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Research Article

# Exhaled Biomarkers of Oxidative Stress and Inflammation in Hookah Smokers: A Public Health Concern

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## Abstract

Current public policy does not reflect health risk awareness associated with hookah smoking. Although, cigarette smoking has gained considerable attention accompanied by public restrictions, hookah smoking continues to increase as an unrestricted, socially acceptable means of recreation. This study identified novel biomarkers altered by hookah smoking compared to cigarette smoking using exhaled breath condensate collected from nonsmokers, hookah smokers and cigarette smokers pre and post smoking. Both cigarette and hookah smokers had higher levels of inflammatory mediators suggestive of inflammasome activation as well as altered expression of *miR-217* and *miR-17*, which are known to regulate inflammasome activation and promote oncogenesis. To assess current public knowledge of risk factors and behaviors associated with hookah smoking, and if knowledge of hookah dangers would influence smoking behaviors, the experimental data were integrated into a survey emphasizing cancer risk associated with hookah smoking. Survey results indicated that 70% of study participants were encouraged to abstain from hookah smoking after gaining knowledge of cancer risk associated with hookah. The identification of behaviors associated with hookah smoking and novel biomarkers of health risk may influence its public health regulation and facilitate community health education.

**Keywords:** Biomarkers; Oxidative stress; miR-217; Inflammasome; Inflammation; miR-17

## Introduction

Unlike cigarette smoking, there are currently no significant restrictions on public hookah smoking. The practice is emerging as a popular social activity among a variety of populations. Lack of public knowledge regarding the addictive potentials, as well as personal health risks associated with hookah smoking, may contribute to its social acceptance especially when compared to cigarette smoking [1,2]. Hookah smoke contains compounds such as carbon monoxide, benzene, polycyclic aromatic hydrocarbons, carbonyls, phenols, phenol derivatives, etc., which initiate oxidative stress and inflammatory pathways that have been implicated in chronic lung disease and cancer [3].

Central to the inflammatory cascade, the inflammasome is a multi-protein complex consisting of nod-like receptor such as pyrin domain-containing 3 (NLRP3), apoptosis-associated speck-like protein containing PYCARD (ASC), and caspase-1. Inflammasome component association and activation is facilitated both directly and indirectly via up regulation of tumor necrosis factor α (TNF-α) and the

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nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). While NF-κB transactivates pro-IL-1β, TNF-α triggers the production of reactive oxygen species (ROS), facilitating inflammasome assembly causing caspase 1 mediated cleavage of pro-IL-1β into its active form IL-1β [4,5]. Additionally, activating and synergistically enhancing the function of IL-1β, oxidative stress induced ROS also upregulates lactose dehydrogenase (LDH). LDH is an oxidoreductase that interconverts pyruvate and lactate with concomitant interconversion of NADH and $NAD^{+}$, and is an accepted biomarker of oxidative stress [6,7].

Importantly, NLRP3, IL-1β, and TNF-α activation are correlated with lung disease and cancer. For example, NLRP3 suppresses natural killer (NK) cell-mediated control of oncogenesis [8]. IL-1β is associated with polymorphisms implicated in carcinogenesis and promotion of metastasis [9,10]; while TNF-α facilitates transformation, cell survival, proliferation, invasion, and angiogenesis [11]. Further, serving as a biomarker of lung damage and a prognostic factor in small cell lung cancer, LDH is currently under investigation as a cancer therapy target [12-14]. The LDHA isoform specifically enhances tumor cell invasion and metastasis. Inhibition of LDHA suppresses non-small cell lung cancer cell proliferation, decreases ROS, and encourages apoptosis and cell cycle arrest [15].

In this study, we measured exhaled breath condensate (EBC) levels of biomarkers indicative of inflammasome activation (LDH, TNF-α and IL-1β) after hookah and cigarette smoking. Using these biomarkers as central nodes, we employed network modeling tools to uncover micro-RNAs (miR-17 and miR-217) that serve to control inflammasome activation, which were subsequently measured. Additionally, through survey analysis, we expose behaviors associated with hookah smoking and the potential knowledge of health risk has on the decision to quit hookah smoking.

