

# Exosomal microRNAs are novel circulating biomarkers in cigarette, waterpipe smokers, E-cigarette users and dual smokers

**Kameshwar P. Singh**

University of Rochester Medical Center

**Krishna P. Maremanda**

University of Rochester Medical Center

**Dongmei Li**

University of Rochester Medical Center

**Irfan Rahman** (✉ [irfan\\_rahman@urmc.rochester.edu](mailto:irfan_rahman@urmc.rochester.edu))

University of Rochester <https://orcid.org/0000-0003-2274-2454>

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## Research article

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# Abstract

## Background

Electronic cigarettes (e-cigs) produce aerosolized substances by heating a liquid, which contains large number of chemicals. The aerosol generated by E-cig may produce serious health effects. Cigarette smoke exposure may causes various diseases including COPD, atherosclerosis, and lung cancer. Waterpipe tobacco smoking also causes various acute and chronic health effects including cardiopulmonary diseases. MicroRNAs are present in higher concentration in exosomes that play a major role in various normal physiological functions and diseases. We hypothesized that the non-coding RNAs transcript may serve as susceptibility to disease biomarkers by smoking and vaping.

## Results

Our data show the enrichment of various non-coding RNAs that include microRNAs, tRNAs, piRNAs, snoRNAs, snRNAs, Mt-tRNAs, and other biotypes in exosomes. The detailed differential expression analysis of microRNAs, tRNAs and piRNA showed significant changes between pairwise comparisons of different groups. The common changes in differential expression of 8 microRNAs that are hsa-let-7a-5p, hsa-miR-21-5p, hsa-miR-29b-3p, hsa-let-7f-5p, hsa-miR-143-3p, hsa-miR-30a-5p, hsa-let-7i-5p, and hsa-let-7g-5p were found when compared with all smoking and vaping groups with non-smoking group. The e-cig group has differentially expressed 7 microRNAs (hsa-miR-224-5p, hsa-let-7c-5p, hsa-miR-193b-3p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-500b-3p, hsa-miR-365a-3p|hsa-miR-365b-3p) that is specific for this group, not expressed in other three groups. Gene set enrichment analysis of microRNA showed significant changes in the top six enriched functions that consisted of biological pathway, biological process, molecular function, cellular component, site of expression and transcription factor in all groups. Further, the pairwise comparison of tRNAs and piRNA in all groups also revealed significant changes in differential expression.

## Conclusions

Plasma exosomes of cigarette smokers, waterpipe smokers, e-cig users and dual smokers have common differential expression of microRNAs (hsa-let-7a-5p, hsa-miR-21-5p, hsa-miR-29b-3p, hsa-let-7f-5p, hsa-miR-143-3p, hsa-miR-30a-5p, hsa-let-7i-5p, and hsa-let-7g-5p), may be biomarker for tobacco exposure. Additionally, the e-cig users have also differential expressed microRNAs (hsa-miR-224-5p, hsa-let-7c-5p, hsa-miR-193b-3p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-500b-3p, and hsa-miR-365a-3p|hsa-miR-365b-3p) that is specific for this group. This study will help to better understand molecular mechanisms of plasma exosome non-coding RNAs and in developing biomarkers that may be useful in diagnosis and therapy of pulmonary injury and disease by smoking and vaping.

## Background

Electronic cigarettes (E-cigs) are battery operated various kind of device, also known as Electronic nicotine delivery system. These devices produce aerosolized substances by heating a liquid, which generates a large number of chemicals due to the presence of propylene glycol, vegetable glycerin, nicotine in various concentrations, flavoring agents and other additive compounds (1). Though the E-cig aerosol contains less number of toxic chemicals than cigarette smoke, the aerosol generated by E-cig may produce serious health effects. The E-cig aerosol contains ultra-fine particles, heavy metals, volatile organic compounds and numerous toxic chemicals including acetaldehyde, acrolein, toluene, and formaldehyde in lower concentration than cigarette smoke (2, 3). Recently, E-cig users are also using this device to deliver some other harmful substances, such as tetrahydrocannabinol, cannabidiol, and butane hash oil (4). There are severe pulmonary illnesses have been reported in users who used nicotine or cannabis extract in E-cig (5–9). E-cig users develop changes in lung function which manifest peripheral obstructive airway involvement (10). E-cig use can produce oxidative stress and endothelial cell dysfunction (11, 12), and compromised innate immune response in lungs (13). E-cig users have increased levels of biomarkers of inflammation and oxidative stress, reduced pro-resolving anti-inflammatory mediators, and endothelial dysfunction (14). Cigarette smoke exposure may causes various diseases including atherosclerosis, chronic obstructive pulmonary disease (COPD) and lung cancer (15–19). Waterpipe tobacco smoking also causes various acute and chronic health effects including cardiovascular disease, chronic bronchitis and cancer (20–22). Similarly, other study has shown that waterpipe smokers may develop COPD after chronic exposure (23). Waterpipe and cigarette smoke exposure described as risk factor for periodontal and cardiopulmonary disease (24, 25).

Exosomes are extracellular vesicles membrane bound particles of 30–150 nm. These are lipid vesicles carrying various types of biological materials such as proteins, RNAs, DNAs, transcription factors, receptors, and lipids. MicroRNAs are present in higher concentration in whole RNAs (26). Exosomes are also present in various body fluids and play a role in various normal physiological functions and diseases (27). The exosomes vesicle deliver molecules to the adjacent cells and via systemic circulation to distant cell population. MicroRNAs are non-coding molecules of 19–23 nucleotides (nt), play major role in genes regulation associated with various biological pathways (28–35). MicroRNAs recognized as molecular markers for different diseases (36–38).

There are studies related to plasma-derived exosomal microRNA in smokers has been reported in the past. Similar is the case with study related to cigarette induced chronic diseases. Cigarette smoke induced release of exosomes which may be involved in the progression of chronic diseases (39–41). Also, the role of exosomes release by cigarette smoke extract have been implicated in producing changes in cellular pathophysiological functions such as endothelial cell function, angiogenesis and inflammatory conditions (40, 42, 43). Dysregulation of microRNAs have been reported in various biological samples of COPD cases including tissues and body fluids (44–46) and plasma exosomes microRNA in cigarette smokers and non-small lung cell cancer patients (47).

Transfer RNAs (transfer ribonucleic acid, tRNAs) are small molecule have multiple role in cellular functions including protein synthesis. The tRNAs are cleaved to fragments which

perform several biological functions. The tRNAs consists of two main types; tRNA-derived fragments (tRFs) and tRNA halves. The tRNA-derived fragments are 14–30 nucleotide (nt) long consists of three subtypes: tRF–5, tRF–3, and tRF–1. The tRFs are 31–40 nt long and generated by cleavage of mature tRNAs anti-codon loop. The tRNAs plays a significant role in in various cellular functions such as cell signaling, proliferation, differentiation, apoptosis and metabolism and in regulation of gene expression at transcription and translation level. Additionally, they have been reported to involve in DNA damage response, viral infection, neurodegeneration and cancer (48–52). PIWI-interacting RNA (piRNA) are 24–31 nt long and form RNA-induced silencing complex with PIWI family proteins and plays a role in stem cell cell division, apoptosis, epigenetic control of transposons, telomeres and translational control. The piRNA serve as gene expression regulators by inducing histone modification and DNA methylation. Additionally, changes in piRNA observed in several types of cancer (53–56). Given the role of tRNAs and piRNA in several important biological functions and diseases, it was thought to determine whether there is any change in plasma exosomes from E-cig users, waterpipe, dual smokers. This is the first study to report the detailed analysis of tRNA and piRNA in plasma exosomes from E-cig users, waterpipe, and dual smokers by small RNA sequencing.

Cigarette smoke exposure promotes release of exosomes and chronic exposure causes chronic obstructive pulmonary disease. To the best of our knowledge, there is no study has been done to identify and characterize the plasma-derived exosomal microRNAs in E-cig users, waterpipe smokers, dual smokers and compared with cigarette smokers. Understanding the changes in plasma-derived exosomes and their microRNA profile will help in developing biomarkers during disease progression in smokers. This study will also be useful in unraveling the molecular mechanisms of disease progression in smokers. Additionally, this study will support in developing novel therapeutic strategies for smoking related diseases in future. Therefore, we did comprehensive analysis of exosomal microRNA expression profile and their biological functions in plasma samples from normal/non-smokers (NS), E-cig users (E-cig), cigarette smokers (SM), waterpipe (WP), and dual smokers (DS) smokers groups.

## Results

### Isolation and characterization of plasma-derived Exosomal vesicles

Plasma EVs were characterized for their size and morphology using TEM. Particles are mostly in exosomal range nonetheless large sized vesicle populations were also observed. Further, nanoparticle tracking analysis did not show any significant difference either in the size range or concentrations across all the groups observed (Fig. 1 B-C). Immunoblot analysis showed the presence of exosomal markers CD63 and CD81 and negative for calnexin and endoplasmic reticulum contamination.

### Input read alignment and small RNA biotype mapping

The reads obtained from sequencing were used for alignment and mapping to the human genome after clipping and quality filtering. The input reads in plasma exosomes were between 5.58 million reads to 21.75 million reads between NS ( $14031359 \pm 2019014$ ), CS ( $10688617 \pm 917325$ ), WP ( $8765589 \pm 768710$ ), E-cig users ( $9570944 \pm 455585$ ) and DS ( $8988900 \pm 798445$ ). Reads were mapped to human rRNA to exclude rRNA sequences before mapping to human genome. The percentage of input reads alignment from each subject in individual group presented in Fig. 2. The input reads were significantly lower in WP and DS in comparison to NS subjects ( $P < 0.05$ ).

The reads mapped to human genome and classified to the various small RNA biotypes. After mapping and excluding rRNA, the microRNAs mapped along with all other RNA transcripts in GENCODE. The microRNA was between 78–81% of all biotype counts in all NS, CS, WP, E-cig and DS groups. There was significant lower counts of Mt-tRNA observed in CS, WP, E-cig and DS ( $P < 0.05$ ) in comparison to NS. Further, snoRNA counts were also significantly ( $P < 0.05$ ) lower in all groups in comparison to NS (Fig. 3 A, B).

## Comparison of microRNAs expression profiles between non-smokers, E-cig users, cigarette smokers, waterpipe smokers and dual smokers

### Principal Component Analysis (PCA) plot

The PCA plots were generated using the microRNAs that exhibited largest variation across all the samples from subjects of the NS, CS, WP, E-cig and DS groups. Majority of the NS samples cluster separately whereas all the smokers (CS, WP and DS) and E-cig users did not cluster together suggesting these effects may be due to common tobacco use (Fig. 4).

