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A meta-analysis of microRNAs expressed in human aerodigestive epithelial cultures and their role as potential biomarkers of exposure response to nicotine-containing products

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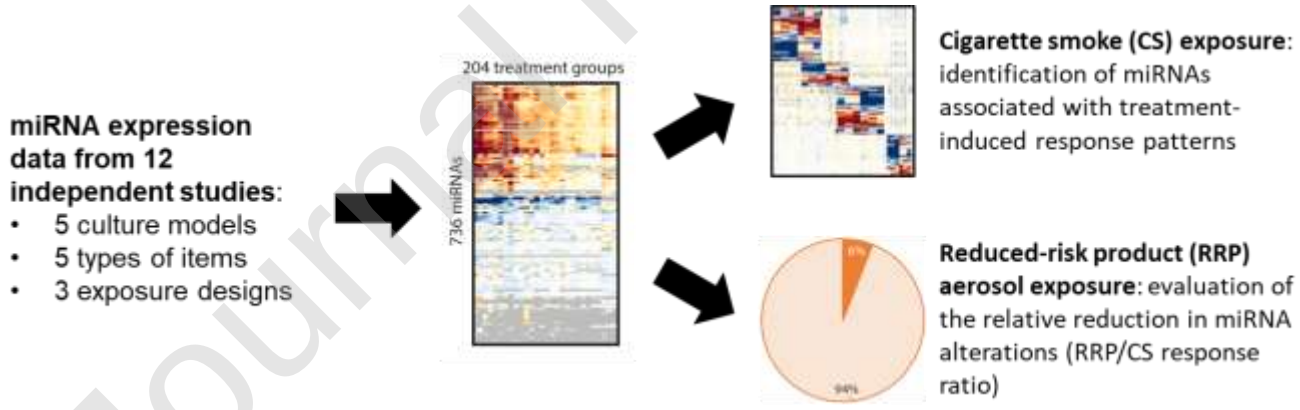
Graphical abstract



Highlights

- This meta-analysis evaluated 12 miRNA expression datasets from aerodigestive tissues

- Treatments included cigarette smoke (CS) and reduced-risk product (RRP) aerosol exposure
- Tissue-dependent changes were observed following CS exposure
- Four miRNAs were identified as potential biomarkers of CS exposure response
- Exposure to RRP aerosols resulted in reduced expression alterations relative to CS

Abstract

The expression of some microRNAs (miRNA) is modulated in response to cigarette smoke (CS), which is a leading cause of major preventable diseases. However, whether miRNA expression is also modulated by the aerosol/extract from potentially reduced-risk products is not well studied. The present work is a meta-analysis of 12 *in vitro* studies in human organotypic epithelial cultures of the aerodigestive tract (buccal, gingival, bronchial, nasal, and small airway epithelia). These studies compared the effects of exposure to aerosols from electronic vapor (e-vapor) products and heated tobacco products, and to extracts from Swedish snus products (in the present work, will be referred to as reduced-risk products [RRPs]) on miRNA expression with the effects of exposure to CS or its total particulate matter fraction. This meta-analysis evaluated 12 datasets of a total of 736 detected miRNAs and 2775 exposed culture inserts. The t-distributed stochastic neighbor embedding method was used to find similarities across the diversity of miRNA responses characterized by tissue type, exposure type, and product concentration. The CS-induced changes in miRNA expression in gingival cultures were close to those in buccal cultures; similarly, the alterations in miRNA expression in small airway, bronchial, and nasal tissues resembled each other. A supervised clustering was performed to identify miRNAs exhibiting

particular response patterns. The analysis identified a set of miRNAs whose expression was altered in specific tissues upon exposure to CS (*e.g.*, miR-125b-5p, miR-132-3p, miR-99a-5p, and 146a-5p). Finally, we investigated the impact of RRPs on miRNA expression in relation to that of CS by calculating the response ratio *r* between the RRP- and CS-induced alterations at an individual miRNA level, showing reduced alterations in miRNA expression following RRP exposure relative to CS exposure (94% relative reduction). No specific miRNA response pattern indicating exposure to aerosols from heated tobacco products and e-vapor products, or extracts from Swedish snus was identifiable.

Abbreviations: 2D, two-dimensional; AKT, protein kinase B; ALI, air-liquid interface; CHTP 1.2, Carbon Heated Tobacco Product 1.2; COPD, chronic obstructive pulmonary disease; CRP, CORESTA Reference Product; CS, cigarette smoke and its TPM fraction; e-vapor, electronic vapor; FDA, Food & Drug Administration; FDR, false discovery rate; GCW, General Classic White; HCI, Health Canada intense; HTP, heated tobacco product; IL-1β, interleukin 1β; miRNA, microRNA; MMP-1, matrix metalloproteinase 1; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; N/A, not applicable; RRP, reduced-risk product; THS 2.2, Tobacco Heating System 2.2; TPM, total particulate matter; t-SNE, t-distributed stochastic neighbor embedding.

Keywords

Organotypic aerodigestive culture; heated tobacco product; e-vapor; miRNA; Tobacco Heating System 2.2; systems toxicology

Introduction

MicroRNAs (miRNA) are small, non-coding RNA species (21–25 nucleotides) that selectively bind to target messenger RNAs (mRNA), affecting protein translation by blocking mRNA access to ribosomes or by accelerating the degradation of mRNA transcripts (Hoefel et al., 2019). Environmental exposure affects the expression of miRNAs (Harrill et al., 2016, Vrijens et al., 2015), altering the levels of their target mRNAs. This deregulation has been linked to specific cellular responses such as cell death, proliferation, metabolism, and inflammation (Yokoi and Nakajima, 2011). The onset and progression of diseases such as cancer, vascular diseases, and periodontal diseases have been shown also to be accompanied by specific miRNA expression changes (Hao et al., 2014, Araldi et al., 2015, Kebschull and Papapanou, 2015).

Several studies have linked particular miRNAs to biological processes related to toxicity or diseases in specific tissues, suggesting their utility as biomarkers. For example, the expression of miRNAs in biofluids has been studied as a potential marker of lung injury (Harrill et al., 2016, Schraml et al., 2017). In a previous study, in blood samples from patients with chronic obstructive pulmonary disease (COPD), the levels of miR-146a/b were inversely correlated with the levels of inflammatory mediators; the authors proposed that miR-146a/b could serve as biomarkers for predicting the risk of acute exacerbation of COPD (Chen et al., 2018). Other studies have proposed that some miRNAs detected in salivary and periodontal tissues could be

biomarkers for periodontal pathologies (Schmalz et al., 2016, Motedayyen et al., 2015, Fujimori et al., 2019).

