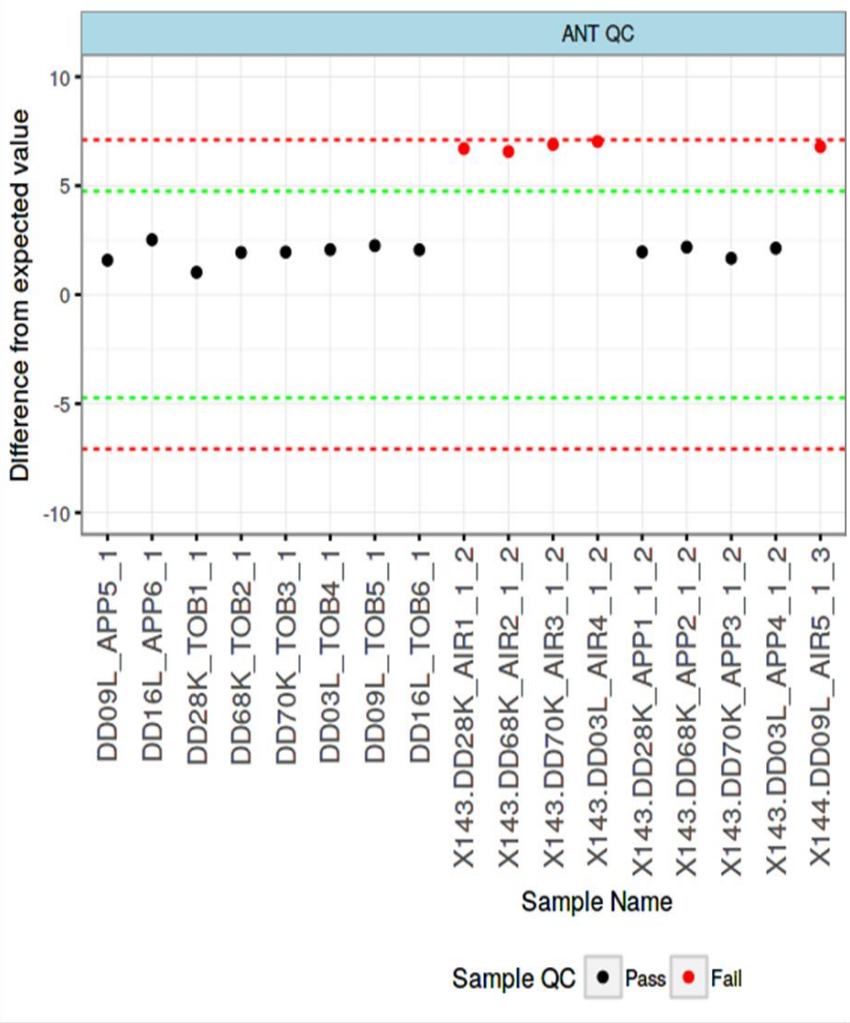


Figure 34: Plot shows the quality control of the samples were processed by HTG EdgeSeq miRNA Whole Transcriptome Assay in which indicates that all air samples group were failed to pass quality control

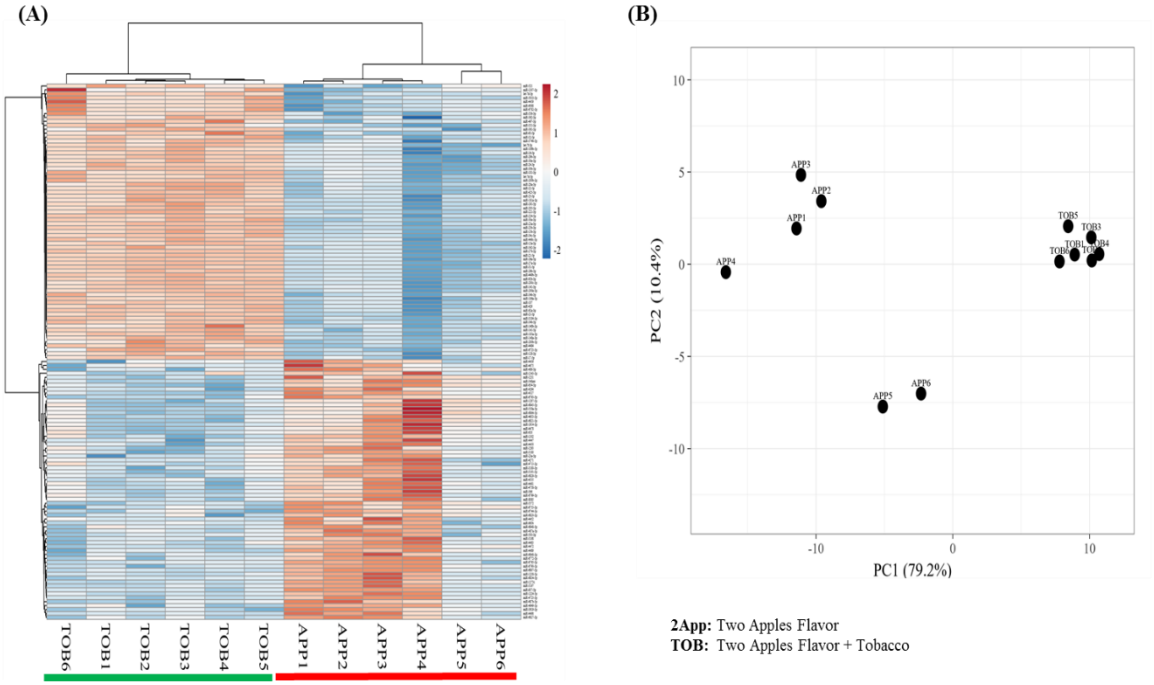


Figure 35: Exosomal miRNA analysis of apical secretion of HTBE cells exposed to waterpipe smoke. **(A)** Heat map, a graphical representation displays the analysis result of clustering exosomal miRNA expression of flavor (2App) and tobacco (TOB) groups. **(B)** Principal component analysis (PCA) of exosomal miRNA expression reveal clustering of the flavor, tobacco flavored-exposed groups.