### Volcano plot

The differentially expressed microRNA from NS vs. CS, NS vs. WP, NS vs. E-cig users, NS vs. DS (Fig. 5 A-D) and CS vs. E-cig, CS vs. WP, CS vs. DS, WP vs. DS (Suppl. Fig. 1 A-D) are presented as volcano plot. The volcano plots are made by plotting the  $-\log_{10}$  of adjusted p-values on the y-axis, and the  $\log_2$  fold change between two groups on the x-axis showing the up- and down regulation appearance similar distance from the center. The microRNA values plotted show two regions with highest magnitude of fold change and high statistical significance. The highlighted spots (red color) with microRNA names are with greatest difference (at least two fold changes) in expression and statistically significant (adjusted  $P < 0.05$ ) after correction for multiple testings.

### Hierarchical Clustering of microRNAs

The hierarchical cluster analyses of differentially expressed microRNAs were done in all different groups. The heat maps were generated using normalized values from individual sample from NS vs. CS, NS vs. WP, NS vs. E-cig users, NS vs. DS (Fig. 6 A-D) and CS vs. E-cig, CS vs. WP, CS vs. DS, WP vs. DS (Suppl. Fig. 2 A-D). In the heatmap, each row represent individual microRNA and each column individual sample. The microRNA clustering on the left indicates hierarchical clustering of significant microRNA.

The color scale at the right side in panel A indicates the relative expression level of microRNA in all samples. The red color indicates lower level than the mean and green a level higher than the mean.

## **Differential expressed microRNAs in E-cig versus non-smokers group**

The microRNAs that were most differentially expressed in plasma exosomes of E-cig users in comparison to NS are presented in Suppl. Table 1. There was upregulation of 17 microRNAs in E-cig users which were as follows: hsa-miR-365a-3p | hsa-miR-365b-3p, hsa-let-7f-5p, hsa-miR-1299, hsa-miR-21-5p, hsa-let-7i-5p, has-let-7a-5p, hsa-miR-30a-5p, hsa-miR-193b-3p, hsa-miR-100-5p, hsa-miR-423-3p, hsa-miR-30c-5p, hsa-miR-143-3p, hsa-miR-224-5p, hsa-let-7g-5p, hsa-miR-125b-5p, hsa-let-7c-5p and hsa-miR-500b-3p. The downregulated expression of 5 microRNAs in E-cig users were as hsa-miR-362-5p, hsa-miR-29b-3p, hsa-miR-451a, hsa-miR-30e-5p, and hsa-miR-10b-5p.

## **Differential expressed microRNAs in cigarette smokers versus non-smokers group**

The significant differential expressed microRNAs between CS versus NS are presented in Suppl. Table 2. The total 26 microRNAs changed significantly of which 16 upregulated and 10 downregulated. The maximum fold change in upregulated microRNAs are hsa-mir-149-5P (20.29 fold), hsa-miR-532-5p (19.79), hsa-miR-2355-5p (19.65) and downregulated are hsa-miR-29b-3p (-23.57), hsa-miR-150-5p (-3.58), hsa-miR-29a-3p (-2.58).

## **Differential expressed microRNAs in waterpipe smokers versus non-smoker group**

The total 15 differentially expressed microRNA in WP versus NS are shown in Suppl. Table 3. The maximum fold change observed in upregulated microRNAs are hsa-miR-2355-5p (39.81 fold), hsa-miR-582-5p (23.15), hsa-miR-1299 (19.61) and downregulated are hsa-miR-362-5p (-45.35), hsa-miR-29b-3p (-24.62) and hsa-miR-320d.

## Differential expressed microRNAs in dual smokers versus non-smokers

The DS vs NS have shown total 23 differential expressed microRNAs that are shown in Suppl. Table 4. The top fold change upregulated microRNAs are hsa-miR-149-5p (29.29 fold), hsa-miR-139-5p (16.41), hsa-miR-424-3p (16.01) and downregulated microRNAs are hsa-miR-362-5p (-44.11 fold), hsa-miR-29b-3p (-21.54), hsa-miR-144-3p (-2.36).

## Differential expressed microRNAs in E-cig users versus cigarette smokers

Total 11 microRNAs changed significantly in E-cig users versus CS of which 7 were upregulated and 4 downregulated (Supplementary Table 5). Among the top fold changed microRNAs, upregulated are hsa-miR-362-5p (19.67 fold), hsa-miR-2355-5p (19.62), hsa-miR-532-5p (19.41) and downregulated microRNAs are hsa-miR-365a-3p (-24.12), hsa-miR-1299 (-24.01), hsa-miR-193b-3p (-7.85).

## Differential expressed microRNAs in waterpipe smokers versus cigarette smokers

Differentially expressed microRNA in WP versus CS presented in Supplementary Table 6. Out of six microRNAs differentially expressed, the upregulated are hsa-miR-532-5p (21.30 fold), hsa-miR-362-5p (20.47), hsa-miR-144-5p (19.42) and downregulated are hsa-miR-1299 (-23.53), hsa-miR-582-5p (-22.63), hsa-miR-1-3p (-6.72).

## Differential expressed microRNAs in dual smokers versus cigarette smokers

The significantly differential expressed 5 microRNAs of DS versus CS are shown in Supplementary Table 7. The downregulated microRNAs with fold change are hsa-miR-144-5p (-21.40 fold), hsa-miR-532-5p (-20.74), hsa-miR-2355-5p (-19.26), hsa-miR-362-5p (-19.22) and upregulated is hsa-miR-424-3p (20.26).

## Differential expressed microRNAs in waterpipe smokers versus dual smokers

The 4 microRNAs differentially downregulated are presented with fold changes [hsa-miR-2355-5p (-39.43), hsa-miR-1299 (-21.29), hsa-miR-582-5p (-21.25), hsa-miR-1-3p (-7.29) and upregulated

microRNAs are hsa-miR-139-5p (22.07), hsa-miR-424-3p (21.71) (Supplementary Table 8).

## Overlap of microRNA expression in plasma exosomes

The overlap of microRNAs expression between all four groups are presented as Venn diagram in Fig. 7 A-C. We have compared microRNAs expressed in all four groups: NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. These groups have common expression of 8 microRNAs that are hsa-let-7a-5p, hsa-miR-21-5p, hsa-miR-29b-3p, hsa-let-7f-5p, hsa-miR-143-3p, hsa-miR-30a-5p, hsa-let-7i-5p, and hsa-let-7g-5p. The E-cig group has expressed 7 microRNAs (hsa-miR-224-5p, hsa-let-7c-5p, hsa-miR-193b-3p, hsa-miR-30e-5p, hsa-miR-423-3p, hsa-miR-500b-3p, hsa-miR-365a-3p|hsa-miR-365b-3p) that is specific for this group, not expressed in other three groups (Fig. 7 A). The comparison of up-regulated microRNAs in all four groups revealed expression 7 microRNA (hsa-let-7a-5p, hsa-miR-21-5p, hsa-let-7i-5p, hsa-let-7f-5p, hsa-miR-143-3p, hsa-miR-30a-5p, hsa-let-7g-5p) common to all groups. However, there are 6 microRNA (hsa-miR-224-5p, hsa-miR-423-3p, hsa-miR-500b-3p, hsa-let-7c-5p, hsa-miR-365a-3p|hsa-miR-365b-3p, hsa-miR-193b-3p) expressed specifically to E-cig group (Fig. 7 B). When these groups compared for down-regulated microRNAs, the only microRNA has-mir-29b-3p was common in all. The microRNA expressed specifically in E-cig was hsa-mir-30e-5p (Fig. 7 C).

## Gene enrichment analysis of differentially expressed microRNAs

The FunRich enrichment analysis of differentially expressed microRNAs performed to explore the potential target genes in NS vs. E-cig, NS vs. CS, NS vs. WP and NS vs DS pairwise comparisons (Fig. 8 A-F). The top six enriched functions with the lowest p values were biological pathway, biological process, molecular function, cellular component, site of expression and transcription factor in all groups. The top 3 biological pathway with the lowest p values were beta1 integrin cell surface interactions, integrin family cell surface interactions, TRAIL signaling pathway common in NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. The proteoglycan-mediated signaling events changed significantly in all three groups except NS vs. E-cig. In addition, endothelin biological pathway with lowest p values were in NS vs. E-cig and NS vs. DS.

The biological process in regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism with lowest p value were present in all four groups. The two molecular functions with highly significant values related to transcription factor activity and extracellular matrix structural constituent were common in all four groups. The top two cellular component related to nucleus and cytoplasm were common in all four groups. The three site of expression of microRNAs with lowest p values were kidney, placenta and skeletal muscle. However, the other site of expression related to lung with significant p values were in NS vs. CS, NS vs. E-cig and NS vs. DS. The transcription factor related EGR1, SP1, SP4 and POU2F1 were highly significant in all four groups while ZFP161 in only NS vs. E-cig and NS vs. DS. Further, pairwise

comparisons were done in between E-cig vs. CS, WP vs. CS, DS vs. CS and WP vs. DS groups are presented in Supplementary Figure 3 A-F.

## Target genes of plasma exosomal microRNA changes

Target genes of plasma exosomal microRNA changes observed in NS vs. E-cig, NS vs. CS, NS vs. WP, NS vs DS and CS vs. E-cig, CS vs. WP, CS vs. DS, WP vs. DS are presented in Supplementary Table 9.

## Differential tRNA fragments identified in plasma exosomes

The changes in tRNAs were calculated based on the trimmed mean of M values (TMM) method using normalized tRNA counts in all NS, CS, WP, E-cig and DS groups. The read counts normalized and subjected to differential expression analysis by DESeq2, to get the change in tRNA expression between different groups by pairwise comparisons. The read raw count data analysis showed 25 different type of tRNAs in plasma exosomes of all groups NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. The pairwise comparison data revealed significant changes in 7 tRNAs in all NS vs. CS, NS vs. WP and NS vs. E-cig groups. However, NS vs. DS group showed changes in eight tRNAs. All four groups have significant increase in six tRNAs (tRNA<sup>Val</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Asp</sup>, tRNA<sup>Gly</sup>, tRNA<sup>Arg</sup> and tRNA<sup>His</sup>) and decrease in tRNA<sup>Cys</sup>. In addition, NS vs. DS group also showed significant increase in tRNA<sup>Ile</sup> (Supplementary Table 10–13). There was no significant change observed in tRNAs counts (total 24 raw counts in each) in all CS vs. DS, CS vs. WP, CS vs. E-cig and WP vs. DS.