Growing evidence indicates that the modulation of miRNA expression by cigarette smoke (CS) could be linked to the onset of pulmonary diseases (Graff et al., 2012, Gross et al., 2014, Izzotti et al., 2009, Rogers et al., 2017). A few publications have linked CS-induced alterations in miRNA expression with non-cancerous changes in oral tissues (Bhat et al., 2018, Zanetti et al., 2016, Zanetti et al., 2019, Zanetti et al., 2018). Nonetheless, evidence on the association between CS exposure and certain miRNA alterations is currently limited (Vrijens et al., 2015). The difficulty in making a clear association results from the heterogeneity of miRNA profiling assay platforms, experimental setups, and study designs. Therefore, identifying an unambiguous signature of CS-induced miRNA alterations that are reproducible has been challenging. Nevertheless, miRNA expression is a promising biomarker of exposure.

For certain United States Food and Drug Administration (FDA)-regulated products, “biomarkers could play an important role across a number of FDA tobacco regulatory activities, including assessing new and modified risk tobacco products and identifying and evaluating potential products standards” (FDA, 2018). While smoking cessation is the most effective way to reduce the harm from smoking-related diseases (Godtfredsen et al., 2008), switching to less harmful products such as modified risk tobacco products (Family Smoking Prevention and Tobacco Control Act, 2009) can be an alternative for smokers who otherwise would continue smoking. A number of heated tobacco products (HTP) have been developed that are designed to reduce the number and levels of harmful and potentially harmful constituents, many of which are formed during combustion of tobacco; these include Tobacco Heating System (THS) 2.2 and Carbon Heated Tobacco System (CHTP) 1.2 (Smith et al., 2016, Titz et al., 2018, Schaller et al., 2016).

Moreover, it has been suggested that electronic vapor (e-vapor) products "may be a unique harm reduction innovation for smoking relapse prevention", and vaping may have "substantial implications for tobacco harm reduction" (Notley et al., 2018). Studies have shown that these products release harmful chemicals and carcinogenic metabolites at substantially reduced levels relative to cigarettes (Phillips et al., 2018, Hecht et al., 2015, Marco and Grimalt, 2015, Goniewicz et al., 2014, Tayyarah and Long, 2014, Schaller et al., 2016). Furthermore, the FDA has granted the modified risk tobacco product status to eight General brand snus smokeless tobacco products. For simplicity, the HTPs and e-vapor products aerosols, and smokeless tobacco products extracts will be termed reduced-risk products (RRP) in the present work.

We conducted the present meta-analysis to investigate the utility of miRNA expression as a biomarker of exposure. Instead of leveraging data from a single study, a meta-analysis – which groups the results from multiple studies – can yield an estimate that is more accurate than that of any individual study, with the uncertainty being typically smaller than that in the estimate of any individual study (McShane and Böckenholt, 2017). Furthermore, a meta-analysis enables identification of recurring response patterns that would otherwise be too weak to pass the statistical significance thresholds in a single study. Finally, it uncovers associations between exposure conditions and miRNA profiles that are much more reliable than if they had been obtained from a single study.

We leveraged miRNA data from 12 independent *in vitro* studies that used human organotypic aerodigestive (buccal, gingival, nasal, bronchial, and small airway) epithelial cultures (Figure 1). These 12 studies were conducted to compare the biological impact of exposure to various RRPs with that of exposure to CS or its total particulate matter (TPM) fraction. The cultures were exposed at the air–liquid interface to smoke from cigarettes or

aerosols from various RRPs or apically to liquid TPM from CS or Swedish snus extracts. The meta-analysis was oriented to assess the global similarities and differences in miRNA profiles across the datasets. The associations between miRNA patterns of exposure or tissue type were also investigated. We leveraged the t-distributed stochastic neighbor embedding (t-SNE) method to evaluate the 12 datasets globally. Subsequently, we performed a supervised clustering to identify CS and its TPM fraction-related miRNA expression changes that were common across the tissue types. Finally, we compared miRNA alterations following exposure to RRPs with those following exposure to CS and its TPM fraction in a comprehensive manner.