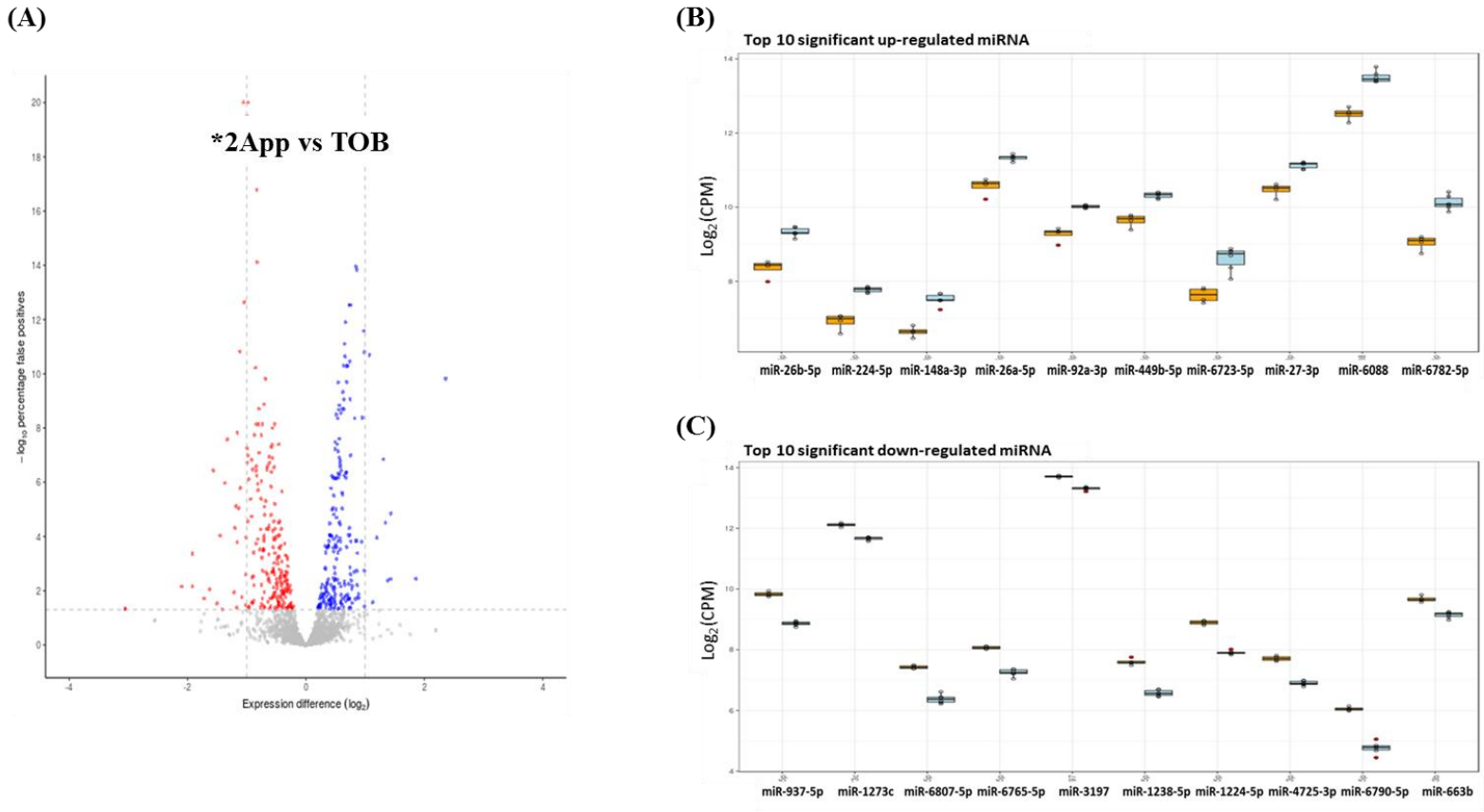


Figure 36: (A) volcano plot showing exosomal miRNA differential expression in HTBE cells exposed to waterpipe Tow Apples flavor (2App) compared to tobacco flavored with Two Apples (TOB). Plots illustrated the significantly top 10 upregulated miRNAs were **(B)** and top 10 downregulated miRNAs **(C)** in the tobacco flavored-exposed groups. *P value < 0.05