The PCA plots were generated using the tRNAs that exhibited largest variation across all the samples from subjects of the NS, CS, WP, E-cig and DU groups. Majority of the samples from all the groups (NS, CS, WP, DS and E-cig) did not cluster together (Suppl. Fig. 4).

The differentially expressed tRNAs from NS vs. CS, NS vs. WP, NS vs. E-cig users, and NS vs. DS (Suppl. Fig. 5 A-D) are presented as volcano plot. The p-values on the y-axis, and the fold change between two groups on the x-axis are plotted showing the up- and down regulation appearance similar distance from the center. The tRNAs values plotted show two regions with highest magnitude of fold change and high statistical significance. The highlighted spots (red color) with tRNAs names are with greatest difference in expression and statistically significant ( $P < 0.05$ ) after correction for multiple measurement.

The hierarchical cluster analyses of differentially expressed tRNAs were done in all different groups. The heat maps were generated using normalized values from individual sample from NS vs. CS, NS vs. WP, NS vs. E-cig users, NS vs. DS (Fig. 6 A-D). In the heat map, each row represent individual tRNA and each column individual sample. The tRNA clustering on the left indicates hierarchical clustering of significant tRNA. The color scale at the right side in indicates the relative expression level of tRNA in all samples. The red color indicates lower level than the mean and green a level higher than the mean.

The overlap of tRNAs expression between all four groups are presented as Venn diagram in Supp. Fig. 7. We have compared tRNAs expressed in all four groups: NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. All the four groups have common changes in 7 tRNAs (tRNA<sup>Val</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Asp</sup>, tRNA<sup>Gly</sup>, tRNA<sup>Arg</sup> and tRNA<sup>His</sup>, and tRNA<sup>Cys</sup>). However, change in tRNA<sup>Ile</sup> was only observed in NS vs. DS.

## Differential expression of piRNA in plasma exosomes

Read counts of piRNAs from NS, CS, WP, E-cig, and DS groups were TMM-normalized and normalized counts were used to generate a PCA plot (Supp. Fig. 8). There was no close clustering observed between individual samples of these groups. Normalized counts were also processed for differential expression analysis by DESeq2 to get the data of different group pairwise comparisons.

The hierarchical cluster analyses of differentially expressed piRNAs were done in all different groups. The heat maps were generated using normalized values from individual sample from NS vs. CS, NS vs. WP, NS vs. E-cig users, NS vs. DS (Supp. Fig. 9 A-D). In the heat map, each row represent individual piRNA and each column individual sample. The piRNA clustering on the left indicates hierarchical clustering of significant piRNA. The color scale at the right side indicates the relative expression level of piRNA in all samples. The red color indicates lower level than the mean and green a level higher than the mean.

Our result exhibit significant changes in piRNA of NS vs. CS (piR-004153, piR-019825), NS vs. WP (piR-004153, piR-019825, piR-000552, piR-014620, and piR-020450), NS vs. E-cig (piR-016658, piR-016659, piR-019825, piR-000552, and piR-017591), and NS vs. DS (piR-020365, piR-000552, and piR-017591). Further, pairwise comparison of piRNA expression in CS vs. WP (piR-000552 and piR-020450), CS vs. E-cig (piR-000552), CS vs. DS (piR-000552), and WP vs. DS (piR-019825, piR-014620, and piR-020450) also showed significant change (Suppl. Table 14). The overlap of piRNAs expression between all four groups are shown as Venn diagram in Supp. Fig. 10. The piRNAs expressed in all four groups were compared: NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. There was no common change of piRNA expression observed in all four groups.

## Discussion

Exosomes are secreted into biological fluids, and communicate with cells by transferring exosomal content including microRNAs to the recipient cells and mediate several cellular and biological processes (57–59). It plays a role in maintaining lung homeostasis. In addition, exosome releases microRNAs during airways lung injury that help in progression of lung diseases (59). microRNA regulate gene expression posttranscriptionally by suppressing translation or through mRNA degradation (60)

In this study, we have investigated small RNA profile of plasma samples of NS, E-cig users, WP and DS using high-throughput sequencing. We have presented comprehensive microRNAs profile of plasma exosomes in all four groups. Our results showed that some microRNAs are common in all tobacco users. Interestingly, we also found group specific microRNAs in different group.

To perform comprehensive analysis of small RNA species, we analyzed our data for several key abundant RNA biotypes. The input reads were lower in all groups, but the significant lower in WP and DS. Several biotypes of known biological importance were identified, such as piRNA, tRNA, snoRNA, snRNA, lincRNA, Mt\_tRNA, Mt\_rRNA and microRNAs. The microRNA was most diverse RNA biotype found in comparison to all biotypes in samples from all groups. Biotypes Mt\_tRNA and snoRNA were significantly lower in all groups in comparison to NS, suggesting nicotine-specific effect. Recent studies of small RNA sequence analysis of exosomes in various body fluid from healthy donors, smokers and COPD patients reported showing changes in relative distribution of biotypes such as microRNA, tRNA, Mt\_tRNA, and snoRNA. (57, 61). We have performed further analysis of tRNA and piRNA in all NS, CS, E-cig, WP and DS groups, showing a differential regulation of these transcripts.

There are several studies related to expression of coding and non-coding RNA in CS exposed cellular model and COPD, have been reported in the past (62–64). Further, reports of dysregulation of microRNAs in the airway epithelial cells, systemic circulation and lung tissues from COPD condition (65–68). The studies related to transcriptome profiling of gene expression in E-cigarette vapor and CS exposed human bronchial epithelial cells and nasal epithelial cells from E-cig users have been published (69, 70). Recently, microRNA expression profiling of E-cig vapor exposed human airway epithelial cells has been reported (71). The waterpipe smokers have differential gene expression in small airway epithelial cells and alveolar macrophages in comparison to non-smokers (72). Further, waterpipe smoker's small airway epithelial cells have epigenetic related changes in gene expression (73). However, the studies related to plasma exosomal microRNA in E-cig users and WP are still unknown.

In plasma exosomes of E-cig users, we identified twenty-two microRNA dysregulated significantly, of which seventeen were upregulated and five microRNA were downregulated in comparison to non-users (Suppl. Table 1). The upregulation of microRNAs in E-cig users were as follows: hsa-miR-365a-3p | hsa-miR-365b-3p, hsa-let-7f-5p, hsa-miR-1299, hsa-miR-21-5p, hsa-let-7i-5p, has-let-7a-5p, hsa-miR-30a-5p, hsa-miR-193b-3p, hsa-miR-100-5p, hsa-miR-423-3p, hsa-miR-30c-5p, hsa-miR-143-3p, hsa-miR-224-5p, hsa-let-7g-5p, hsa-miR-125b-5p, hsa-let-7c-5p and hsa-miR-500b-3p. The two-fold change three upregulated microRNAs (has-miR-365a-3p/has-miR-365b-3p, hsa-miR-1299 and has-miR-193b-3p) are associated with tumor growth and metastasis, rheumatic heart disease and cancer (74–76). The downregulated expression of microRNAs in E-cig users: hsa-miR-362-5p, hsa-miR-29b-3p, hsa-miR-451a, which play role in neuroblastoma, human natural killer cell function, non-small cell lung cancer metastasis, epithelial-mesenchymal transition and metastasis of bladder cancer, lung adenocarcinoma, suppression of cell migration and invasion in non-small cell lung cancer NSCLC) (77–81). Further, expression of hsa-miR-30e-5p and hsa-miR-10b-5p downregulated in NSCLC tissues in comparison to non-cancerous adjacent tissues (82).

The total 15 differentially expressed microRNA in WP versus NS presented in Suppl. Table 3. The three top upregulated microRNAs are hsa-miR-2355-5p, hsa-miR-582-5p, hsa-miR-1299 and downregulated are hsa-miR-362-5p, hsa-miR-29b-3p and hsa-miR-320d. The microRNA hsa-miR-2355-5p is upregulated in endothelial colony-forming cell, the compromise function of this cell may lead to onset of

cardiovascular disorder (83). The hsa-miR-582-5p is upregulated in monocytes of patient with active tuberculosis and inhibits monocyte apoptosis (84). Third upregulated hsa-miR-1299 suggested as diagnostic marker for rheumatic heart disease (85). The two downregulated has-miR-362-5p and has-miR-29b-3p are also downregulated similarly in E-cig users. The downregulated hsa-miR-320b suggested predicting reduced survival of COPD patients and hsa-miR-320d as biomarker for aortic dissection (86, 87).

We examined the differential expression of microRNA in cigarette smokers in comparison to non-smokers. The maximum number of total 26 microRNAs were differentially expressed in cigarette smokers, of which 16 upregulated and 10 downregulated (Suppl. Table 2). The top three upregulated microRNAs are hsa-miR-149-5p, hsa-miR-532-5p, hsa-miR-2355-5p and down regulated hsa-miR-29b-3p, hsa-miR-150.5p, hsa-miR-29a-3p. The upregulated hsa-miR-2355-5p is also upregulated in waterpipe smokers and hsa-miR-29b-3p downregulated similarly in E-cig users and waterpipe smokers. The microRNA has-miR-532-5p overexpression increases the colony formation and migration of gastric cancer cells (88). The miR-149-5p is upregulated in the HCC827/GR-8-1 cells and involved in the acquired gefitinib resistance (89). The downregulation microRNA hsa-miR-29a-3p and hsa-miR-150-5p in plasma has suggested circulatory biomarkers for NSCLC radiation therapy and hsa-miR-29a-3p associated with fibrosis in human heart, lung and kidney (90).

The dual smokers differentially expressed total 23 microRNA of which 14 upregulated and 3 downregulated (Suppl. Table 4). The top three upregulated based on fold change were hsa-miR-149-5p, hsa-miR-424-3p, hsa-miR139-5p and downregulated were hsa-miR-362-5p, hsa-miR-29b-3p, hsa-miR-144-3p. The hsa-miR-362-5p downregulated in WP and hsa-miR-29b-3p downregulated in both WP and E-cig users. The upregulated hsa-miR149-5p is also upregulated similarly in cigarette smokers. Downregulated has-miR-144-3p associated with progression of lung adenocarcinoma (91). However, upregulation of has-miR-139-5p suggested as a serum biomarker for recurrence and metastasis of colorectal cancer (92) and has-miR-424-3p related to resistance to chemotherapy (93).