# Materials and Methods

## Study participants

This study was approved by Loma Linda University Institutional Review Board (IRB). Participants in the biomarker quantification were recruited from local hookah cafes and included healthy non-smokers, current healthy hookah smokers, and current healthy cigarette smokers. All participants were males over the age of 18 with no known history of cardiopulmonary disease or any major health issue. Informed consent was obtained from all participants during the data collection. Survey only participants were recruited through an online survey distributed through email and social media.

## EBC collection

EBC was collected from the following subject groups: non-smokers, smokers both pre and post 60 minute of a hookah smoking session, or pre and post completion of 1 cigarette. Exhaled breath condensates were collected using the RTube™ (Respiratory Research, Inc., USA). EBC samples were concentrated and stored per published recommendations [16-18].

## Biomarker quantification

EBC samples were analyzed for exhaled IL-1β using the eBioscience, Human IL-1β Enzyme-Linked Immunosorbent Assay (ELISA) 88-7010, TNF-α using the Cayman Chemical, TNF-α ELISA Kit -500850, and LDH using the Thermo Scientific, Pierce LDH Cytotoxicity Assay Kit -88953) per manufacturers guidelines.

## miR-17 and miR-217 quantification

RNA was isolated using miRNeasy® Mini Kit (Qiagen) from EBC. Reverse transcription was performed using miScript II RT Kit (Qiagen cat# 218160) following quantification of miRNA via quantitative polymerase chain reaction (qPCR) with miScript SYBR Green PCR Kit (Qiagen cat # 218073) and miScript Primer Assay kit for miRs-217 and -17 (Qiagen Cat # MS00003843 & MS0002927). Relative expression was calculated using the 2-(ΔCT) method with SNORD44 as loading control. Mean values and the standard deviation of the dCT values.

## Network modeling

A data base of predicted and known interactions with

inflammatory biomarkers measured in EBC samples was created using String open source data base (http://string-db.org/). This database was annotated using thorough literature reviews and The Gene Ontology (GO) database (http://geneontology.org/). The resulting database was then integrated into Gaggle software to reveal potential novel, measurable biomarkers related to inflammatory mediated oncogenic pathways [19].

## Data analysis

Biological data are expressed as means ± standard error of means ± SEM of all subjects of each respective study group. Comparisons of mean values between two groups were evaluated using a two-tailed Student's t test, Wilcoxon signed-rank test or Mann-Whitney U test depending on normality and data assumptions. Survey data were imported from https://llu.co1.qualtrics.com into IBM SPSS™. Unless otherwise indicated, *p<0.05 was considered statistically significant.

## Results

Biomarkers can be used to predict oncogenic pathway activation in the lung using respiratory tract tissue samples. These include sputum, saliva, nasal/bronchial airway epithelial cells, as well as EBC. Of these, the most promising is the EBC, currently used to monitor/measure lung inflammatory processes in a variety of chronic pulmonary disorders. In this study, we show that EBC samples from both hookah and cigarette smokers yield signature markers of inflammasome activation predisposing smokers to oncogenic pathway initiation.