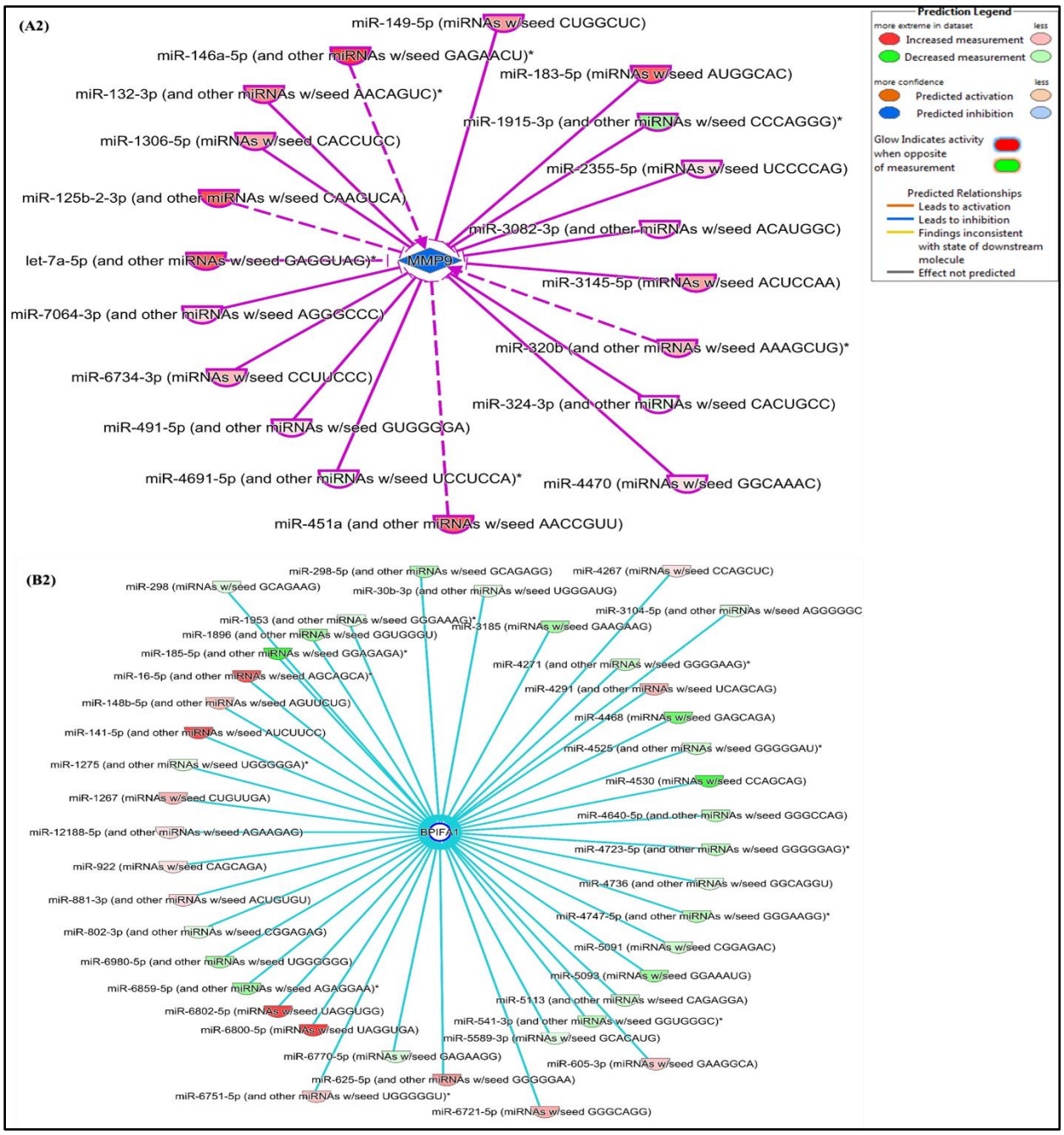


Figure 37: Example of the relationships between MMP9 **(A)** and PIGR **(B)** proteins and their putative miRNAs targeted targets their genes and related seed complementary sequence may bind to the mRNA. The list of differentially expressed miRNA profiles in the all three NETPs smoke exposure, and fold-change was uploaded into the ingenuity pathway analysis (IPA) application to generate the network. The intensity of the node color indicates the degree of upregulation (red) or down regulation (green).