To our surprise, when we analyzed the overlap of microRNAs in all four groups, there was 7 upregulated (hsa-let-7a-5p, hsa-miR-21-5p, hsa-let-7i-5p, hsa-let-7f-5p, hsa-miR-143-3p, hsa-miR-30a-5p, hsa-let-7g-5p) common to all groups. The microRNAs hsa-let-7a-5p and hsa-let-7f-5p are involved in NSCLC and typical and atypical carcinoid tumors of the lung respectively (94, 95). Circulating plasma microRNA hsa-let-7g-5p suggested as one of the biomarkers for Alzheimer's disease (96). The hsa-miR-21 play a role in proliferation of various cells and tumors (97-99). Further, exosomal microRNA-21 derived from bronchial epithelial cells play a role in myofibroblast differentiation in COPD induced by cigarette smoking (100). Exosomes microRNA profile of human gastric cancer revealed upregulation of has-miR-30a-5p and hsa-miR-143-3p is associated with hepatic fibrosis in humans infected with *Schistosoma japonicum*. Further, when groups compared for down-regulated microRNAs, the only microRNA has-miR-29b-3p was common in all (Fig. 7 C) which is involved in NSCLC metastasis and regulate epithelial-mesenchymal transition and metastasis of bladder cancer (79, 80). As changes in these microRNAs expression are common in all group, suggesting may be tobacco specific effect. There

are six-upregulated microRNA (hsa-miR-224-5p, hsa-miR-423-3p, hsa-miR-500b-3p, hsa-let-7c-5p, hsa-miR-365a-3p|hsa-miR-365b-3p, and hsa-miR-193b-3p) and downregulated hsa-miR-30p expressed specifically to E-cig group (Fig. 7 B, C). These microRNAs may be specific biomarkers for E-cig users.

Next, we did FunRich enrichment analysis of differentially expressed microRNAs to explore the potential target genes in NS vs. E-cig, NS vs. CS, NS vs. WP and NS vs DS pairwise comparisons. The top six enriched functions with the lowest p values were biological pathway, biological process, molecular function, cellular component, site of expression and transcription factor in all groups. The top 3 biological pathway with the lowest p values were beta1 integrin cell surface interactions, integrin family cell surface interactions, TRAIL signaling pathway common in NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. The proteoglycan-mediated signaling events changed significantly in all three groups except NS vs. E-cig. In addition, endothelin biological pathway with lowest p values were in NS vs. E-cig and NS vs. DS.

The biological process of regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism with lowest p value were in all four groups. The two molecular functions with highly significant values related to transcription factor activity and extracellular matrix structural constituent were common in all four groups. The top two cellular component related to nucleus and cytoplasm were common in all four groups. The three site of expression of microRNAs with lowest p values were kidney, placenta and skeletal muscle. However, the other site of expression related to lung with significant p values were in NS vs. CS, NS vs. E-cig and NS vs. DS. The transcription factor related EGR1, SP1, SP4 and POU2F1 were highly significant in all four groups while ZFP161 in only NS vs. E-cig and NS vs. DS. Our analysis revealed that many vital biological functions associated genes are potential target of selective plasma exosomal microRNAs in all four groups, which may be associated with pathophysiological conditions in future.

To explore the target genes of differentially expressed microRNAs in different groups, we identified 3244 (NS vs. CS), 2428 (NS vs. WP), 2223 (NS vs. E-cig users), 2887 (NS vs. DS), 538 (CS vs. WP), 784 (CS vs. E-cig users), 111 (CS vs. DS and 532 (DS vs. WP) and presented in Suppl. Table 9. The large number of genes are the target of differentially expressed microRNAs in all the groups suggesting compromised several biological functions in these groups.

Studies have shown that tRNA and piRNA various biological function and associated with several human diseases. The top six enriched functions with the lowest p values were biological pathway, biological process, molecular function, cellular component, site of expression and transcription factor in all groups. Our differential expression analysis showed 25 different type of tRNAs in plasma exosomes of all groups NS vs. CS, NS vs. WP, NS vs. E-cig and NS vs. DS. The pairwise comparison data revealed significant changes in 7 tRNAs in all NS vs. CS, NS vs. WP and NS vs. E-cig groups. However, NS vs. DS group showed changes in 8 tRNAs. All four groups have significant increase in 6 tRNAs (tRNA<sup>Val</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Asp</sup>, tRNA<sup>Gly</sup>, tRNA<sup>Arg</sup> and tRNA<sup>His</sup>) and decrease in tRNA<sup>Cys</sup>. In addition, NS vs. DS group also showed significant increase in tRNA<sup>Ile</sup> (Supplementary Table 10–13). There is no information available

regarding the tRNA expression in plasma exosomes of E-cig users, WP, and DS. A recent study from our lab have shown that five tRNAs differentially expressed (tRNA<sup>Lys</sup>, tRNA<sup>Gly</sup>, tRNA<sup>Tyr</sup>, tRNA<sup>Glu</sup> and tRNA) in non-smokers vs COPD and five tRNAs (tRNA<sup>Gly</sup>, tRNA<sup>Tyr</sup>, tRNA<sup>Leu</sup>, tRNA and tRNA<sup>Met</sup>) in smoker vs. COPD pairwise comparison among the 25 enriched tRNAs. tRNAs plays important role in tumor development and associated with the clinicopathological characteristics of lung adenocarcinoma and cancer-specific survival (101). Previous literature suggest that tRNAs involved in gene regulation, signaling dynamics and human diseases (52, 102, 103).

The significant differential expression of piRNA in plasma exosomes of NS vs. CS (piR-004153, piR-019825), NS vs. WP (piR-004153, piR-019825, piR-000552, piR-014620, and piR-020450), NS vs. E-cig (piR-016658, piR-016659, piR-019825, piR-000552, and piR-017591), and NS vs. DS (piR-020365, piR-000552, and piR-017591) by pairwise comparisons were found. Further, pairwise comparison of piRNA expression in CS vs. WP (piR-000552 and piR-020450), CS vs. E-cig (piR-000552), CS vs. DS (piR-000552), and WP vs. DS (piR-019825, piR-014620, and piR-020450) also showed significant change (Suppl. Table 14). piRNA form RNA-induced silencing complex with PIWI family proteins and plays a role in stem cell cell division, apoptosis, epigenetic control of transposons, telomeres and translational control. The piRNA serve as gene expression regulators by inducing histone modification and DNA methylation. Further, changes in piRNA observed in several types of cancer (53–56). Additionally, recent studies have suggested differentially expressed piRNA molecule as potential biomarkers of several cancers (104–107). No study done in the past to explore the differential expression of piRNAs in plasma exosomes of WP, E-cig users, and DS. In a recent publication differential expression of piRNA-004153 observed in non-smokers vs. smokers, similar to our finding in NS vs. CS (61).

We mapped input reads to human genome and classified to the various small RNA biotypes. To our surprise, significant lower counts of Mt-tRNA and snoRNA were observed in CS, WP, E-cig and DS ( $P < 0.05$ ) in comparison to NS (Fig. 3 A, B). Small nucleolar RNAs are non-coding RNAs consist of 60–300 nt long which accumulate mostly in nucleoli. snoRNAs are involved in various pathophysiological processes. The aberrant expression or mutation of snoRNA may induce cell transformation, tumorigenesis and tumor metastasis. snoRNA genes are transcribed by RNA polymerase II and synthesizes messenger RNA (mRNA). The synthesized mRNA exported to cytoplasm and translated. However, snoRNA remains in nucleus. As the snoRNAs are involved in posttranscriptional modification of ribosomal RNAs, any alteration in level of snoRNAs may affect normal functions of the cells and lead to various diseases (108–110). Mt-tRNA play a role in mitochondrial protein synthesis and oxidative phosphorylation (OXPHOS) to produce energy (ATP) and reactive oxygen species (ROS). Any alteration in the level of Mt-tRNA may have impact on mitochondrial function. The mitochondrial dysfunction has been associated with several lung diseases such as asbestos-related lung fibrosis and COPD (111, 112). The Mt-tRNA gene mutations reported in idiopathic pulmonary fibrosis, lung cancer and hypertension (113–115). We did not do detailed analysis of Mt-tRNA and snoRNA genes in our samples. In our future studies, we will do detailed analysis of Mt-tRNA, snoRNA, snRNA and other differentially expressed small RNA enriched biotypes.

## Conclusions

In conclusion, we have performed comprehensive plasma exosomes small RNA-sequence analysis that includes exosome isolation, purification, RNA extraction, library preparation, RNA sequencing, and RNA annotation in samples from NS, CS, WP, E-cig users and DS. The isolated RNAs from plasma exosomes were used for small RNA-sequencing analysis. Our data show the enrichment of various non-coding RNAs that include microRNAs, tRNAs, piRNAs, snoRNA, snRNAs, Mt-tRNAs, and other biotypes. Further, the detailed differential expression analysis of microRNAs, tRNAs and piRNA showed significant changes between pairwise comparisons of different groups. Gene set enrichment analysis showed significant changes in the top six enriched functions that were biological pathway, biological process, molecular function, cellular component, site of expression and transcription factor in all groups. The pairwise comparison of tRNAs and piRNA also revealed significant changes in differential expression in all groups. We mapped input reads to human genome and classified to the various small RNA biotypes that showed significant lower counts of Mt-tRNA and snoRNA in all groups. Our comprehensive transcriptome analysis done in this study will help to better understand molecular mechanisms of plasma exosome non-coding RNAs and in developing biomarkers that may be helpful in diagnosis and therapy of lung injury and disease mechanisms in smokers and vapers.

## Methods

### Ethic statements

The study subjects were recruited at the General Clinical Research Center of the University of Rochester. All protocols, procedures and subject recruitment described in this study were approved by the ethical Institutional Review Board (IRB)/Research Subject Review Board (RSRB) committee of the University of Rochester Medical Center, Rochester, NY. All subjects provided written informed consent before collecting samples. The selected plasma samples from non-smokers, E-cig users, waterpipe, cigarette smokers and dual smokers (Cigarette and waterpipe smokers) used in this study as described previously (14, 116).

### Collection of blood plasma

The venous blood (20–25 ml) was collected from cigarette smokers, waterpipe smokers and dual smokers (cigarette and waterpipe) in vacutainer tubes containing EDTA. Plasma was separated by centrifugation for 10–15 minutes at 2000g within 60 minutes of collection. The supernatants were collected. The plasma samples were stored immediately at  $-80^{\circ}\text{C}$  until analyzed.