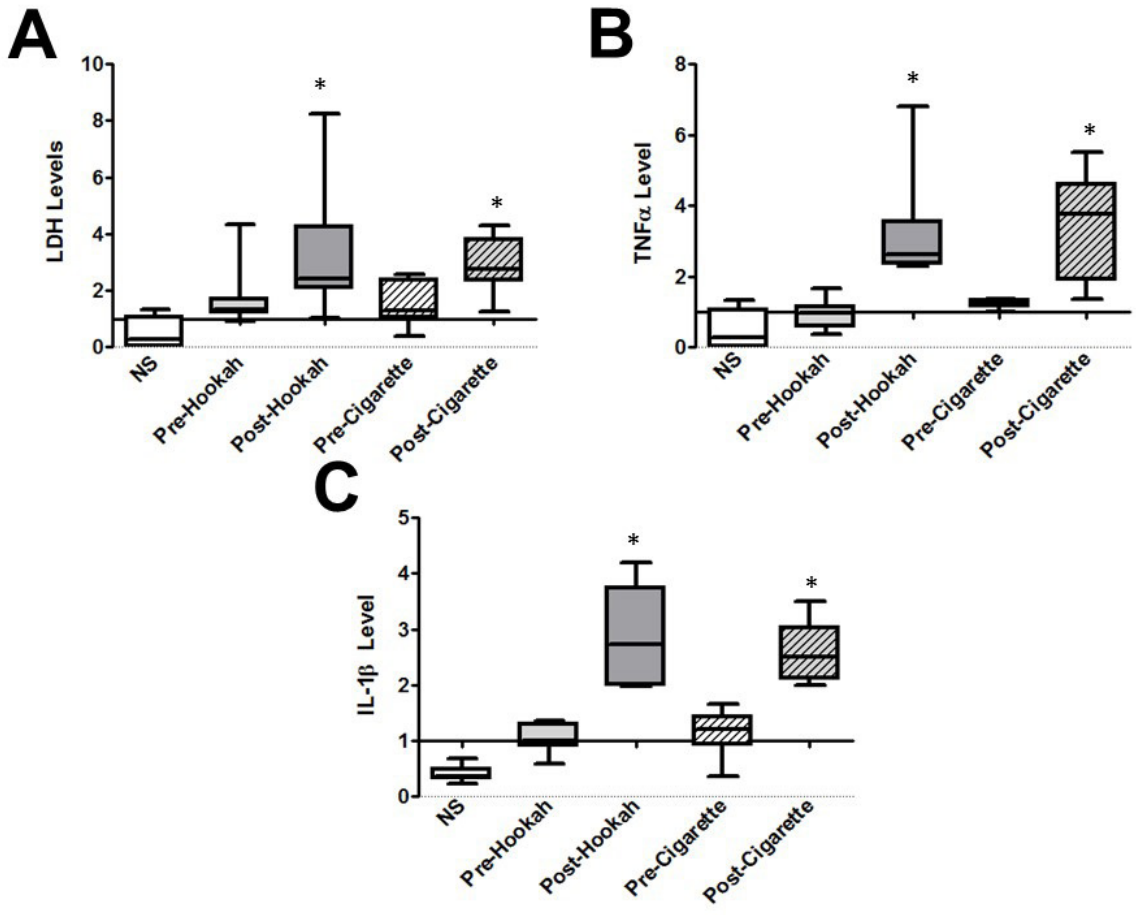


**Figure 1:** Hookah smoking increases expression of IL-1β, LDH, and TNF-α.
Box plot of enzyme-linked immunosorbent assay (ELISA) showing the gene expression of exhaled (A) LDH, (B) IL-1β and (C) TNF-α levels prior to or 2 hours after hookah or cigarette smoke inhalation. N=24, *P <0.5.

## Hookah smoking increases oxidative stress and inflammation

EBC samples were collected from nonsmokers, cigarette smokers (pre and post), and hookah smokers (pre and post) to determine the degree of relative pulmonary inflammation. LDH, TNF-α, and IL-1β were chosen as inflammatory-oncogenic mediators due to their known expression in early tumor-inducing responses. EBC LDH and IL-1β levels were significantly higher in the hookah smokers relative to the cigarette smokers (Figure 1A and B). TNF-α levels were increased similarly in both post hookah and cigarette smoking groups (Figure 1C).

## Network modeling reveals miR-217 and miR-17 as potential biomarkers of inflammasome activation

After validating that LDH, TNF-α, and IL-1β are indeed higher after hookah smoking, network modeling was employed to unveil other important regulators of the inflammasome as potential measurable candidates in the EBC samples. The original biological network database was created using extensive literature searches and String Version 9.1 (http://string-db.org/). The generated database was integrated into Gaggle software to display predicted oxidative and inflammatory pathways induced by hookah. Figure 2 depicts a portion of the inflammatory network predicted that includes components of the inflammasome, NLRP3, ASC, and caspase-1, serving as central nodes. This database led us to investigate the relative expression of micro RNA's (miRNAs) involved in inflammasome regulation [20]. From this subnetwork, m*iR-217* and m*iR-17* were further analyzed.

Figure 3 represents a schematic of the overall regulatory pathways underlying the mediators measured in the EBC samples. Figure 3A illustrates *miR-217* acting as a transcriptional repressor of sirtuin 1 (Sirt1) by binding to its 3' untranslated region (3' UTR) ultimately inhibiting its expression [21]. Sirt1 is a class III deacetylase that targets NF-κB to inhibit its nuclear translocation. Inhibition of Sirt1 via miR-217 activates NF-κB, which transactivates NLRP3 leading to IL-1β production. Considering Sirt1 as a negative regulator of the inflammasome, it was predicted that miR-217 would be upregulated (higher) in the post hookah and cigarette smoking EBC samples.