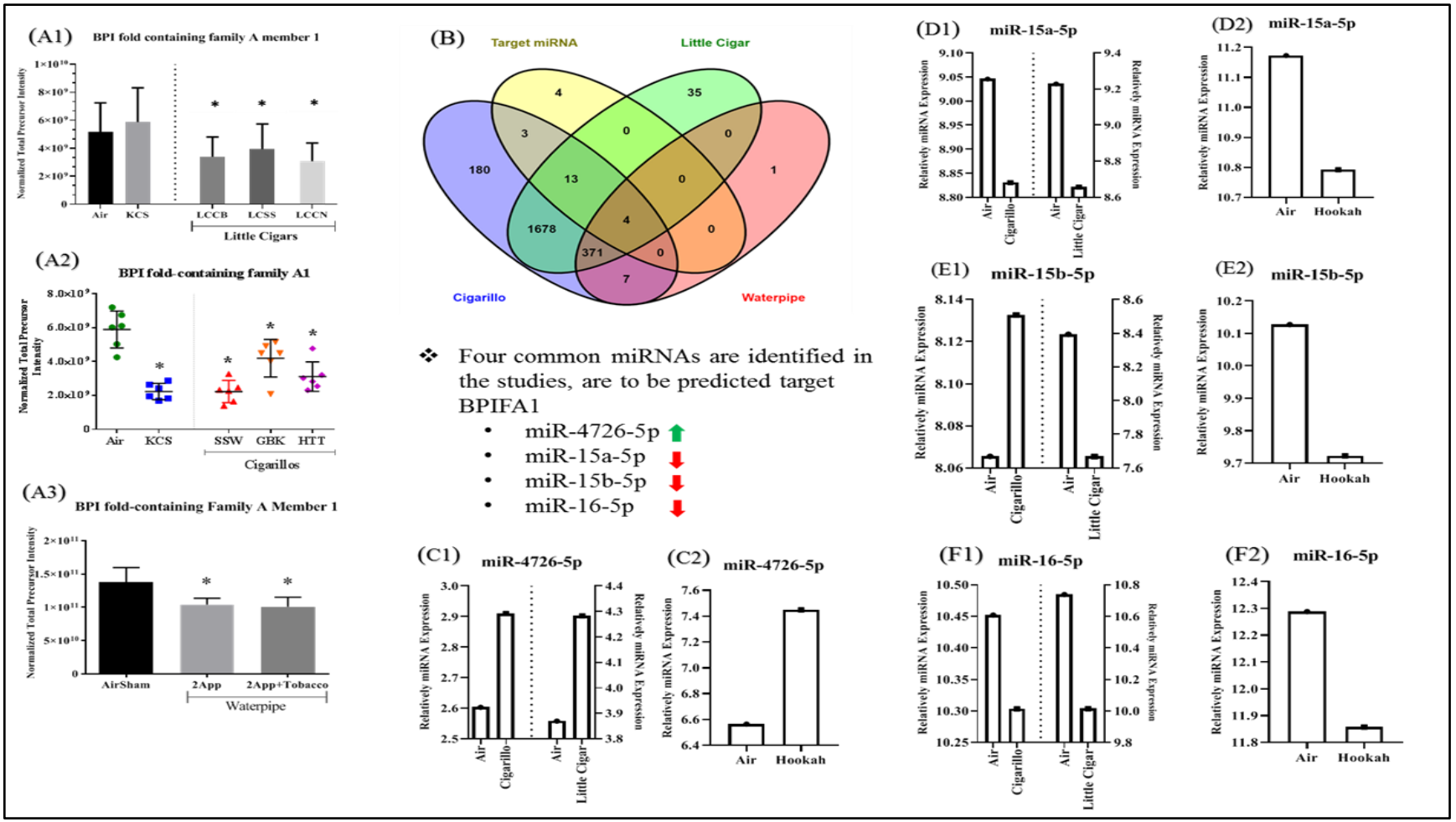


Figure 38: miRNAs predicate to target BPI fold containing family A member 1 (BPIFA1): downregulation of BPIFA1 was associated with NETPs smoke include little cigars **(A1)**, cigarillo **(A2)** and waterpipe **(A3).** Venn diagram **(B)** generated by overlay a list of miRNAs predicted to target BPIFA1 (http://www.mirbase.org) and lists miRNA profile form three different independent studies due to HTBE cell exposed to little cigars, cigarillo and waterpipe smoke. Four common miRNAs were identified in the studies that to be predicted target BPIFA1, miR-4726-5p **(C1-3)** was upregulated in all three studies while miR-15a-5p **(D1-2)**, miR-15b-5p **(E1-2)** and miR-16-5p **(F1-2)** were downregulated.

Table 6: Characterization of exosome-like vesicles derived from the apical secretion of HTBE cells exposed to air, Kentucky cigarette (KCS) or NETPs smoked-group were measured by Nanoparticle Tracking Analysis (NTA) method.

	Little Cigar Study		**Cigarillo Study**		**Waterpipe study**	
	Average Size (nm)	Concentration (Particles/ml)	Average Size (nm)	Concentration (Particles/ml)	Average Size (nm)	Concentration (Particles/ml)
Air	226.35	2.28e+11	295.5	8.60e+11		
KCS	255.25	1.78e+11	275.5	8.98e+11		
LCSS	245	1.19e+11				
SSW			292.8	6.94e+11		
Air-sham					272.5	7.40e+11
2App					269.8	6.26e+11
TOB					276.4	5.73e+11

Table 7: Partial list of top biological processes and functional pathways predicted to be altered by waterpipe smoke exposure.

KEGG pathway[1]	**P-value**	**# Genes**	**# miRNAs**
Mucin type O-Glycan biosynthesis	5.57E-13	26	51
Proteoglycans in cancer	5.57E-13	163	87
Pathways in cancer	5.57E-13	311	91
ECM-receptor interaction	2.40E-12	63	69
Mucin type O-Glycan biosynthesis	9.95E-11	12	10
ErbB signaling pathway	6.09E-08	76	83
Axon guidance	4.55E-07	52	13
Hippo signaling pathway	7.08E-07	120	85
Focal adhesion	5.21E-06	164	85
Thyroid hormone signaling pathway	5.77E-06	96	82
Glioma	9.29E-06	54	77
Rap1 signaling pathway	1.77E-05	164	87
Regulation of actin cytoskeleton	2.72E-05	74	15
Renal cell carcinoma	0.000529	28	10
Glycosaminoglycan biosynthesis - keratan sulfate	0.000658	7	8
Transcriptional misregulation in cancer	0.000658	58	14
Glutamatergic synapse	0.000959	39	15
Endocytosis	0.001385	64	15
Lysine degradation	0.00312	15	12
Thyroid hormone synthesis	0.0057	22	13

[1] Diana Tools, mirPath v.3. Vlachos, Ioannis S., Konstantinos Zagganas, Maria D. Paraskevopoulou, Georgios Georgakilas, Dimitra Karagkouni, Thanasis Vergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou. "DIANA-miRPath v3. 0: deciphering microRNA function with experimental support." Nucleic acids research (2015): gkv403.

Supplement Table 6: Partial list of biological processes and functional pathways that predicted to be altered by little cigar swisher-sweets (LCSS).

KEGG pathway [1]	**P-value**	**# Genes**	**# miRNAs**
TGF-beta signaling pathway	4.13E-05	14	4
MAPK signaling pathway	0.000164088	47	4
Neurotrophin signaling pathway	0.003509097	24	4
Glycosphingolipid biosynthesis - lacto and neolacto series	0.004586994	3	3
Ras signaling pathway	0.004586994	33	4
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	0.006333444	13	3
Hepatitis B	0.009434859	19	4
Hippo signaling pathway	0.013698094	20	4
Prolactin signaling pathway	0.013698094	15	4
N-Glycan biosynthesis	0.017151258	7	4
TNF signaling pathway	0.020781874	21	4
Signaling pathways regulating pluripotency of stem cells	0.023602761	21	4
B cell receptor signaling pathway	0.037884447	14	4
Pancreatic cancer	0.037884447	12	4
T cell receptor signaling pathway	0.038952764	19	4
Chagas disease (American trypanosomiasis)	0.040812445	18	4

[1] Diana Tools, mirPath v.3. Vlachos, Ioannis S., Konstantinos Zagganas, Maria D. Paraskevopoulou, Georgios Georgakilas, Dimitra Karagkouni, Thanasis Vergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou. "DIANA-miRPath v3. 0: deciphering microRNA function with experimental support." Nucleic acids research (2015): gkv403.