### Plasma exosome purification

Plasma samples stored at  $-80^{\circ}\text{C}$  were thawed on ice for the exosomal isolation as per the manufacturer's instructions (Norgen Bioteck Corporation, Cat# 57400). Briefly, aliquoted samples were

centrifuged at 400 x g (~2000 rpm) for 2 min and one ml of clear supernatant were transferred subsequently into 15 ml tube for exosomal isolation. To this 3 ml nuclease-free water was added, followed by addition of 100 µl ExoC buffer. To this mixture 200 µl of slurry E was added. The tube was mixed well by vortexing for 10 seconds and left at room temperature for 5 minutes. The samples were mixed well by vortexing again for 10 seconds and centrifuged at 2000 rpm for 2 minutes. The supernatant was discarded and 200 µl of ExoR buffer was added to the slurry pellet in the tube and mixed well by vortexing for 10 seconds. The slurry pellet mixture was incubated at room temperature for 5 minutes. After the incubation, the slurry was mixed well by vortexing for 10 seconds followed by centrifugation at 500 rpm for 2 minutes. The supernatant from this was transferred to a mini filter spin column assembled in an elution tube and centrifuged for 1 minute at 6000 rpm. The eluted fraction containing purified exosomes were collected and used for further characterization and RNA isolation.

## **Transmission electron microscopy**

Transmission electron microscopy (TEM) was used to visualize exosomes as reported earlier (117). In short, the exosomes (2–4µl) were fixed in 4% paraformaldehyde and deposited onto carbon-coated electron microscopy grids. The grid was washed with 1 x PBS containing glycine twice and there after once with 1 x PBS containing glycine (50 mM) each for three minutes. Then again washed with 1 x PBS containing BSA (0.5%) for 10 minutes. The grids were stained with 25 uranyl acetate and viewed with Hitachi 7650 Analytical TEM.

## **Nanoparticle tracking analysis (NanoSight NS300) for size and particle concentrations**

NanoSight Technology NS300 was used for the analysis of particle size and concentration of plasma derived EVs by nanoparticle tracking analysis (NTA). The diluted exosomes samples were mixed and transfer into the sample chamber with a syringe with fixed flow rate. Three video recordings for every 60 seconds was taken to obtain the measurements. The optimal visualization of the maximum number of exosomes was done by manual focusing of NanoSight Technology NS300 which uses a combination of shutter speed and gain. The post-acquisition settings of NTA were optimized and kept constant between exosome samples and analyzed as reported earlier (117, 118).

## **Exosomal RNA isolation**

Exosomal RNA was isolated using exosomal RNA isolation kit (Norgen Bioteck Corporation, Cat# 58000). Briefly, 300 µl lysis of buffer A and 37.5 µl of lysis buffer additive B were added to the 200 µl ExoR buffer in the tube containing purified exosomes as mentioned above. The tubes were mixed well by vortexing for 10 seconds then incubated at room temperature for 10 minutes. After incubation, 500 µl of 96–100% ethanol was added to the mixture in the tube and mixed well by vortexing for 10 seconds. The 500 µl of

the mixture was transferred to mini spin column and centrifuged for 1 minute at 3,300 x g. The flow through was discarded and spin column was reassembled with its collection tubes to transfer the remaining mixtures for additional mini spin column. Next 600 µl of wash solution A was added to the column and centrifuge for 30 seconds at 3,300 x g (~6000 rpm), this was repeated for a total of two washings. The flow through was discarded and spin column was reassembled with its collection tubes. Column was spin for 1 minute at 13,000 x g (~14,000 rpm) and collection tube was discarded. Spin column was transferred to a fresh 1.7 ml elution tube and 50 µl of elution buffer was added to the column and centrifuged for 1 minute at 2000 rpm, followed by 2 minutes at 8,000 rpm. The eluted buffer was transferred back to the column, incubated at room temperature for 2 minutes and then centrifuged for 1 minute at 400 x g (~2,000 rpm), followed by 2 minutes at 8,000 rpm. The eluted purified buffer containing RNA was collected, quantified and used for downstream applications.

## Small RNA library construction and sequencing

Exosomal RNA isolated from each sample [non-smokers (8), cigarette smokers (7), E-cigarette users (7) and waterpipe smokers (7)] was used for small RNA library preparation using the small RNA library preparation kit for Illumina (Small RNA library prep kit, cat# 63600, Norgen Biotek Corp., Canada) as reported previously (57). The prepared libraries were eluted overnight in nuclease free water and cleaned (Ambion, USA). Then library was quantified using the High Sensitivity DNA Analysis kit on the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA). Libraries were diluted, pooled, and sequenced on the Illumina MiSeq or Illumina NextSeq 500 sequencing platform using the MiSeq reagent kit v3 or NextSeq 500/550 High Output kit v2 (51 cycles using a 75-cycle kit) at the Norgen Biotek. Corp.

## Sequence read mapping and RNA annotation

The sequence raw data from the Illumina HiSeq 4000 were converted to fastq format. The converted files were used in the Genboree Workbench's exceRpt small RNA-seq pipeline (version 4.6.2) for read mapping to the hg38 human genome version (119). This phenomenon allows to single mismatched base down to 18 nucleotides. The adapter trimming was carried out and the read quality was assessed by FASTQC to filter out reads with a quality score lower than 30 on the PHRED scale. The reads were mapped to the UniVec and human ribosomal RNA (rRNA) sequences to exclude before mapping to databases of miRBase version 21, gtRNadb and piRNABank to assign reads to miRNAs, tRNAs and piRNAs, respectively. Identified tRNAs are tRNA-derived RNA fragments because the library insert size is below 50 nt. The other remaining sequences were then annotated to gencode version 24 (hg38) which includes protein coding transcripts (protein\_coding), mitochondrial rRNA (Mt\_rRNA), mitochondrial tRNA (Mt\_tRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), long intergenic noncoding RNA (lincRNA) and miscellaneous RNA (misc\_RNA) (57).

# Extracellular vesicle/exosome microRNA database searches

The miRNAs identified from plasma-derived EVs in various groups were searched for in online databases, using the extracellular vesicles miRNA database (<http://bioinfo.life.hust.edu.cn/EVmiRNA>), Vesiclepedia (<http://microvesicles.org/>) (120), ExoCarta (<http://exocarta.org/>) (121) and miRDB (<http://mirdb.org/>) (122). The databases provided a compendium of miRNAs identified in several EVs/exosome preparations and from other sample types. Venn diagrams were generated using the `venn.diagram` function in the `VennDiagram` package in statistical analysis software R version 3.6.1 to compare lists of miRNAs, in order to find the common EV/exosome-enriched miRNA biomarkers.

## Data and statistical analysis

The RNA-Seq fastq files processed for quality control, and the summarized RNA-Seq count data was analyzed to filter out genes with summed counts across all samples less than 10 to generate the data for further statistical analysis. Then, the trimmed mean of M values (TMM) method was used for count normalization across the non-smokers, E-cig users, waterpipe, cigarette smokers groups and dual smokers for miRNA analysis (123). The DESeq function in the DESeq2 package in the statistical analysis software R/Bioconductor was used to identify significant miRNAs that have different expression levels across different groups. The pairwise differences in miRNA expression levels among the non-smokers, E-cig users, waterpipe, cigarette smokers and dual smokers groups were examined using the linear contrast in the DESeq2 RNA-Seq differential analysis method (124). The false discovery rate (FDR) was controlled for at 5% using the Benjamini-Hochberg method for each pairwise comparison. A heatmap of significant miRNA selected from the DESeq2 method was generated using the `pheatmap` function with the `ward.D2` clustering method in the statistical analysis software R to show the differences between groups (NS, E-cig, WP, CS, and DS) based on their pairwise comparisons. Pathway and gene enrichment analyses were conducted using the FunRich software to examine the potential functionalities and roles of significant miRNAs in the gene regulation network (125).

## Functional over-representation analysis and gene set enrichment analysis of microRNA

The functions of the differentially expressed microRNA from non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cig. Smokers and non-smokers vs. dual smokers were analyzed by using microRNA enrichment analysis and annotation tool (MiEAA; [http://ccb-compute2.cs.uni-saarland-de/mieaa\\_tool/](http://ccb-compute2.cs.uni-saarland-de/mieaa_tool/); accessed in August 2019) (126). Further, MiEAA a web-based application used for commonly applied statistical test over-representation analysis (ORA) and miRNA set enrichment analysis. MiEAA used to analyze rich functional test of microRNA categories such as gene ontology, pathways and disease. This also test whether a category is significantly enriched (FDR

adjustment) in a specific microRNA set with respect to the reference using statistical tests implemented in the gene set analysis toolkit, GeneTrail.

## List Of Abbreviations

E-cigs: E-cigarettes

COPD: Chronic obstructive pulmonary disease

NS: Normal/non-smokers

SM: Cigarette smokers

WP: Waterpipe

DS: Dual smokers

## Declarations

## Author Contributions

KPS, KPM, DL, IR conceived and designed the experiments, wrote and edited the manuscript. IR obtained research funding and study design and experimental plans/assays. IR recruited the volunteers, KPS, performed the experiments and analyzed data. All authors contributed to manuscript preparation and approved the final version before the submission.

## Conflict of Interest Disclosures

No conflict of interests.

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## Role of the Funder/Sponsor

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or the FDA.

## **Ethics approval and consent to participate**

The protocols were approved by the Institutional Review Board (IRB) at the University of Rochester Medical Center, Rochester, NY. Written informed consent was obtained from all study participants.

## **Disclaimer: None**

## **Meeting Presentation: None**

## **Additional Contributions: None**

## **Competing interests:**

The authors have no competing interests.

## **Availability of data and materials:**

All authors confirm the availability of data and materials online/free access to readers.

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## Supplementary Files Legend

*Supplementary Table 1.* Differential expressed microRNAs from plasma exosomes of E-cigarette users in comparison to non-smokers pairwise comparison.

*Supplementary Table 2.* Differential expressed microRNAs from plasma exosomes of cigarette smokers in comparison to non-smokers.

*Supplementary Table 3.* Differential expressed microRNAs from plasma exosomes from waterpipe smokers in comparison to non-smokers.

*Supplementary Table 4.* Differential expressed microRNAs from plasma exosomes of dual smokers in comparison to non-smokers.

*Supplementary Table 5.* Differential expressed microRNAs from plasma exosomes of E-cig users in comparison to cigarette smokers.