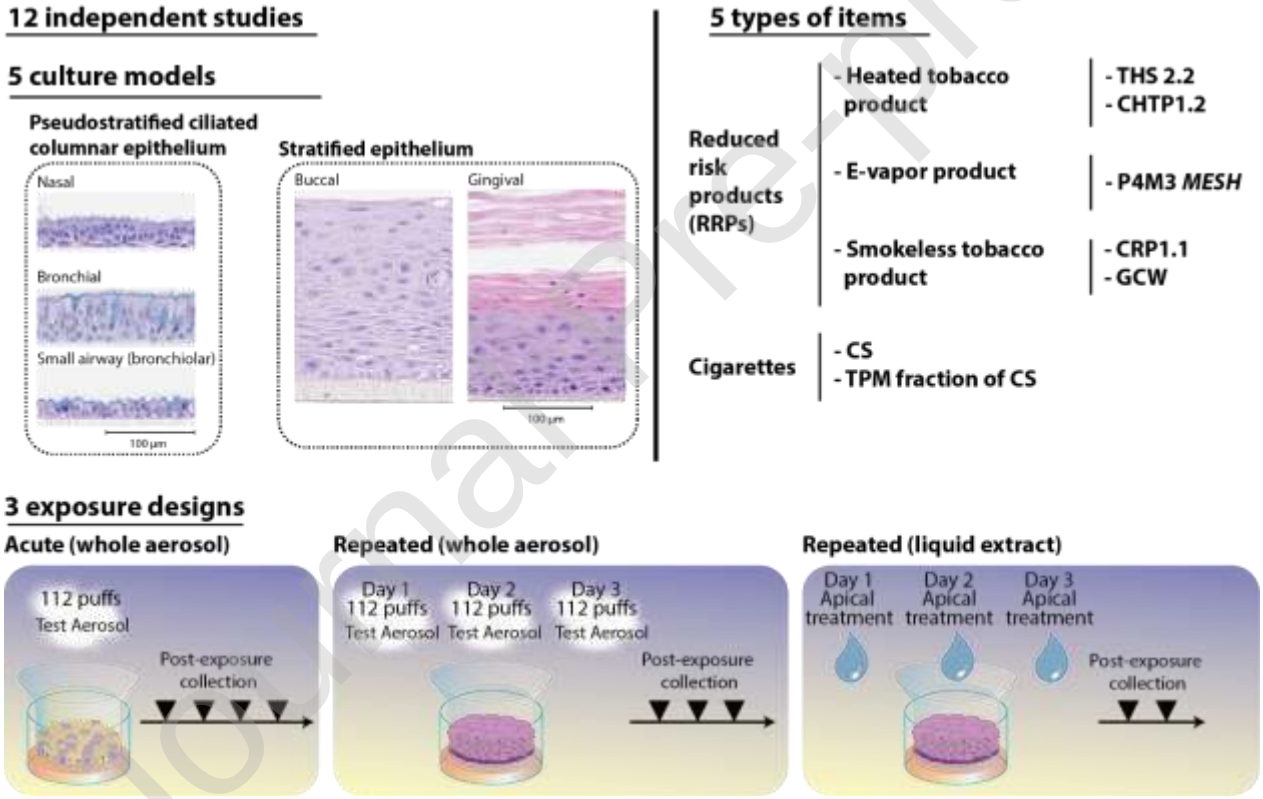


Figure 1. Characteristics of the studies included in the meta-analysis. The meta-analysis included 12 independent studies (see Table 1). Each study used one of the five models of organotypic human epithelial cultures (upper left panel).The cultures were exposed to aerosols from heated tobacco products or an e-vapor product or to the extract of a smokeless tobacco product (Swedish snus) (upper right panel). The impact of these exposures on miRNA expression profiles was compared with that of exposure to CS or the TPM fraction of CS. Whole smoke or aerosol exposures were tested acutely (1 day) or repeatedly (3 days), while exposure to Swedish snus or TPM was tested repeatedly (bottom panel). CHTP, Carbon Heated Tobacco Product;

CRP, CORESTA reference product; CS, 3R4F cigarette smoke; GCW, General Classic White; miRNA, microRNA; P4M3, e-vapor product; THS, Tobacco Heating System; TPM, total particulate matter.

Materials and methods

Studies used in the meta-analysis

We considered 12 independent *in vitro* studies on a wide range of test systems and exposure treatments that we have recently published (Figure 1 and Table 1). The human organotypic aerodigestive (buccal, gingival, nasal, bronchial, and small airway) epithelial cultures represent the various tissues exposed to inhaled aerosols in the *in vivo* situation. The variety of RRPs tested in these studies allowed us to draw conclusions about the response to exposure by product type (*i.e.*, heated tobacco, e-vapor, and smokeless tobacco products). The experimental details and data-generation process are described in the respective publications. The corresponding protocols are available in the IDF file of the ArrayExpress submissions, and the treatment details are available in the ArrayExpress SDRF files (Table 1).

Table 1. List of studies used in the meta-analysis

Study identifier	Exposure item	Exposure regimen	Aerosol generation parameter	Reference	Dataset repository
THS-Bronchial	• 3R4F[(1)] • HTP-THS2.2	A 112-puff exposure	HCI[(5)]	(Iskandar et al., 2017c)	E-MTAB-5318
THS-Nasal	• 3R4F[(1)] • HTP-THS2.2	A 112-puff exposure	HCI[(5)]	(Iskandar et al., 2017b)	E-MTAB-6613
THS-Small airway	• 3R4F[(1)] • HTP-THS2.2	A 112-puff exposure	HCI[(5)]	(Iskandar et al., 2017a)	E-MTAB-6004
THS-Buccal	• 3R4F[(1)] • HTP-THS2.2	A 112-puff exposure	HCI[(5)]	(Zanetti et al., 2016)	E-MTAB-6545
THS-Gingival	• 3R4F[(1)] • HTP-THS2.2	A 112-puff exposure per day for 3 days	HCI[(5)]	(Zanetti et al., 2017)	E-MTAB-5609
CHTP-Nasal	• 3R4F[(1)] • HTP-CHTP1.2	A 112-puff exposure	HCI[(5)]	(Iskandar et al., 2018)	E-MTAB-6609
CHTP-Small airway	• 3R4F[(1)] • HTP-CHTP1.2	A 112-puff exposure	HCI[(5)]	(Iskandar et al., 2018)	E-MTAB-6610
CHTP-Buccal	• 3R4F[(1)] • HTP-CHTP1.2	A 112-puff exposure	HCI[(5)]	(Zanetti et al., 2018)	E-MTAB-6543
CHTP-Gingival	• 3R4F[(1)] • HTP-CHTP1.2	A 112-puff exposure per day for 3 days	HCI[(5)]	(Zanetti et al., 2018)	E-MTAB-6538
Snus-Gingival	• 3R4F[(1)] TPM • Snus CRP1.1[(2)] • Snus GCW[(3)]	Continuous exposure for 72 h	N/A	(Zanetti et al., 2019)	E-MTAB-7580
P4M3-Small airway	• 3R4F[(1)] • E-vapor-P4M3[(4)]	A 28/112-puff exposure	• HCI • CORESTA[(6)]	(Iskandar et al., 2019)	E-MTAB-7912
P4M3-Buccal	• 3R4F[(1)] • E-vapor-P4M3[(4)]	A 112/228-puff exposure	• HCI • CORESTA[(6)]	(Iskandar et al., 2019)	E-MTAB-7912

CHTP, carbon heated tobacco product; HCI, Health Canada Intense; HTP, heated tobacco product; N/A, not applicable; THS, Tobacco Heating System; TPM, total particulate matter. [(1)]3R4F reference cigarettes (Kentucky Tobacco Research & Development Center; University of Kentucky, Lexington, KY, USA). [(2)]Swedish snus-type CORESTA Reference Product 1.1 (CRP1.1, Tobacco Analytical Services Laboratory, North Carolina State University, Raleigh, NC, USA). [(3)]Swedish snus-type General Classic White (GCW, Swedish Match, Stockholm, Sweden). [(4)]An e-vapor product with *MESH* technology (P4M3 generation 1.0, Philip Morris International with Classic Tobacco flavor). [(5)]Puff generation from one cigarette was conducted for a puff volume of 55 mL, puff duration of 2 s, and puff interval of 30 s (Health Canada, 1999). [(6)]Puff generation from one P4M3 device was conducted for a puff volume of 55 mL, puff duration of 3 s, and puff interval of 30 s (CORESTA, 2015).

Exposure setup

Briefly, all studies in Table 1 (except the Snus-Gingival study) used the VITROCELL® 24/48 exposure system (VITROCELL Systems GmbH, Waldkirch, Germany) for the experiments. Cultures were placed in the wells of the base module. A dilution/distribution module located on top of the base module allows delivery of aerosols directly to the apical side of the cultures. Aerosols were generated either by a smoking machine (in case of CS or HTPs) or a programmable single syringe pump (in case of e-vapor products) and then injected into the system.