Supplement Table 7: List of biological processes and functional pathways that predicted to be altered by cigarillo (SSW).

GO Category [1]	p-value	#genes	# miRNAs
organelle	1.39E-51	869	4
ion binding	9.49E-40	578	4
cellular nitrogen compound metabolic process	4.52E-31	445	4
biosynthetic process	1.05E-22	377	4
cellular protein modification process	1.41E-19	240	4
nucleic acid binding transcription factor activity	1.10E-18	130	4
neurotrophin TRK receptor signaling pathway	3.44E-16	45	4
molecular_function	1.94E-13	1355	4
Fc-epsilon receptor signaling pathway	4.76E-13	31	4
toll-like receptor TLR1:TLR2 signaling pathway	3.26E-10	18	4
toll-like receptor TLR6:TLR2 signaling pathway	3.26E-10	18	4
cytosol	4.41E-10	255	4
TRIF-dependent toll-like receptor signaling pathway	1.56E-09	18	4
gene expression	4.80E-09	61	4
toll-like receptor 4 signaling pathway	4.89E-09	22	4
enzyme binding	5.87E-09	129	4
toll-like receptor 10 signaling pathway	7.68E-09	16	4
MyD88-independent toll-like receptor signaling pathway	9.72E-09	18	4
protein binding transcription factor activity	1.69E-08	59	4
toll-like receptor 2 signaling pathway	4.74E-08	18	4
toll-like receptor 5 signaling pathway	5.99E-08	16	4
toll-like receptor 3 signaling pathway	8.58E-08	18	4
protein complex	1.04E-07	320	4
toll-like receptor signaling pathway	1.26E-07	22	4
nucleoplasm	1.26E-07	117	4
toll-like receptor 9 signaling pathway	2.29E-07	16	4
symbiosis, encompassing mutualism through parasitism	3.99E-07	54	4
cell death	4.45E-07	94	4
transcription, DNA-templated	4.56E-07	235	4
cellular_component	4.72E-07	1339	4
viral process	9.12E-07	48	4
catabolic process	9.12E-07	166	4

[1] Diana Tools, mirPath v.3. Vlachos, Ioannis S., Konstantinos Zagganas, Maria D. Paraskevopoulou, Georgios Georgakilas, Dimitra Karagkouni, Thanasis Vergoulis, Theodore Dalamagas, and Artemis G. Hatzigeorgiou. "DIANA-miRPath v3. 0: deciphering microRNA function with experimental support." Nucleic acids research (2015): gkv403.

response to stress	1.66E-06	195	4
cell-cell signaling	2.25E-06	71	4
synaptic transmission	4.02E-06	49	4
epidermal growth factor receptor signaling pathway	4.89E-06	29	4
stress-activated MAPK cascade	5.62E-06	13	4
MyD88-dependent toll-like receptor signaling pathway	5.62E-06	18	4
immune system process	2.58E-05	142	4
biological_process	3.93E-05	1291	4
transcription initiation from RNA polymerase II promoter	5.79E-05	30	4
enzyme regulator activity	6.08E-05	80	4
cellular lipid metabolic process	0.000225194	19	4
nervous system development	0.000312204	52	4
cytoskeletal protein binding	0.000326817	72	4
blood coagulation	0.000437245	42	4
regulation of transcription from RNA polymerase II promoter in response to hypoxia	0.000818336	7	4
innate immune response	0.001189732	68	4
negative regulation of type I interferon production	0.00122378	8	4
cellular component assembly	0.00122378	106	4
cell junction organization	0.001646193	20	4
small molecule metabolic process	0.002142506	171	4
fibroblast growth factor receptor signaling pathway	0.002183232	23	4
nucleobase-containing compound catabolic process	0.002183232	75	4
energy reserve metabolic process	0.002425497	14	4
leukocyte migration	0.002723669	16	4
protein polyubiquitination	0.002723669	23	4
nucleotide-binding oligomerization domain containing signaling pathway	0.002833697	6	3
mitotic cell cycle	0.006221696	33	4
phosphatidylinositol-mediated signaling	0.008531995	17	3
nucleotide-binding domain, leucine rich repeat containing receptor signaling pathway	0.014061041	7	3
T cell receptor signaling pathway	0.014813842	18	4
transcription factor binding	0.018264143	61	4
JNK cascade	0.021072549	14	3
G1/S transition of mitotic cell cycle	0.02496505	21	4
apoptotic signaling pathway	0.035559549	16	4
cell motility	0.039168833	50	4

glycoprotein metabolic process	0.041291315	6	3
transcription from RNA polymerase II promoter	0.042634931	59	4
intracellular transport of virus	0.044615519	4	3
post-translational protein modification	0.046991725	15	4
NLS-bearing protein import into nucleus	0.047496038	5	2
axon guidance	0.049294351	43	4