*Supplementary Table 6.* Differential expressed microRNAs from plasma exosomes of waterpipe smokers in comparison to cigarette smokers.

*Supplementary Table 7.* Differential expressed microRNAs from plasma exosomes of dual smokers in comparison to cigarette smokers.

*Supplementary Table 8.* . Differential expressed microRNAs from plasma exosomes of waterpipe smokers in comparison to dual smokers.

*Supplementary Table 9.* List of target genes of differentially changed microRNA in non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cigarette smokers, non-smokers vs. dual smokers, cigarette smokers vs. waterpipe smokers, cigarette smokers vs. E-cigarette smokers, cigarette smokers vs. dual smokers, and dual smokers vs. waterpipe smokers.

*Supplementary Table 10.* Differential expressed tRNAs from plasma exosomes of cigarette smokers in comparison to non-smokers.

*Supplementary Table 11.* Differential expressed tRNAs from plasma exosomes of waterpipe smokers in comparison to non-smokers.

*Supplementary Table 12.* Differential expressed tRNAs from plasma exosomes of E-cigarette users in comparison to non-smokers.

*Supplementary Table 13.* Differential expressed tRNAs from plasma exosomes of dual smokers in comparison to non-smokers.

*Supplementary Table 14.* Differentially expressed piRNAs pairwise comparison between non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cig users, non-smokers vs. dual smokers, cigarette smokers vs. waterpipe smokers, cigarette smokers vs. E-cig users, cigarette smokers vs. dual smokers and waterpipe smokers vs. dual smokers.

*Supplementary Fig. 1. Volcano plot.* Volcano plot showing the relation between P-values of the changes in differentially expressed microRNA, and fold change in cigarette smokers, waterpipe smokers, dual smokers and e-cigarette users. (A) Cigarette smokers versus E-cig users. (B) Cigarette smokers versus waterpipe smokers. (C) Cigarette smokers versus dual smokers. (D) Dual smokers versus waterpipe smokers.

*Supplementary Fig. 2. Hierarchical cluster analysis of differentially expressed miRNAs.* (A) Heatmap clustering of the differentially expressed miRNAs significant among cigarette smokers vs. waterpipe smokers. (B) Heatmap clustering of the differentially expressed miRNAs significant among cigarette smokers vs. E-cig users. (C) Heatmap clustering of the differentially expressed miRNAs significant among cigarette smokers vs. dual smokers. (D) Heatmap clustering of the differentially expressed miRNAs significant among waterpipe smokers vs. dual smokers. These top miRNAs were identified based on individual pairwise comparisons (with adjusted  $p$ -value;  $P < 0.01$ ). The analysis generated using Z scores of the most differentially expressed significant miRNAs. The dendrogram shows clustering of sample groups (cigarette smokers vs. waterpipe smokers, cigarette smokers vs. E-cig users, cigarette smokers vs. dual smokers and waterpipe smokers vs. dual smokers).

*Supplementary Fig. 3. FunRich gene enrichment analysis for the differentially expressed miRNAs.* Here we provide the top 6 enriched: (A) Biological process, (B) Molecular function, (C) Cellular component, (D) Biological pathway, (E) Site of expression, and (F) Transcription factors for the significant miRNAs and possible target genes in cigarette smokers vs. waterpipe smokers, cigarette smokers vs. E-cig users, cigarette smokers vs. dual smokers and dual smokers vs. waterpipe smokers pairwise comparisons.

*Supplementary Fig. 4.* Principal component analysis based on differential tRNA expression in individual samples of non-smokers, cigarette smokers, waterpipe smokers, E-cig users and dual smokers.

*Supplementary Fig. 5.* Volcano plot showing the relation between P-values of the changes in differentially expressed tRNA, and fold change in non-smokers, cigarette, waterpipe, E-cigarette users and dual smokers. (A) Non-smokers versus cigarette smokers. (B) Non-smokers versus waterpipe smokers. (C) Non-smokers versus E-cig users. (D) Non-smokers versus dual smokers.

*Supplementary Fig. 6. Hierarchical cluster analysis of differentially expressed miRNAs.* (A) Heatmap clustering of the differentially expressed tRNAs significant among non-smokers vs. cigarette, smokers. (B) Heatmap clustering of the differentially expressed tRNAs significant among non-smokers vs. waterpipe

smokers. (C) Heatmap clustering of the differentially expressed tRNAs significant among non-smokers vs. E-cigarette users. (D) Heatmap clustering of the differentially expressed tRNAs significant among non-smokers vs. dual smokers. These tRNAs were identified based on individual pairwise comparisons (with unadjusted raw  $p$ -value;  $P < 0.05$ ). The analysis was generated using Z scores of the most differentially expressed significant tRNAs. The dendrogram shows clustering of pairwise comparisons among the different groups (non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cigarette users and non-smokers vs. dual smokers).

*Supplementary Fig. 7.* Venn diagram showing the overlap of differentially expressed tRNAs identified from comparing four groups: non-smokers vs. cigarette smokers, non-smokers vs. waterpie smokers, non-smokers vs. E-cig users and non-smokers vs. dual smokers.

*Supplementary Fig. 8.* Principal component analysis based on differential piRNA expression in individual samples of non-smokers, cigarette smokers, waterpipe smokers, E-cig users and dual smokers.

*Supplementary Fig. 9. Hierarchical cluster analysis of differentially expressed piRNAs.* Heatmap clustering of the differentially expressed piRNAs significant among (A) non-smokers vs. cigarette smokers, (B) non-smokers vs. waterpipe smokers, (C) non-smokers vs. E-cigarette users, (D) non-smokers vs. dual smokers (E) cigarette smokers vs. waterpipe smokers, (F) cigarette smokers vs. E-cigarette smokers, (G) cigarette smokers vs. dual smokers, and (H) dual smokers vs. waterpipe smokers. These piRNAs were identified based on individual pairwise comparisons (with unadjusted raw  $p$ -value;  $P < 0.05$ ). The analysis was generated using Z scores of the most differentially expressed significant piRNAs. The dendrogram shows clustering of pairwise comparisons among the different groups (non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cigarette users and non-smokers vs. dual smokers).

*Supplementary Fig. 10.* Venn diagram showing the overlap of differentially expressed piRNAs identified from comparing four groups: non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cig users and non-smokers vs. dual smokers.

## Figures

Figure 1.

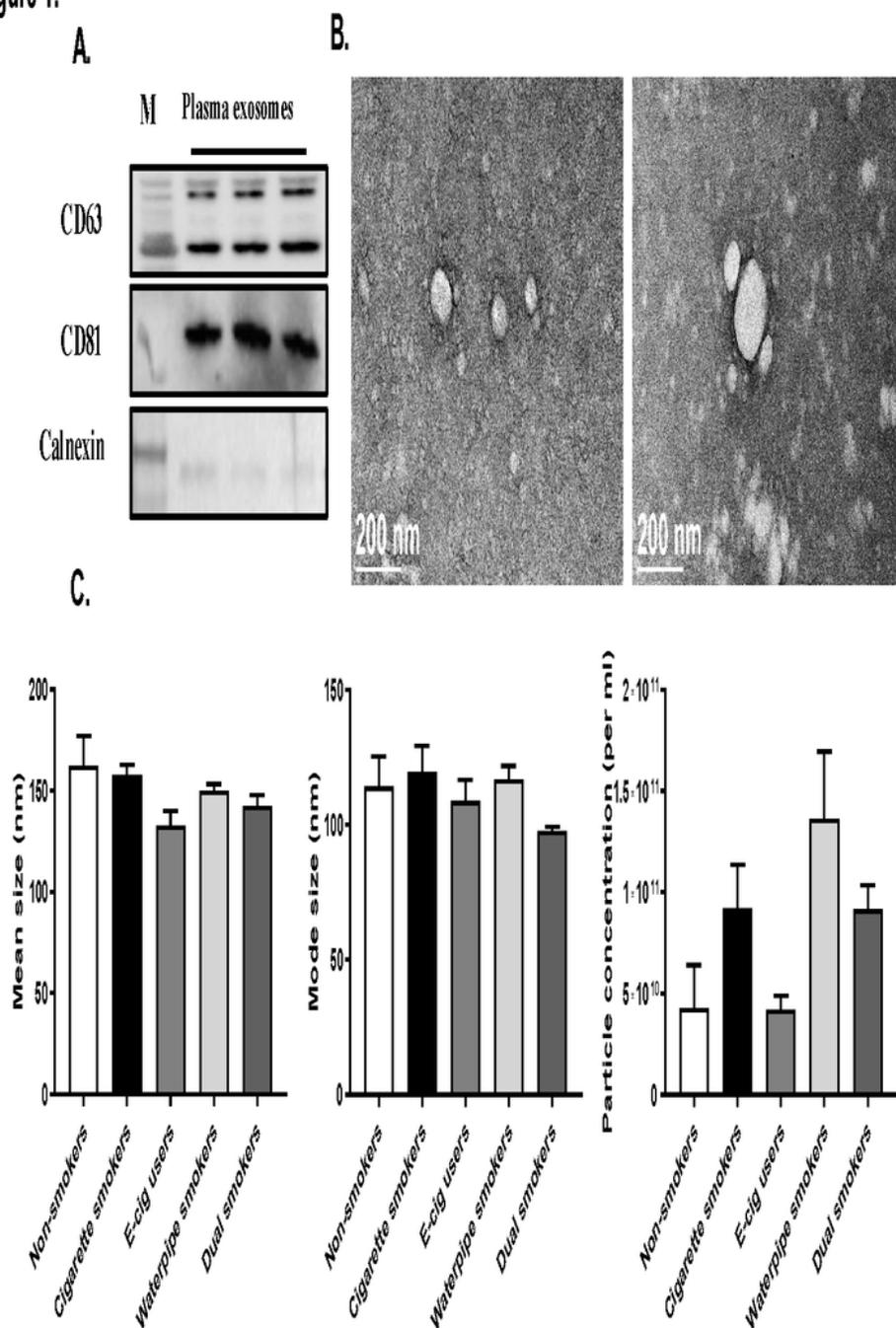


Figure 1

Isolation and characterization of plasma-derived EVs/Exosomes. (A) Immunoblot analysis of proteins isolated from plasma EVs. (B) Representative TEM images of plasma-derived EVs/Exosomes. (C) Particle size depicted as mean and mode, and particle concentration were estimated using NanoSight NS300 (n=3/ group).

Figure 2.