For the Snus-Gingival study, a TPM fraction was prepared from 3R4F CS as previously described (Zanetti et al., 2019). For snus, the CORESTA reference product (CRP) 1.1 (Tobacco Analytical Services Laboratory, North Carolina State University, Raleigh, NC, USA) and General Classic White (GCW, Swedish Match, Stockholm, Sweden) extracts were prepared in phosphate-buffered saline as previously described (Zanetti et al., 2019). TPM and extracts of CRP1.1 and GCW were added directly to the apical side of the cultures for 72 h (the treatment was renewed every 24 h). In the present study, exposure to CS or the TPM fraction of CS will be referred to as "CS exposure".

Exposure to the different products aerosols/extracts was performed at different concentrations selected on the basis of nicotine content during previous dose-range-finding experiments. In this study, these concentrations are indicated as "Low", "Medium", and "High." In case of the e-vapor studies (*i.e.*, P4M3-Small airway and P4M3-Buccal (Iskandar et al., 2019)), two undiluted aerosols were tested: the complete *MESH* Classic Tobacco formulation (containing propylene glycol, glycerol, nicotine, and flavor ingredients) and the Base aerosol (containing propylene glycol, glycerol, and nicotine). For simplicity, these distinct aerosols are referred to as the "High" and "Low" concentrations, respectively.

Calculation of miRNA response to exposure treatments

As all 12 studies had uniformly implemented a block-based paired experimental design (Iskandar et al., 2017c), we preprocessed and analyzed the raw expression data as one single "meta-study." This approach differs from the meta-analyses of less homogeneous studies in which the results (and not the raw data) are aggregated and analyzed by using ad-hoc statistical models.

The miRNA response to each treatment (Figure 2, *upper panel*) was computed by using the miRNA differential expression data obtained by running the same pipeline used in the individual studies (Table 1). The detailed data preprocessing steps are described in the Supplementary Materials and Methods. Briefly, 2775 raw expression data CEL files obtained with the Affymetrix® GeneChip™ miRNA 3.0 and 4.0 array platforms (Thermo Fisher Scientific, Waltham, MA, USA) were collected mainly from the ArrayExpress public repository (Athar et al., 2019) (see Supplementary Material and Methods). The normalized expression data were obtained by applying robust multiarray normalization (Bolstad et al., 2003) without background

correction. To distinguish the "detected" miRNAs from background noise, a binary detection call was assigned to each expression value by following the recommendations of the platform manufacturer (Thermo Fisher Scientific).

After retaining only those human mature miRNAs that were detected in at least 75% of the samples in at least one of the 292 experimental groups, we obtained a normalized expression data matrix of 736 rows and 2775 columns. The block-based paired design of the 12 studies enabled evaluation of the two versions of the miRNA differential expression data by "treatment vs. control" pairwise comparisons. The first sample-based version, containing 736 rows and 1857 columns, was obtained by subtracting the expression value of the control (air-exposed sample) from that of the treated samples of the same block. The second experimental group-level version, containing 736 rows and 204 columns, resulted from the differences in mean expression values between the treatment and control groups. This was accompanied by statistical significance calculations using moderated *t* statistics (Smyth, 2004).

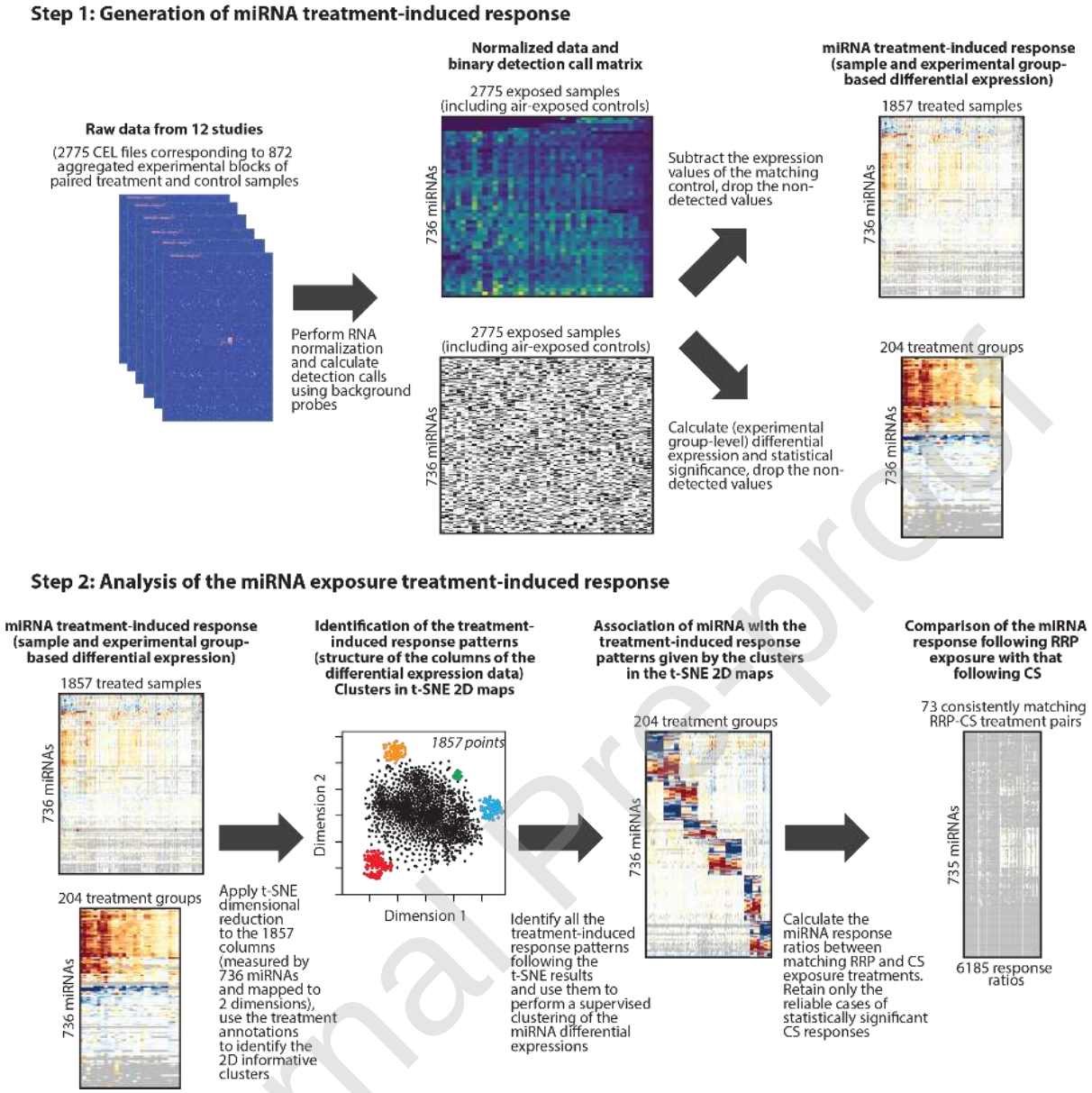


Figure 2. Computational workflows used in the meta-analysis. *Upper panel*, calculation of miRNA treatment-induced response given by the differential expression data. *Lower panel*, analysis of miRNA response. The description of and rationale for the calculations underlying each arrow are given in the corresponding subsections of the Materials and Methods and in the Supplementary Materials and Methods. The graphical representations of the data are schematic and not meant to reproduce the actual results. miRNA, microRNA; CS, CS, 3R4F cigarette smoke and its total particulate matter fraction; RRP, reduced-risk product; t-SNE, t-distributed stochastic neighbor embedding.