Figure 3B depicts the role of *miR-17* in inflammasome regulation. *MiR-17* translationally represses thioredoxin-interacting protein (TXNIP) also by binding to its mRNA 3' UTR stopping the interactions that lead to NLRP3 inflammasome activation [22]. TXNIP is necessary to facilitate NLPR3 complex formation with ASC and caspase-1 prompting cleavage of pro- IL-1β to active IL-1β [23]. Because *miR-17* inhibits inflammasome activation, it was predicted that *miR-17* would be down regulated (lower) in the post hookah and cigarette smoking EBC samples.

## MiR-217 and miR-17 are differentially expressed during Hookah

As expected, a significant overexpression of *miR-217* was detected in post-hookah and post-cigarette samples compared to the other samples (Figure 4A). On the other hand, *miR-17* was significantly higher in non-smoker and pre-smoker groups (Figure 4B).

## Online survey

Of the 650 participants in the survey, the majority of the hookah smokers (N=323) were between 18 to 36 years old with a mean age being 24 years, and 88% were college students or graduates. Reasons for smoking hookah are quantified in Figure 5A: 51% smoke hookah to pass free time; 39% for socializing and report the local hookah café is amongst their most favorable places; 31% for the pleasant smell of hookah smoke; 26% for peer effect (peer-pressure); 24% because they think it imparts a legal "high" or is intoxicating; 15% as a safer alternative to/and in order to quit cigarette smoking; and 10% reported other reasons which included stress relief, mood changes, and possible addiction. Figure 5B illustrates subject perception of health risk associated with cigarette vs. hookah smoking: 35% reported both the same; 31% reported hookah is more dangerous; 31% reported cigarette smoking is more dangerous; and 1% reported that neither have any health risks. Survey participates were also asked to gauge their perception of the validity