Supplement Table 8: List of the exosomal miRNAs and the mean value that significant differentially expressed after air, Kentucky cigarette (KCS) and little ciagr (LCSS) smoke exposure**.**

miR	Air-mean	KC-mean	LCSS-mean	log2FoldChange	lfcSE[1]	P-value
miR-4732-3p	2.33503	2.499528	3.295274	1.666756	0.366348	5.37E-06
miR-1269b	2.445108	2.761572	3.587483	1.802291	0.438577	3.97E-05
miR-4502	3.501833	3.609544	4.113072	1.190434	0.305843	9.93E-05
miR-8060	1.167738	1.339309	1.886826	1.929977	0.508254	0.000146
miR-5684	2.444942	2.711878	3.231057	1.46789	0.394224	0.000196
miR-449a	10.70886	10.68075	10.20609	-0.82984	0.228943	0.000289
miR-3619-3p	2.590914	2.63442	3.274078	1.38105	0.388825	0.000383
miR-449b-5p	9.914685	9.811565	9.261162	-0.84937	0.239666	0.000394
miR-8085	1.848806	2.297418	2.690714	1.843099	0.520733	0.000401
miR-449b-3p	6.39882	6.26802	5.871063	-0.70103	0.199492	0.000441
miR-5698	1.140778	1.213687	2.156754	1.8042	0.517913	0.000495
miR-554	1.295778	1.087978	2.010514	1.815757	0.521814	0.000502
miR-4496	3.043634	3.06862	3.652878	1.29793	0.374069	0.000521
miR-1322	3.386587	3.545672	4.265727	1.263401	0.365079	0.000539
miR-7111-5p	5.35745	5.485769	6.336639	1.250061	0.363179	0.000577
miR-573	0.2580695	0.6013378	1.2742988	2.683286	0.781085	0.000592
miR-4647	0.4763835	0.4128495	1.1948987	2.523372	0.734612	0.000593
miR-125b-1-3p	0.2797253	0.4368138	1.0045254	2.78503	0.819929	0.000682
miR-4531	0.6713406	0.7775476	1.5052706	2.423065	0.714264	0.000693
miR-490-5p	0.5612333	0.478619	1.1116502	2.368096	0.701084	0.000731
miR-4452	0.59337	0.9906833	1.6413808	2.143508	0.63555	0.000744
miR-3064-5p	1.665027	1.693595	2.331293	1.526713	0.452818	0.000747
miR-1227-3p	3.625792	3.534066	4.218818	1.00684	0.300366	0.000802
miR-5581-3p	0.718286	0.91837	1.5069829	2.184046	0.661618	0.000963
miR-8054	1.070648	0.9512497	1.7092176	1.934703	0.589766	0.001036
miR-4311	0.8210195	0.9843079	1.6773499	2.0717	0.633488	0.001074
miR-520a-5p	-0.009	0.2176609	0.95771763	2.697317	0.826143	0.001095
miR-4297	1.476197	1.733059	2.677975	1.766829	0.542842	0.001135
let-7c-3p	1.0266765	0.930921	1.4282609	2.093881	0.644141	0.001151
miR-1183	0.6393005	0.5646213	1.5044435	2.27534	0.701445	0.00118
miR-3674	3.760469	4.06333	4.950675	1.422822	0.439881	0.001218
miR-889-5p	0.4044841	0.7305104	1.2686465	2.137263	0.661924	0.001243
miR-449c-5p	7.20007	7.198034	6.880721	-0.94499	0.292686	0.001244
miR-8086	0.3123762	1.015444	1.1895423	2.190486	0.687025	0.001431
miR-3679-3p	0.9626513	1.1874204	1.4921468	1.961905	0.615929	0.001446
miR-3675-3p	0.9552723	1.0107794	1.642646	1.825452	0.573191	0.001449

[1] lfcSE (log fold change Standard Error), Analysis of miRNA-seq data with DESeq2 software.

HK_RNU47	0.4161978	0.5110485	1.1440976	2.588608	0.820048	0.001596
miR-503-5p	0.4509114	0.4359258	1.2166693	2.440752	0.77366	0.001606
miR-4283	1.366676	1.273279	1.984866	1.638518	0.519516	0.001611
miR-488-5p	0.9152758	0.6003602	1.5868101	2.018085	0.640363	0.001625
miR-6792-3p	4.304069	4.19692	4.673508	0.756251	0.240193	0.001641
miR-130a-5p	0.2419002	0.398526	0.9723998	2.175178	0.691185	0.001649
miR-2355-3p	0.1282953	0.5485779	0.9446056	2.264698	0.72294	0.001733
miR-323b-3p	0.8550907	0.9141671	1.4808824	2.066468	0.665234	0.001894
miR-1306-3p	1.007466	1.066258	1.586212	1.982167	0.638807	0.001916
miR-548b-5p	3.383975	3.554365	3.772744	1.115032	0.359845	0.001944
miR-744-3p	1.381749	1.431613	1.797931	1.482371	0.479004	0.00197
miR-4682	1.681227	1.586931	2.036876	1.438105	0.46536	0.002
miR-208a-3p	1.539846	1.619992	2.112932	1.622378	0.525745	0.00203
miR-4510	0.5933982	0.6555645	1.1614145	2.146599	0.69839	0.002115
miR-6801-3p	3.199775	3.22477	3.575162	0.938571	0.306949	0.00223
miR-3690	1.124156	1.538709	1.953908	1.790677	0.58629	0.002256
miR-634	0.7365787	1.1628441	1.7333668	1.921541	0.632112	0.002367
miR-1236-3p	0.756161	0.8754363	1.4835319	1.801223	0.592836	0.002379
miR-6895-5p	3.377602	3.402946	3.797093	1.015134	0.334518	0.002408
miR-4256	0.5311778	0.5751317	1.397061	2.095417	0.692229	0.002469
miR-370-3p	1.635895	1.907229	2.626123	1.408915	0.465628	0.002479
miR-4446-3p	2.219273	2.3713	2.869993	1.257675	0.416641	0.002539
miR-6886-3p	3.50736	3.280535	3.945032	0.85982	0.285937	0.002638
miR-4705	0.4046182	0.5830217	0.9621444	2.384449	0.794571	0.002692
miR-6859-5p	1.682442	1.723608	2.227969	1.563051	0.521421	0.00272
miR-34c-3p	9.963915	9.685888	8.868769	-0.69408	0.232512	0.002835
miR-6847-5p	1.008819	1.132745	1.608797	1.941911	0.650788	0.002846
miR-1289	2.674853	2.128585	2.957396	1.222857	0.410128	0.002867
miR-3934-5p	2.452443	2.564105	3.247687	1.337417	0.450109	0.002965
miR-5581-5p	0.4399907	0.5350738	0.8890317	2.432827	0.8192	0.00298
miR-138-5p	8.20193	8.094221	7.435335	-0.80836	0.272657	0.003029
miR-2682-3p	2.337625	2.222185	2.733889	1.189354	0.401421	0.003048
miR-4656	2.42124	2.790217	3.097912	1.202679	0.406977	0.003125
miR-4632-3p	2.504195	2.727094	3.122655	1.131037	0.382972	0.003144
miR-6131	10.480465	10.307528	9.437286	-0.98379	0.333678	0.003195
miR-6807-3p	3.07197	2.975437	3.419312	0.996191	0.338305	0.003233
miR-2392	4.253283	4.302261	3.853374	-0.95371	0.325369	0.003377
miR-4254	0.905032	0.7089998	1.3194749	1.816428	0.621406	0.003466
miR-4421	1.796313	2.131894	2.40555	1.59107	0.546608	0.003605
miR-4646-5p	2.436174	2.597861	3.361995	1.391426	0.478343	0.003628
miR-7106-3p	2.544312	2.32453	2.994478	1.157624	0.400616	0.003857