Analysis of miRNA response to exposure treatments

Given the two versions of the miRNA differential expression data, the analysis of exposure treatment-induced response consisted of a three-step pipeline (Figure 2, lower panel). The detailed procedures are described in the Supplementary Materials and Methods. The analytical methods were selected for their sensitivity and specificity in identifying certain patterns of miRNA expression as potential biomarkers of exposure. Briefly, we first applied the unsupervised t-SNE dimensional reduction algorithm to identify similarities among the 1857 treatment-induced miRNA responses. To ensure that the clustering results were not dependent on the free perplexity parameter of the t-SNE algorithm, several values were considered, and only the common features were retained. The experimental factors underlying the relevant miRNA expression alteration patterns were deduced from the annotation of the clusters appearing in the resulting t-SNE two-dimensional (2D) maps.

After identifying the experimental factors that most strongly contributed to miRNA expression alterations, we considered the association patterns between individual miRNA alterations and the relevant combinations of experimental factors. This step enabled us to rearrange the 736 detected miRNAs into distinct groups exhibiting similar response patterns, a feature that fitted well to the main objective (*i.e.*, to identify biomarkers of exposure).

Calculation of miRNA response ratios between RRP- and CS-exposure treatments

Because the block-based paired experimental designs of the 12 studies were similar, we obtained 73 consistently matching RRP–CS exposure treatment pairs from the 204 available exposure treatments (*i.e.*, matching date of exposure, nicotine concentration, culture batch, and post-

exposure measurement time points). A total of 735 miRNAs were detected in these 2 × 73 = 146 exposure treatment groups, one less than the number in the complete set of 204 treatment groups (Figure 2, lower panel).

For the consistently matching RRP–CS exposure treatment pairs, we calculated the miRNA response ratios *r* to compare the miRNA alterations following RRP exposure (relative to the air controls) with those following CS exposure (relative to the air controls); *r* is obtained by dividing the RRP response by the CS response. To ensure reliable results, we retained only those *r* values of the miRNAs that exhibited a statistically significant CS response (see Materials and methods: Calculation of miRNA response to exposure treatments and Figure 2). This approach enabled us to distinguish three cases in evaluating *r*, which differed by the type of RRP response: (1) undetected, (2) detected but statistically not significant, or (3) detected and statistically significant.

Subsequently, we estimated the overall response ratio ($r_{average}$) for each miRNA by averaging the available *r* values regardless of the product type, tissue type, dose, and post-exposure measurement time.

Finally, following an approach used previously (Zanetti et al., 2018, Iskandar et al., 2018), we evaluated the relative reduction in miRNA expression following exposure to RRPs (*i.e.*, the difference between CS- and RRP-induced effects) relative to the CS-induced effect, which was taken as 100%.

A reduction of more than 100% (observed, for example, when the two effects had opposite signs) was truncated to 100% reduction to achieve a conservative estimate. For a given miRNA, the relative reduction was equivalent to $100 \times (1 - \max(0, r_{average}))$, where the "max"

operation selects the larger of its arguments. The overall percentage reduction for the RRP was obtained by averaging the relative reductions across all available miRNAs.

Results

Assessing global miRNA alterations across 12 studies

We first examined the miRNA response to exposure in the 12 studies. Figure 3 shows a heatmap of the experimental group-based differential expression of all miRNAs detected in comparison with the expression in the air-exposed (sham) controls (204 comparisons). The global view indicates a highly diverse response with multiple patterns of clustering. The high proportion of weakly detected miRNAs (almost one third) showed that proper treatment of signal detection was necessary to obtain reliable expression data. Among the organotypic cultures investigated in the meta-analysis, gingival and buccal cultures exhibited the highest miRNA expression alterations in response to CS or its TPM fraction. The responses to CS and TPM exposure were comparable (Figure 4B). The response of the cultures to RRPs was much lower than that to CS. Among the RRPs, the highest miRNA expression alterations were observed in gingival cultures in response to Swedish snus extracts and CHTP 1.2 aerosol exposure at high concentrations.