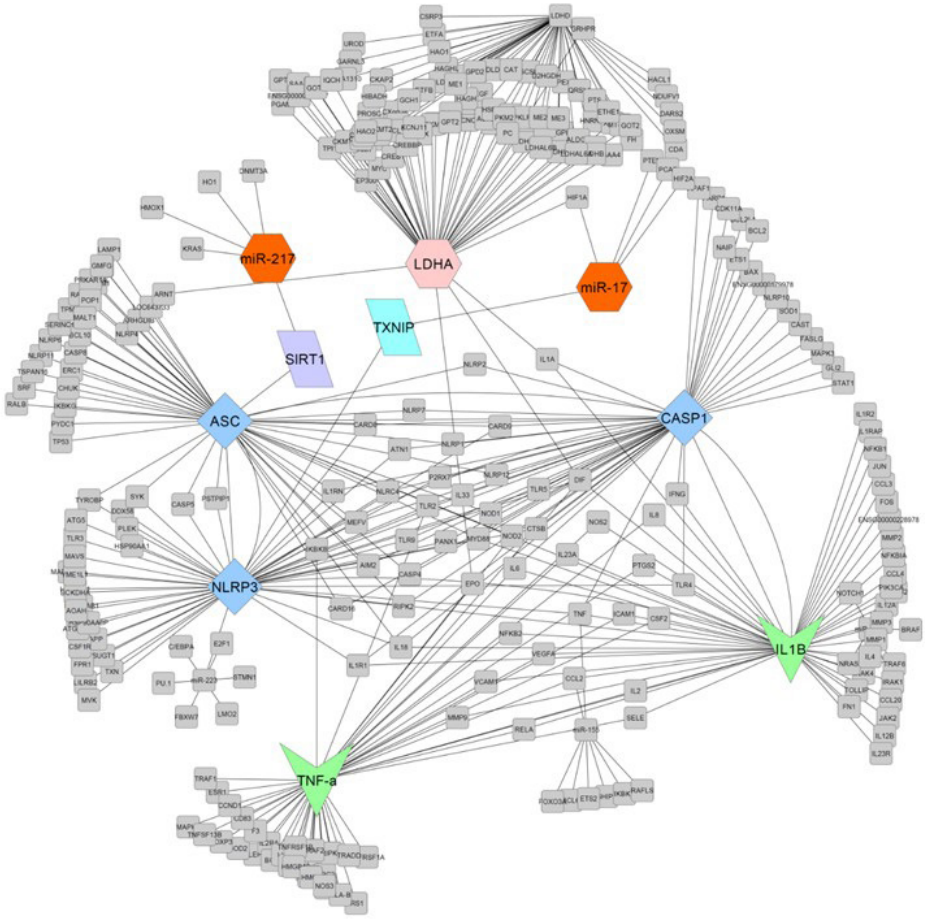


**Figure 2:** Predicted hookah induced inflammatory network.
Network modeling displaying regulatory pathway of the inflammasome represented by its components NLRP3, ASC, and Casp1 (blue). TNF-α (green), IL-1β (green), and LDHA (pink) are shown with their relative association with inflammasome components. MiR-217 and miR-17 are illustrated in red and associated with their targets, Sirt1 (purple) and TXNIP (light blue), respectively. Gray boxes represent network partners that were not explored in this study.

of the EBC results presented to them as an indication that hookah could possibly cause cancer (Figure 5C): 39% reported that they believed the results to be valid; 27% reported that they neither agree nor disagree; 13% users disagreed; 11% users strongly agree and only 7% users strongly disagree. Interestingly, 70% reported that exposure to the experimental results encouraged them to consider quitting; 24% reported had no effect on the decision to quit; and 5% reported that the data decreases the probability of quitting (Figure 5D).

## Discussion

In this study we have used EBC sampling to demonstrate that hookah smoking results in measurable oxidative stress and inflammation (Figure 1). LDH levels increase in response to aberrant mitochondrial ROS production, making it an ideal indicator of oxidative stress. Importantly, LDHA is often upregulated in oncogenic environments and cancer cells serving a protective role by reducing ROS, subsequent DNA damage, and apoptosis. This encourages oncogenic pathway persistence, cancer cell growth and proliferation [6,7].

Oxidative stress initiates a subsequent inflammatory response also measurable in the EBC samples from hookah and cigarette smokers. Hookah and cigarette smoking increase the expression of inflammatory

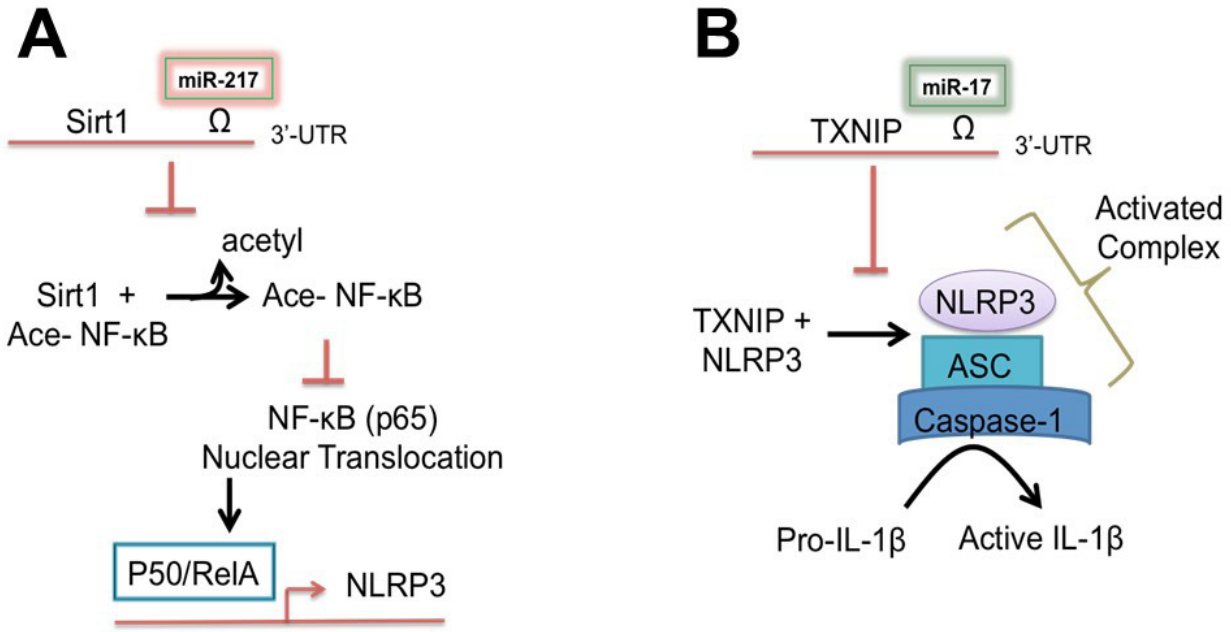


**Figure 3:** A schematic representation of miR-217 and miR-17 in the regulation of inflammasome components in response to hookah smoking.