miR-214-5p	0.9049528	1.222377	1.9249161	1.607376	0.556459	0.00387
miR-6076	1.973334	1.959129	2.866127	1.733788	0.60253	0.004008
miR-34b-3p	10.121212	9.894469	9.228332	-0.50004	0.173776	0.004008
miR-4251	2.109179	2.341756	2.572831	1.253737	0.435835	0.004019
miR-4664-5p	0.657143	0.6546507	1.1094967	1.968127	0.684636	0.004044
miR-4422	0.5986423	0.4465478	1.1705019	2.008075	0.700066	0.004125
miR-4476	1.139149	1.184079	1.63318	1.682271	0.588411	0.00425
miR-30a-5p	10.493826	10.251405	9.647315	-0.71132	0.249124	0.0043
miR-4440	1.260823	1.533229	2.087949	1.747314	0.612538	0.004337
HK_GAPDH	2.831879	3.01438	3.517983	1.079417	0.378659	0.004363
miR-6816-3p	3.843668	3.910537	4.558264	0.73134	0.256684	0.004383
miR-3116	0.4053384	0.519809	1.0335422	2.207882	0.775712	0.004424
miR-6874-3p	2.203058	2.511924	3.039078	1.204475	0.424565	0.004555
miR-92b-3p	10.756714	10.710114	10.05995	-0.63386	0.223534	0.004573
miR-34a-5p	8.547628	8.353346	7.669468	-0.67395	0.23798	0.004627
miR-4662a-3p	0.6412566	0.4103284	0.9312489	2.192007	0.775443	0.004702
HK_PPIA	2.448891	2.468426	3.093272	1.460256	0.517434	0.004771
miR-6831-5p	1.304412	1.014946	1.586498	1.785725	0.632879	0.004779
miR-6792-5p	1.444454	1.84734	1.992526	1.386314	0.491746	0.004815
miR-1587	2.26166	2.764954	3.167389	1.172362	0.416738	0.004905

Supplement Table 9: List of the exosomal miRNAs and the mean value that significant differentially expressed after air, Kentucky cigarette (KCS) and cigarillo (SSW) smoke exposure**.**

miR	Air-mean	KCS-mean	SSW-mean	log2FoldChange	lfcSE	P-adj value
miR-664b-5p	0.941978	0.694622	3.653103	5.074062	0.684891	1.17E-10
miR-4507	4.611238	4.507238	6.206728	2.59556	0.519855	0.000194
miR-1303	2.020227	2.587301	3.528616	2.907618	0.588007	0.000194
miR-6741-5p	8.028275	7.313286	10.22177	2.97583	0.604335	0.000194
miR-1291	0.39099	0.590302	2.164723	4.091383	0.848931	0.000264
miR-8078	4.566475	3.781699	6.69881	2.744276	0.574733	0.000275
miR-1254	4.070661	4.323053	5.332118	2.11413	0.478228	0.001288
miR-4695-5p	3.734461	3.655729	4.938303	2.018936	0.460414	0.001294
miR-4530	4.478899	3.532783	6.453644	2.930745	0.671376	0.001294
miR-1273h-5p	7.779505	7.678065	9.184113	2.244471	0.520587	0.001487
miR-4463	5.248434	4.338908	6.733939	2.373809	0.559925	0.001867
miR-3197	9.918033	9.010046	11.8467	2.730418	0.660846	0.002752
miR-1269b	0.927416	0.974493	2.603959	2.9542	0.719767	0.002793
miR-1273d	7.83375	7.816942	9.05397	1.97883	0.484602	0.002793
miR-6821-5p	6.041836	5.634602	7.28899	2.087955	0.512168	0.002793
miR-4763-3p	5.130574	4.81299	6.281604	1.913003	0.473129	0.002908
miR-664a-5p	1.650506	1.845448	3.107867	2.757463	0.683387	0.002908
miR-937-5p	1.609712	1.950587	3.176328	2.658984	0.661823	0.002908
miR-1255b-2-3p	5.987788	5.906319	7.550425	2.434451	0.608574	0.002908
miR-1299	3.40396	3.633848	5.036516	2.630691	0.657716	0.002908
miR-4430	1.027185	1.011026	2.479552	2.777275	0.706077	0.003422
miR-4539	2.636324	2.315327	3.867611	2.151578	0.548258	0.003422
miR-1285-5p	7.20025	7.058649	8.489111	2.025246	0.517561	0.003422
miR-1273g-5p	2.666694	2.642419	3.720213	2.032452	0.51988	0.003422
miR-5090	0.459038	0.645922	2.123596	3.092147	0.791349	0.003422
miR-3674	3.336968	3.18115	4.633552	2.374804	0.622765	0.004836
miR-616-3p	1.193738	1.42126	2.768834	2.71229	0.726674	0.006218
miR-1322	1.584662	1.771682	2.950578	2.586559	0.693049	0.006218
miR-2110	5.080644	5.316747	5.618733	0.87488	0.2383	0.007311
miR-4646-5p	1.494938	1.28499	2.830899	2.316155	0.631434	0.007311
miR-7845-5p	4.170932	4.155837	5.053438	1.422733	0.388176	0.007311
miR-3687	3.147493	2.747611	4.384146	1.879772	0.518106	0.00818
miR-649	2.776171	2.805795	4.366031	2.518874	0.696069	0.008227
miR-6516-5p	0.114122	-0.00878	1.888123	2.988633	0.830354	0.008415
miR-5585-3p	6.968422	6.747932	8.029599	1.750379	0.486543	0.008415
miR-3607-5p	1.215833	0.857	3.110521	2.762203	0.773035	0.008983