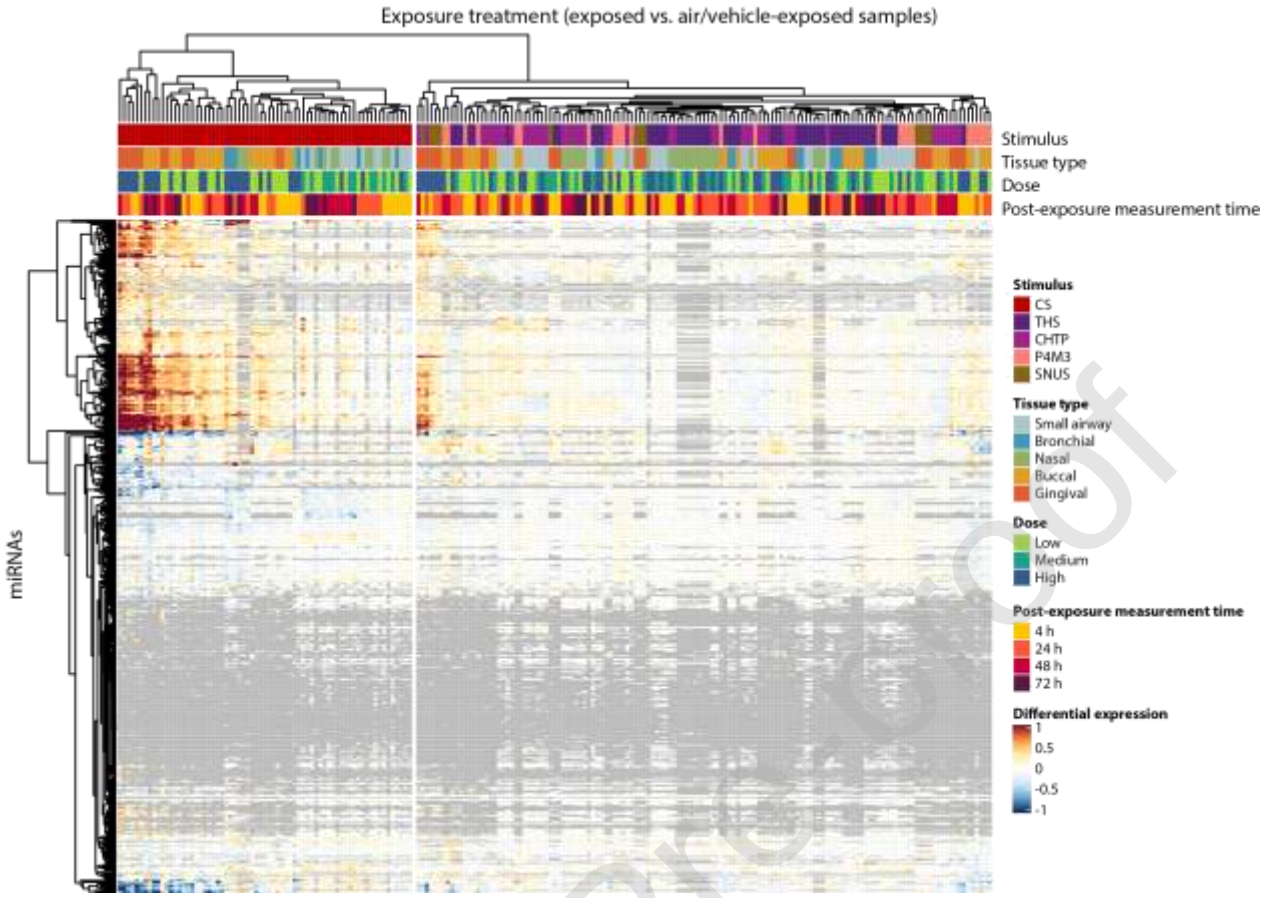


Figure 3. Heatmap of experimental group-based miRNA differential expression profiles. The columns correspond to the 204 "treatment vs. control" pairwise comparisons in the 12 studies included in the meta-analysis. The rows correspond to the 736 miRNAs detected above background noise in at least one of the considered comparisons (see Materials and methods). When a differential expression value was not available because miRNA expression was not detected, grey was used instead of the color map. The vertical grey stripes correspond to the "THS-Nasal" study, which had used the earlier GeneChip™ miRNA 3.0 array platform with a lower coverage of human miRNAs. The clustering was performed by using Euclidean distances with 0 replacing the missing values and the complete-linkage method. CHTP, Carbon Heated Tobacco Product; CS, 3R4F cigarette smoke or its total particulate matter fraction; miRNA, microRNA; P4M3, e-vapor product; THS, Tobacco Heating System.

Identifying patterns of miRNA alterations across the 12 in vitro studies

To better understand the patterns of miRNA alterations in the 12 studies, we first examined the structure of the exposure treatment dimension. Our goal was to identify similarities in the larger variety of miRNA responses to exposure treatments obtained from the 204 included combinations of five tissues, five stimuli, three relative doses, and four post-exposure measurement times. For

this, we applied the t-SNE dimensional reduction technique, which mapped the original 736 dimensional coordinates of miRNA-based treatment responses into the 2D coordinates of a point on a plane (Figure 2, lower panel). The visualization of the 2D distribution of the mapped points enabled us to extract similarity relationships by their mutual distance and construct clusters of similar responses to treatment.

Figure 4A shows the t-SNE results with three annotating color schemes based on tissue type, stimulus, and post-exposure measurement time. The reduced dimensional distribution showed a large cluster around the center, comprising samples with mixed annotations, and four peripheral clusters (*a*, *b*, *c*, and *d*), grouped for defined stimulus, tissue type, and post-exposure measurement time. In interpreting t-SNE results, it is important to consider that only the *local* structure of the original space will be preserved: Neighboring points in two dimensions do correspond to neighboring points in the original space, but small/large and close/distant clusters in two dimensions do not necessarily reflect the same property of the original clusters.

A central cluster of samples exposed to CS and measured at the early post-exposure times (4 and 24 h post-exposure) and those exposed to RRPs was observed (Figure 4A). This indicated a non-specific response to the corresponding exposure treatments, which was globally too weak to be split into distinct parts.

Moreover, Figure 4A shows three small clusters (*a*, *b*, and *c*) that were associated with exposure to CS (middle panel). A separation of responses in buccal and gingival tissue cultures was detected as clusters *b* and *c*, respectively (left panel). These responses were also generally linked to the later post-exposure measurement times (*i.e.*, 48 and 72 h post-exposure; right panel). Additionally, cluster *a* was observed in all three pseudostratified epithelial tissue types (nasal,

bronchial, and small airway cultures; left panel). This cluster mainly included samples exposed to CS and measured 24, 48, and 72 h post-exposure (middle panel). An additional cluster, *d*, although fairly smaller than the other clusters, was linked to small airway samples exposed to P4M3 e-vapor and CS.

To confirm the observed dominance of the tissue type-based separation of responses to CS obtained from the t-SNE calculations, we examined their similarity relationships using a different approach (Figure 4B). The heatmap in Figure 4B shows that the miRNA profiles of gingival samples exposed to CS correlated better with the profiles of buccal samples than with those of nasal, bronchial, or small airway samples, regardless of the study. Moreover, miRNA expression in nasal, bronchial, and small airway samples was also well correlated among the studies, although this correlation was slightly less than that between the buccal and gingival culture responses. These results indicate a conserved pattern of miRNA expression alterations in response to CS.