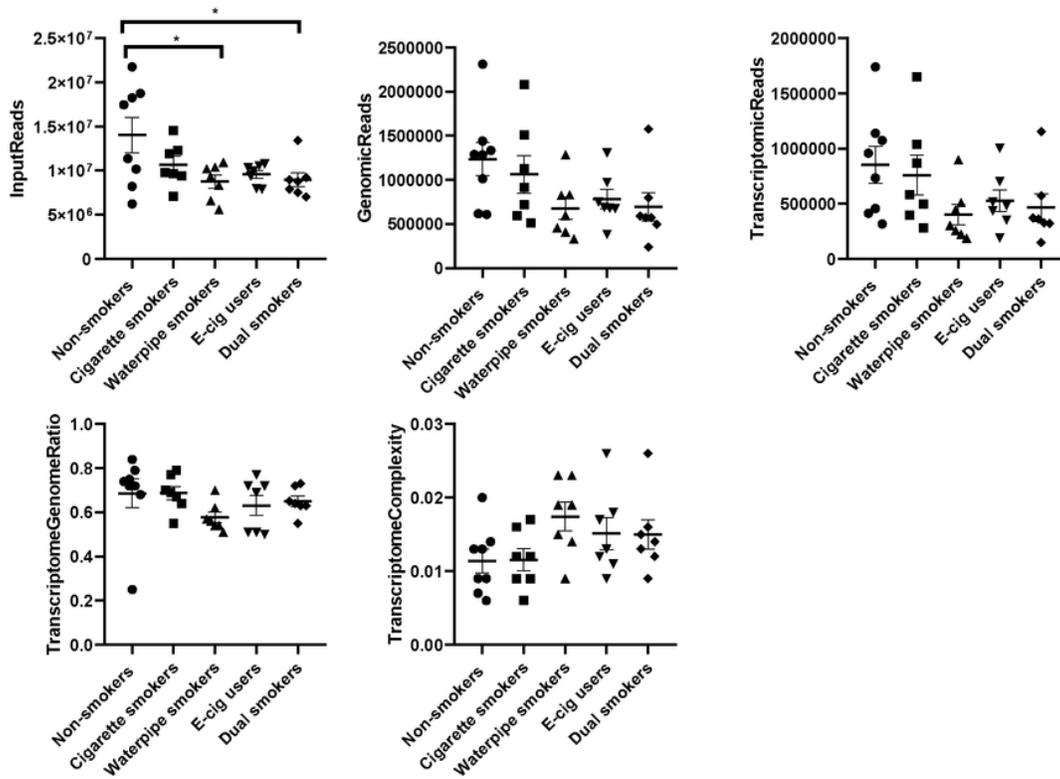
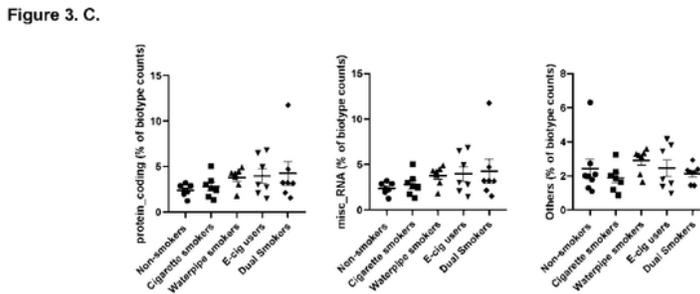
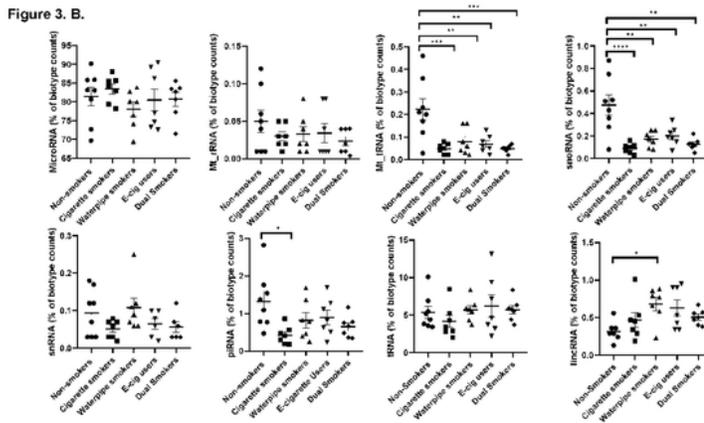
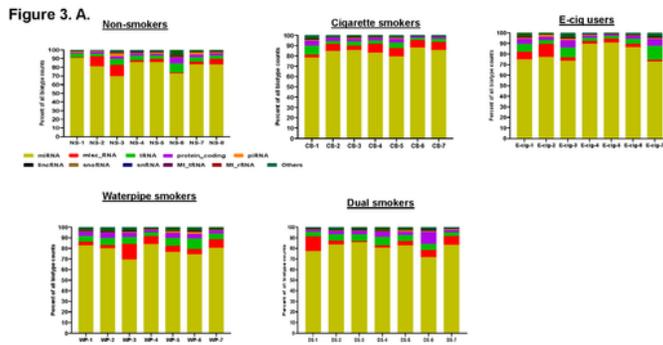


Figure 2

The small RNA analysis quality control results of individual samples in non-smokers, cigarette smokers, waterpipe smokers, E-cig users and dual smokers.



**Figure 3**

Relative biotype distribution from each sample. (A) This graph represents average percentage of biotype counts of each sample from non-smokers, cigarette, waterpipe smokers and E-cigarette smokers. (B) Comparison of relative biotype distribution of non-smokers, cigarette smokers, waterpipe smokers, E-cigarette users and dual smokers.

Figure 4.

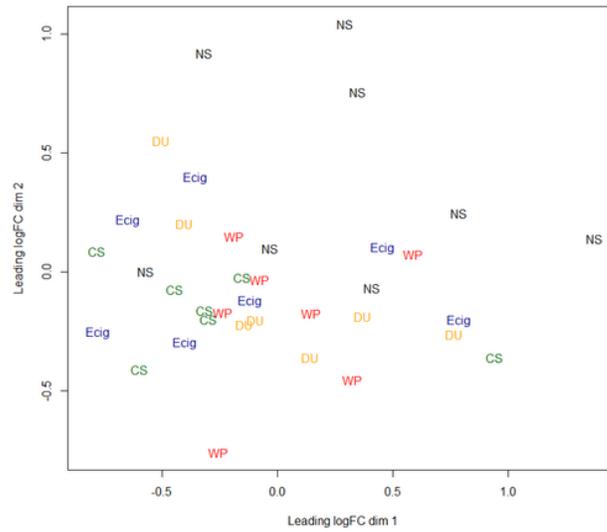
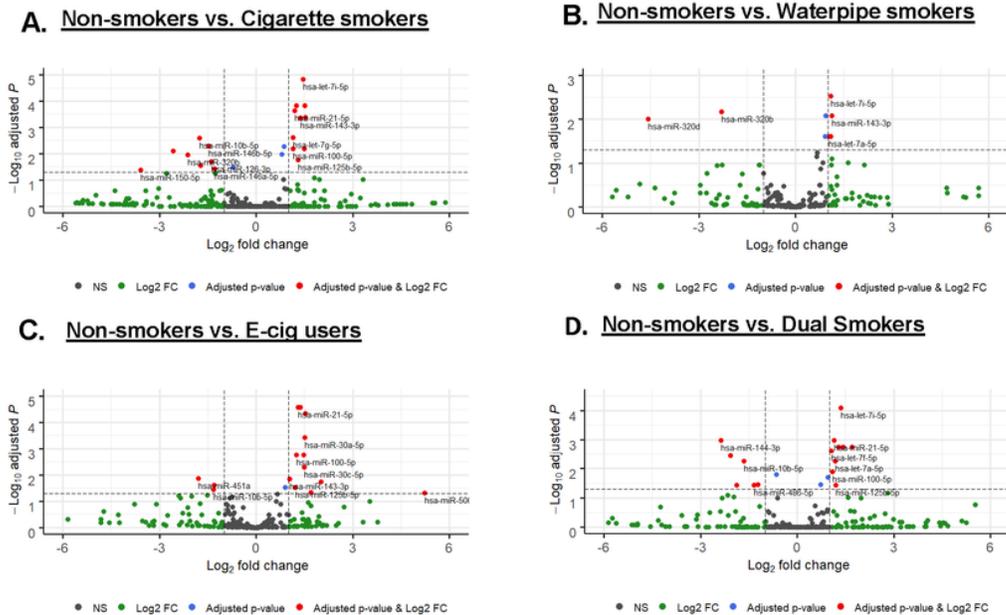


Figure 4

Principal component analysis based on differential microRNA expression in individual samples of non-smokers, cigarette smokers, waterpipe smokers, E-cig users and dual smokers.

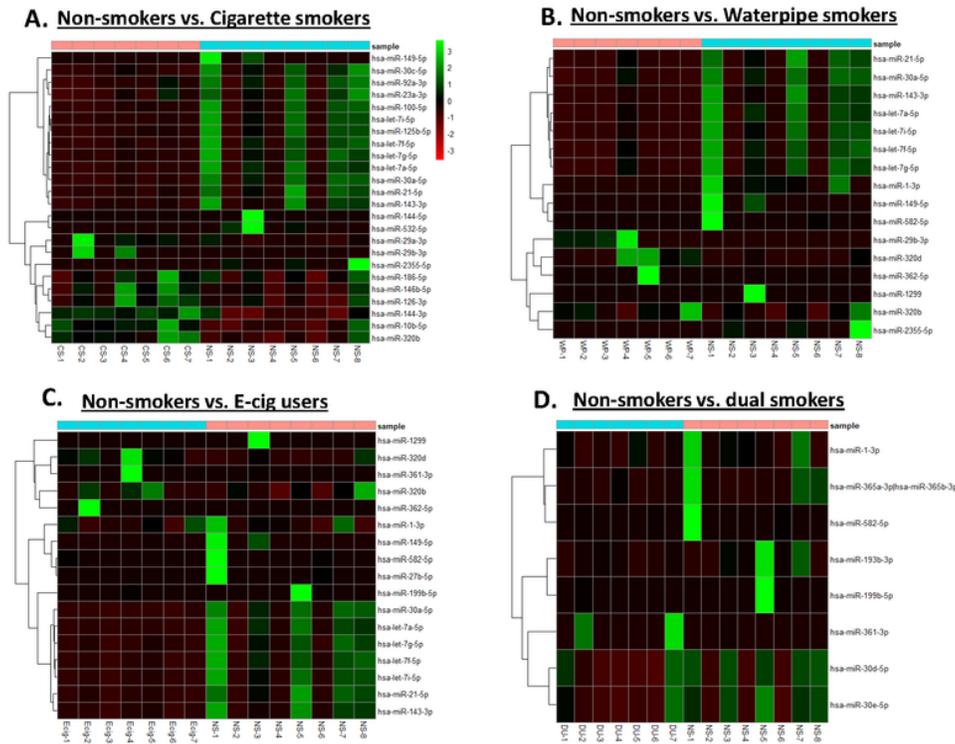
Figure 5.