miR-1202	2.664558	2.418829	3.815167	1.961596	0.550473	0.00907
miR-548d-5p	4.461069	4.354608	5.83305	2.214568	0.626917	0.009935
miR-6798-5p	4.227716	3.432618	5.434713	2.187086	0.626426	0.011299
miR-4461	4.878002	3.778831	6.255569	2.075573	0.611926	0.015555
miR-4725-3p	2.066956	2.027348	3.278343	2.070326	0.610611	0.015555
miR-1587	0.91143	1.045199	2.040359	2.461337	0.727184	0.015555
miR-574-3p	7.088404	7.264445	7.729142	1.061567	0.315831	0.01655
miR-566	2.996846	3.235147	4.007628	1.811204	0.547113	0.019411
miR-3934-5p	1.261188	1.555956	2.453531	2.326866	0.704645	0.01955
miR-4732-3p	1.093152	1.324955	2.244831	2.336181	0.712519	0.020745
miR-320a	7.090957	7.401697	7.498772	0.643221	0.196816	0.020745
miR-1273e	6.027428	5.914602	6.978357	1.592399	0.487378	0.020745
miR-6894-5p	3.165858	2.372946	4.132526	1.577159	0.483591	0.020751
miR-548e-5p	1.484892	1.536777	2.610456	2.251282	0.691519	0.020755
miR-6088	8.819463	7.465696	10.06775	2.147553	0.667096	0.023109
miR-664b-3p	2.23624	2.571063	3.322468	2.314178	0.722036	0.023257
miR-150-3p	1.522752	0.962478	2.935623	2.414067	0.753687	0.023257
miR-3652	3.508431	2.814709	4.435778	1.606785	0.501964	0.023257
miR-1275	7.315825	5.931365	8.450633	1.881228	0.597403	0.027314
miR-1304-3p	2.891625	2.957479	3.783556	1.749489	0.559316	0.028829
miR-92b-5p	5.277213	5.272409	5.810622	0.900452	0.289077	0.0296
miR-548w	2.58831	2.03794	3.736494	2.003188	0.645498	0.030255
miR-8071	2.955565	3.094893	3.935521	1.80086	0.584987	0.031835
miR-3690	-0.23823	0.036527	1.059286	2.663534	0.865918	0.031835
miR-8060	0.013531	0.248915	1.339568	2.638431	0.858628	0.031835
miR-4800-3p	2.785125	2.407081	3.78693	1.640675	0.534709	0.031835
miR-7150	7.452851	6.433081	8.439021	1.912082	0.627058	0.033388
miR-6756-5p	7.609158	7.011205	8.539474	1.741454	0.579062	0.037757
miR-4758-5p	4.402285	3.643216	5.433766	1.822063	0.61052	0.038526
miR-3609	1.140186	0.753827	2.649285	2.256122	0.756036	0.038526
miR-4478	3.711244	3.019204	4.609548	1.531371	0.513795	0.038526
miR-4687-3p	1.122288	1.218924	2.048068	1.953077	0.655448	0.038526
miR-1290	6.682283	6.09557	7.875811	1.78666	0.5999	0.038526
miR-6833-5p	-0.08294	-0.04134	0.986247	2.366022	0.797566	0.039452
miR-3662	-0.63798	-0.35196	0.734085	2.731318	0.924244	0.040358
miR-548ay-5p	2.75585	2.345539	3.961155	1.90425	0.650277	0.042873
miR-561-3p	3.699075	3.222004	2.784281	-1.63788	0.55941	0.042873
miR-8085	1.004056	0.914205	2.015059	1.935732	0.663922	0.043804
miR-4685-5p	0.605039	0.470776	1.554701	2.054992	0.706011	0.043804
miR-4512	2.93238	2.881779	3.763625	1.427532	0.491463	0.043804
miR-6856-5p	-0.11501	0.094037	0.926784	2.387855	0.822117	0.043804

miR-6782-5p	4.767206	3.699094	5.664644	1.85664	0.64371	0.046122
miR-6893-5p	0.789272	0.702567	1.829926	2.099561	0.733514	0.048627
miR-4525	3.415536	3.101533	4.350502	1.715806	0.60107	0.048627
miR-1273c	4.351058	4.070448	5.28986	1.500597	0.526305	0.048627
miR-6788-3p	-0.1726	0.626983	0.877814	2.473633	0.868161	0.048627
miR-6775-5p	5.270205	4.791929	6.059205	1.257357	0.441509	0.048627
miR-4253	1.506279	2.0767	2.296742	1.726604	0.608068	0.049108
miR-6750-3p	0.672615	0.859266	1.733606	2.208732	0.778507	0.049108

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