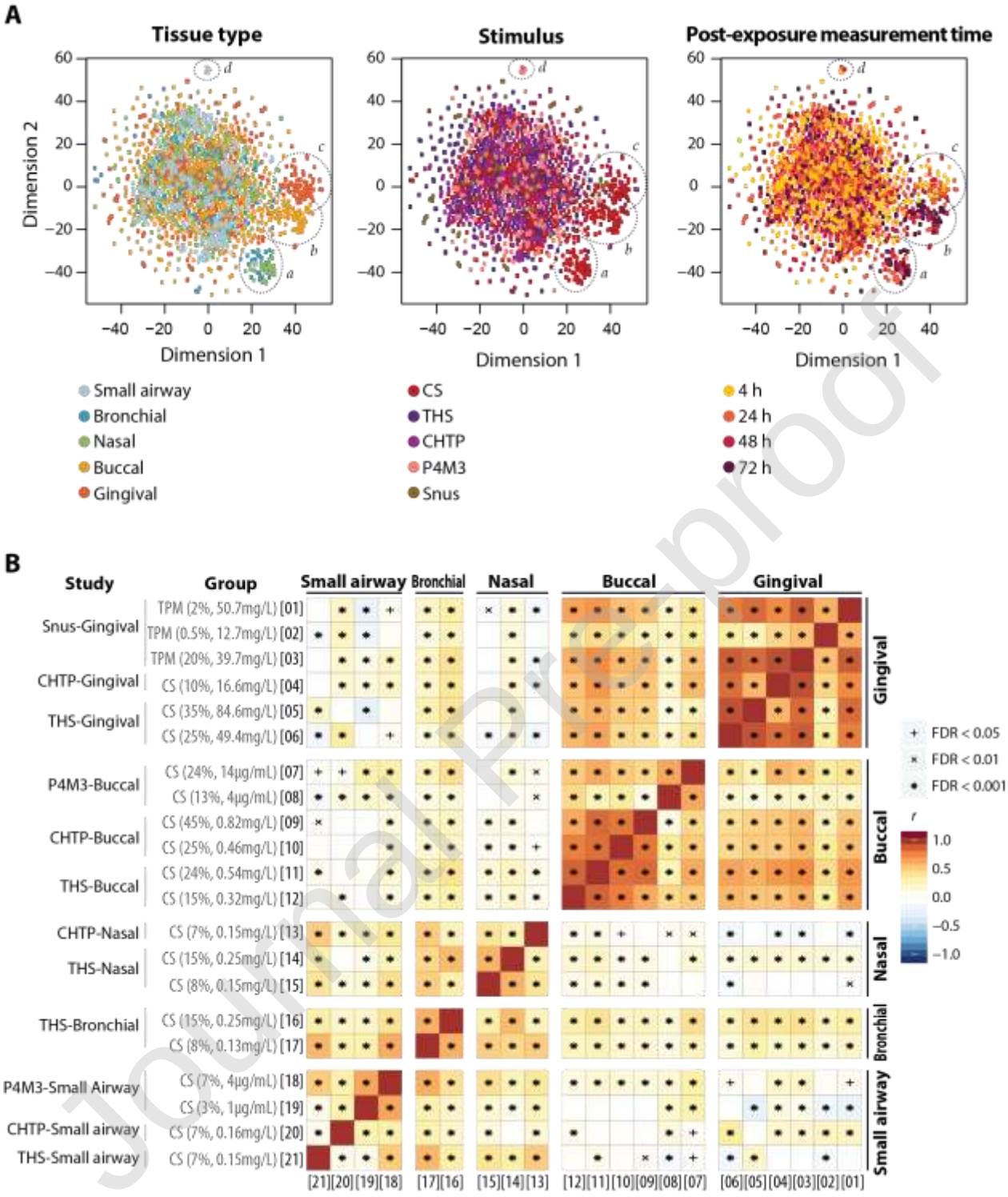


Figure 4. t-SNE dimensional reduction plot and Pearson correlation coefficients of alterations in miRNA expression following exposure. (A) Two-dimensional distribution of the miRNA-based treatment responses obtained by the t-SNE dimensional reduction technique (perplexity = 55). Instead of the 204 experimental group-based miRNA differential expression profiles shown in Figure 3, we used the wider 1857 sample-based miRNA differential expression profiles to offer optimal input to the t-SNE method (see Materials and methods). The three plots

display the same mapped points but are colored by tissue type, stimulus, and post-exposure measurement time (from left to right). The four clusters (*a–d*) were identified after we observed a common annotation ("tissue type" in our case) in some denser groups of points. (B) Heatmap of the Pearson correlation coefficients among the 21 experimental group-based and post-exposure time-merged miRNA differential expression profiles following exposure to CS. The forced tissue type-based ordering of the rows and columns enables visualization of the similarities across tissue types and confirms the dominance of the tissue type variable in the high-level separation of miRNA-based treatment responses. CHTP, Carbon Heated Tobacco Product; CS, CS, 3R4F cigarette smoke or its total particulate matter fraction; FDR, false discovery rate-adjusted *p* values; miRNA, microRNA; P4M3, e-vapor product; *r*, ratio of the corresponding differential expression values for each detected miRNA between reduced-risk products and CS; THS, Tobacco Heating System; t-SNE, t-distributed stochastic neighbor embedding.

Analyzing the commonly altered miRNAs across the tissues following exposure to CS

After clustering the miRNA expression profiles by stimulus, tissue type, and post-exposure measurement time, we identified the miRNAs involved in the tissue-specific responses observed. We performed supervised clustering of the experimental group-based miRNA differential expression profiles (Figure 3) by grouping the miRNAs in accordance with the tissue specificity of their response pattern. This feature was determined by deciding, for each tissue, whether or not each miRNA was exhibiting a response. The process yielded $2^5 = 32$ potential categories (see Materials and methods). Supplementary Figure 1 shows the number of miRNAs in each category. To focus on the cases that were more likely to exert a biological effect, we isolated the "high-confidence mature miRNAs" (de Rie et al., 2017, Kozomara and Griffiths-Jones, 2014), which represented 10–30% of the total number.

Figure 5 presents the resulting reordered response structure and shows that the response of gingival cultures to CS included the highest number of differentially expressed miRNAs (23). These miRNAs alterations were conserved across all studies in which gingival culture models were used. Moreover, a substantial number of differentially expressed miRNAs (18) were observed in both gingival and buccal cultures following exposure to CS. We detected only 12

miRNAs that exhibited altered expression following exposure to CS in buccal cultures. The analysis also identified 3 miRNAs with conserved differential expression in bronchial, buccal, and gingival cultures following CS exposure (Figure 5).

We did not detect any miRNA that was differentially expressed and conserved in all five tissues (Figure 5). Following CS exposure, only one miRNA (miR-146a-5p) showed altered expression specifically in small airway cultures, and none showed differential expression specifically in nasal cultures. The response of two miRNAs (miR-132-3p and miR-149-5p) was conserved in four tissues (small airway, bronchial, nasal, and buccal), and the differential expression of only one miRNA (miR-708-5p) was conserved in bronchial, nasal, buccal, and gingival cultures. Bronchial and small airway cultures shared only one differentially expressed miRNA (miR-34b-5p) in response to CS.