**Figure 5**

Volcano plots. Volcano plot showing the relation between P-values of the changes in differentially expressed microRNA, and fold change in non-smokers, cigarette, waterpipe, E-cigarette users and dual smokers. (A) Non-smokers versus cigarette smokers. (B) Non-smokers versus waterpipe smokers. (C) Non-smokers versus E-cig users. (D) Non-smokers versus dual smokers.

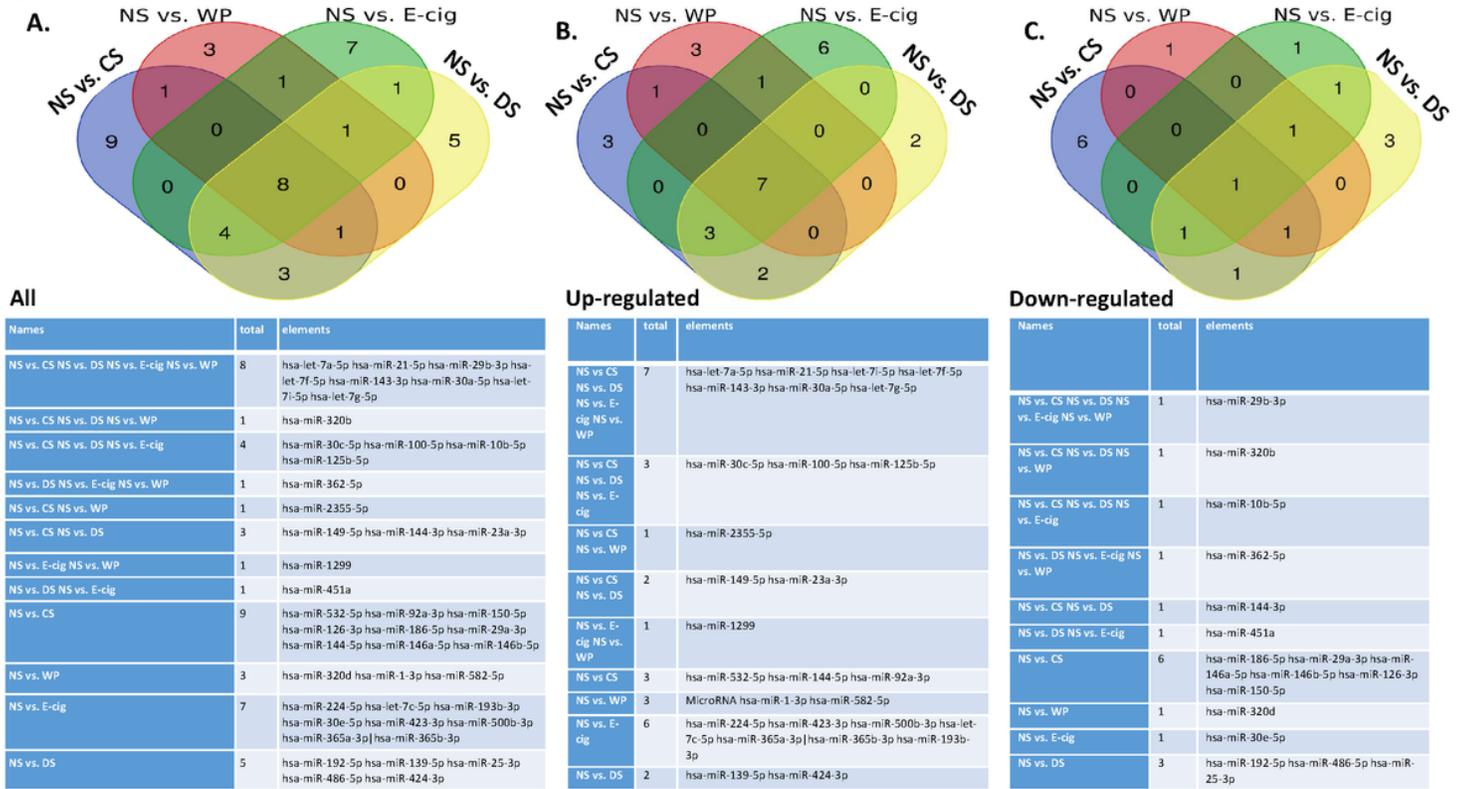
**Figure 6.**



**Figure 6**

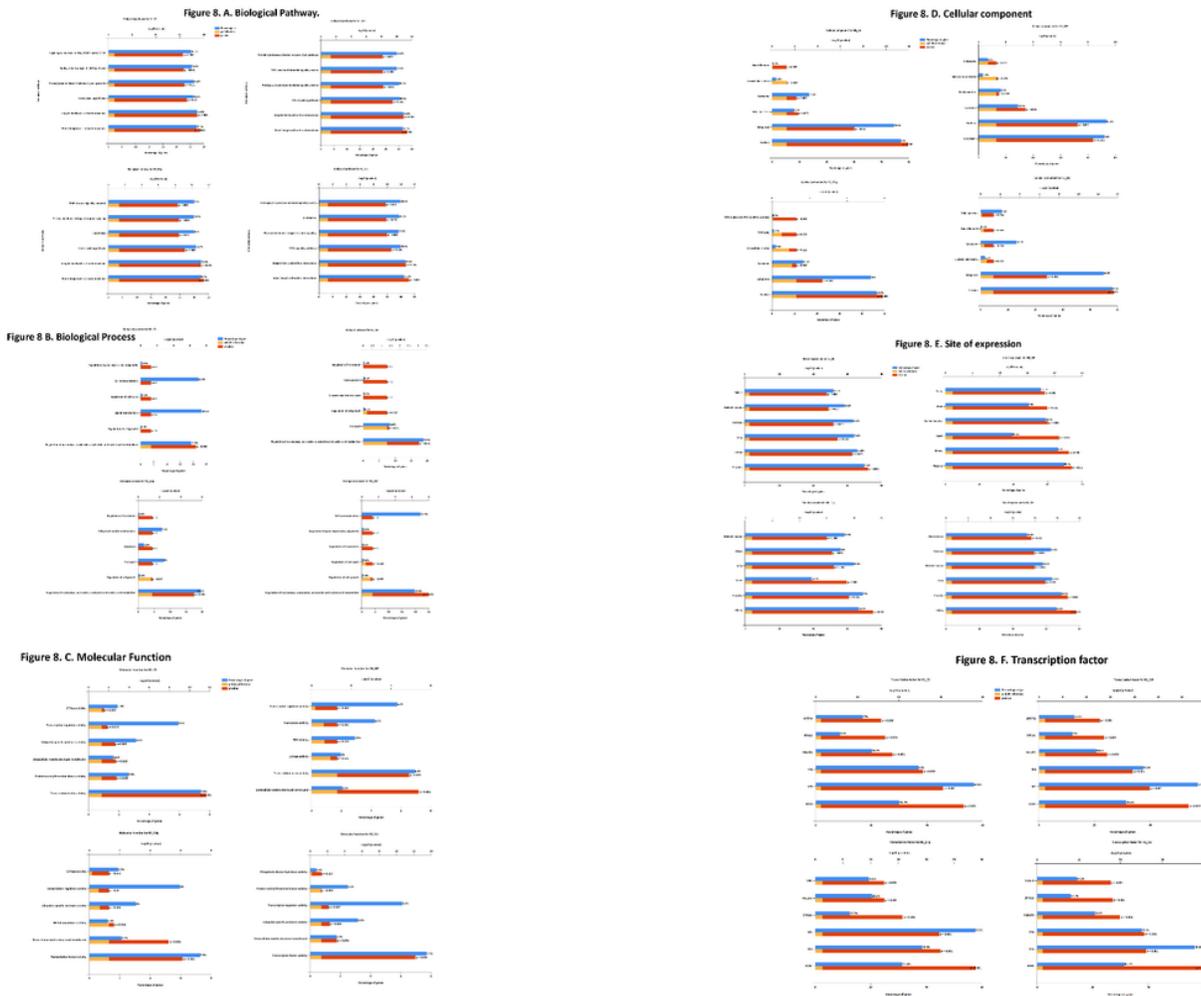
Hierarchical cluster analysis of differentially expressed miRNAs. (A) Heatmap clustering of the differentially expressed miRNAs significant among non-smokers vs. cigarette, smokers. (B) Heatmap clustering of the differentially expressed miRNAs significant among non-smokers vs. waterpipe smokers. (C) Heatmap clustering of the differentially expressed miRNAs significant among non-smokers vs. E-cigarette users. (D) Heatmap clustering of the differentially expressed miRNAs significant among non-smokers vs. dual smokers. These miRNAs were identified based on individual pairwise comparisons (with unadjusted raw p-value;  $P < 0.05$ ). The analysis was generated using Z scores of the most differentially expressed significant miRNAs. The dendrogram shows clustering of pairwise comparisons among the different groups (non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs. E-cigarette users and non-smokers vs. dual smokers).

**Figure 7.**



**Figure 7**

Venn diagram showing the overlap of differentially expressed microRNAs identified from comparing four groups: non-smokers vs. cigarette smokers, non-smokers vs. waterpie smokers, non-smokers vs. E-cig users and non-smokers vs. dual smokers. (A) The overlap of all differentially expressed microRNAs, (B) The overlap of up-regulated differentially expressed microRNAs, and (C) The overlap of down-regulated differentially expressed microRNAs



**Figure 8**

FunRich gene enrichment analysis for the differentially expressed miRNAs. Here we provide the top 6 enriched: (A) Biological process, (B) Molecular function, (C) Cellular component, (D) Biological pathway, (E) Site of expression, and (F) Transcription factors for the significant miRNAs and possible target genes in non-smokers vs. cigarette smokers, non-smokers vs. waterpipe smokers, non-smokers vs e-cigarette users, and non-smokers vs. dual smokers pairwise comparisons.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarymaterials.pdf](#)