A) *miR-217* binds to Sirt1 3'UTR to repress translation stopping Sirt1 deacetylation of NF-κB (p65, P50/RelA) allowing it to dissociate and translocate to the nucleus to increase NLRP3 transcription. B) *miR-17* translationally represses thioredoxin-interacting protein (TXNIP) stopping NLPR3 complex formation with ASC and caspase-1, and cleavage of pro- IL-1β to active IL-1β.

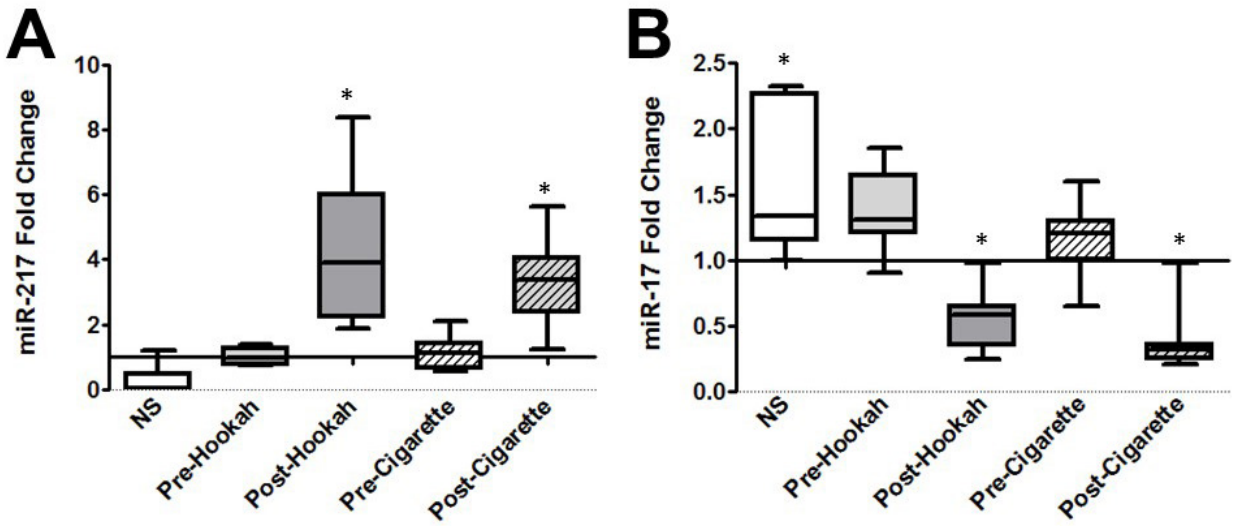


**Figure 4:** Hookah regulates mir-217 and miR-17 expression in EBC.

Real-time PCR analysis of A) miRNA-217 and B) miR-17 (as RNA copy number/ml of ECB) in non-smoking college subjects, pre- hookah college smokers, post- hookah college smokers (after 2 hours); pre-cigarette college smokers, and post- cigarette college smokers (completed one cigarette). N=24, *P <0.5

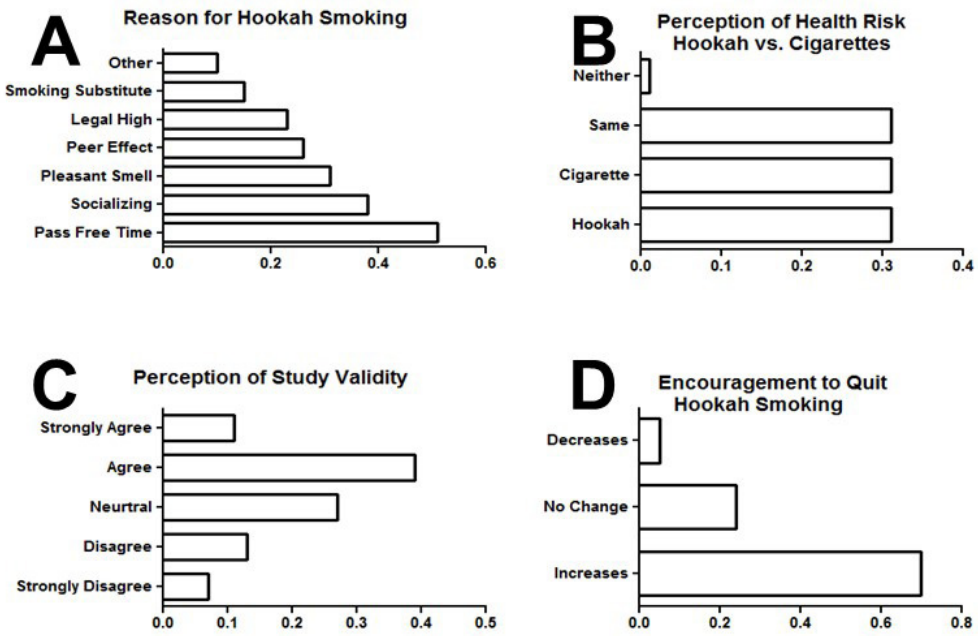


**Figure 5:** Survey results.

A) Displays the respondent hookah smokers' rational for smoking: 51% to pass free time; 39% for socializing; 31% for pleasant smell of hookah smoke; 26% due to peer effect (peer-pressure); 24% for legal "high"; 15% as a safer alternative to/and in order to quit cigarette smoking; and 10% reported other reasons which included stress relief, mood changes, and possible addiction. B) Illustrates the subject perception of health risk associated with cigarettes vs. hookah smoking: 35% reported same; 31% hookah more dangerous; 31% cigarette smoking more dangerous; and 1% reported that neither has any health risks. C) Represents the responses on perception of validity of EBC studies that linked hookah risk: 39% agreed; 27% neither agree nor disagree; 13% disagreed; 11% strongly agree; and 7% strongly disagree. D) Reflects reported encouragement to quit hookah smoking upon learning of EBC sample data: 70% were more encouraged; 24% were unchanged; and 5% less encouraged.

mediators upstream (TNF-α) and downstream (IL-1β) of the inflammasome (Figure 1B and C). Further, differential expression of *miR-217* and *miR-17* during smoking provides further inference for inflammasome activation. Specifically, smoking increases *miR-217* (Figure 4A), which plays a pro-inflammatory role by repressing the anti-inflammatory deacetylase, Sirt1 (Figure 3A). Impaired Sirt1 expression allows the TNF-α/NF-κB pathway to transactivate the NLRP3 inflammasome. On the other hand, smoking decreases *miR-17* expression, which serves an opposing role to *miR-217* through inhibition of NLRP3 activating partner, TXNIP.

Here we demonstrate quantitatively that hookah smoking poses a public health risk by activating oxidative and inflammatory pathways that are reported to be involved in oncogenesis. We also demonstrate through survey responses that public health policy needs to be addressed, through regulation or awareness, the health risk associated with hookah smoking. Public perceptions can be influenced when presented with compelling evidence of risks associated with certain lifestyle behaviors. As stated in a 2009 publication from the American Journal of Public Health; *"It has long been known that public health policy, in the form of laws, regulations, and guidelines, has a profound effect on public health status".* The three most important components to consider when attempting to develop new public health policies are; process, content, and outcome [24]. Process refers to how data is collected and incorporated into public policy. Content refers to elements of that data are most likely to be effective. Outcome is determined by the overall impact

the policy has on public health status [24]. This leads to the significance of our study. Data was obtained directly from the populations experiencing the greatest risk from hookah smoking. Not only did the data reveal significant risk but also perceptions related to current social hookah smoking (reasons and risks), which can lead to further study aimed at creating effective public health policy and education.

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## Competing Interests

The authors declare that they have no competing interests.

## Author's Contributions

Arwa Alnashwan and Ali Hakamy contributed equally to the manuscript. They conceived and designed the study, obtained EBC samples, performed molecular assays and bioinformatics analysis, drafted the manuscript and performed statistical analysis. Brendan Gongol performed molecular assays, participated in experimental design, and coordinated with the draft development. Abdullah Alismail participated in the survey administration and the manuscript writing. Michael Horn participated in the manuscript draft. Traci Marin participated in the design, coordination and final draft of the manuscript.

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