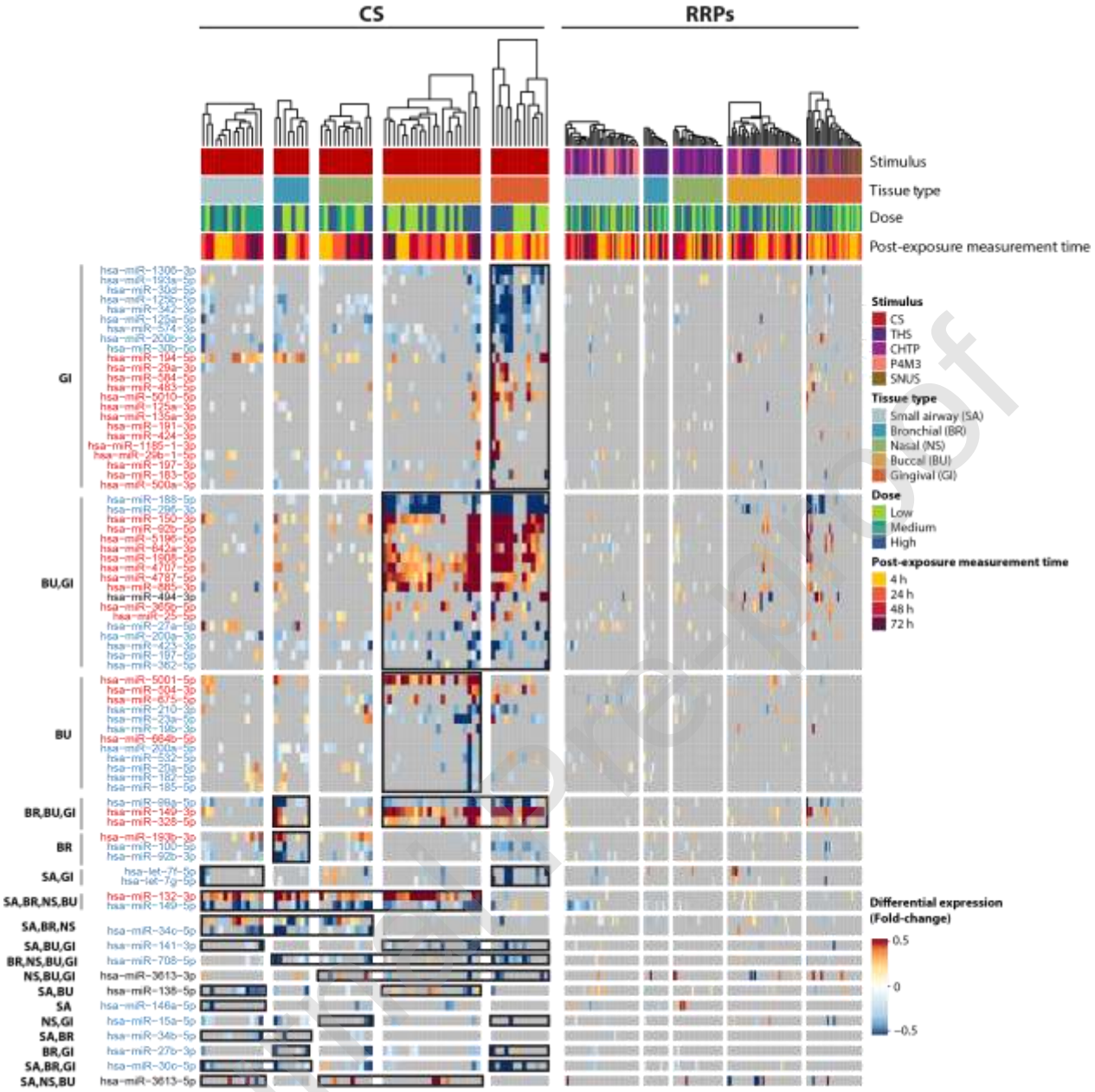


Figure 5. Heatmap of high-confidence treatment-induced alterations in the expression of miRNAs, grouped by tissue type. The rows and columns of the experimental sample group-based differential expression matrix are grouped to highlight the aspect of tissue specificity. miRNAs are associated with the tissue type combination in which they show a response (*i.e.*, they contain at least one "treatment vs. control" pairwise comparison with an absolute differential expression ≥ 0.5 and a corresponding p value ≤ 0.05; see Materials and methods). The "non-reliable" comparisons with p values > 0.05 are displayed in grey for readability, and the color map saturates at ±0.5, so that all "responding" cases appear in the darkest colors. miRNA labels are colored in blue or red when their downregulated or upregulated expression is unambiguous in all relevant tissues. Frames highlight portions of the heatmap corresponding to the tissue types where the conserved high-confidence miRNA expression changes are measured. CHTP, Carbon Heated Tobacco Product; CS, CS, 3R4F cigarette smoke or its total particulate matter fraction;

miRNA, microRNA; P4M3, e-vapor product; RRP, reduced-risk products; THS, Tobacco Heating System.

Comparing miRNA alterations following exposure to RRPs with those following CS exposure

Next, we investigated the impact of RRPs on miRNA expression in relation to that of CS exposure by calculating the response ratios (*r*) between the RRP- and CS-induced alterations at an individual miRNA level. The calculation of *r* between the RRP and CS exposures was based on the 73 consistently matching RRP–CS exposure treatment pairs that were assembled from the meta-analysis data. A treatment pair (*i.e.*, consistently matching RRP–CS exposure) shares all experimental factors except the product type (*i.e.*, the date of exposure, nicotine concentration, culture batch, and post-exposure measurement time point).

We restricted ourselves to the reliable significant CS-induced miRNA alterations and extracted 6185 *r* values from the meta-analysis of data on miRNA alterations (see Materials and methods: Calculation of miRNA response ratios between RRP- and CS-exposure treatments). Figure 6A shows the distribution of the RRP response-types obtained when evaluating these 6185 *r* values. We found that the majority (91.71%) corresponded to cases without significant miRNA alterations following RRP exposure. Furthermore, RRP exposure response was not detected in 8.16% of the cases (because the signal was too low), and, therefore, the *r* value was set to 0. Finally, a very small percentage (0.13%) corresponded to the 8 cases of significant miRNA alterations following RRP exposure. A visualization of the miRNAs and the consistently matching RRP–CS exposure treatment pairs associated with these *r* values is shown in Supplementary Figure 2.

To better understand the response ratio *r*, we averaged the available *r* values to obtain a single $r_{average}$ value for each miRNA. These values were calculated by aggregating the available *r* values regardless of the product type, tissue type, dose, and post-exposure duration. The resulting distribution of $r_{average}$ values is shown in Figure 6B. The values ranged from -1.0 to 1.0, demonstrating that the alterations of miRNAs following RRP exposure were generally less than those following CS. Moreover, 90% of the values of $r_{average}$ were present in the narrower interval, ranging from -0.25 to 0.25, indicating nearly zero average alterations following RRP exposure.

Finally, we evaluated the relative reduction in miRNA expression alterations in response to RRPs compared with CS (see Materials and methods: Calculation of miRNA response ratios between RRP- and CS-exposure treatments). We obtained a value of 94% for the overall relative reduction in miRNA **alterations** following RRP exposure with respect to CS (Figure 6C).

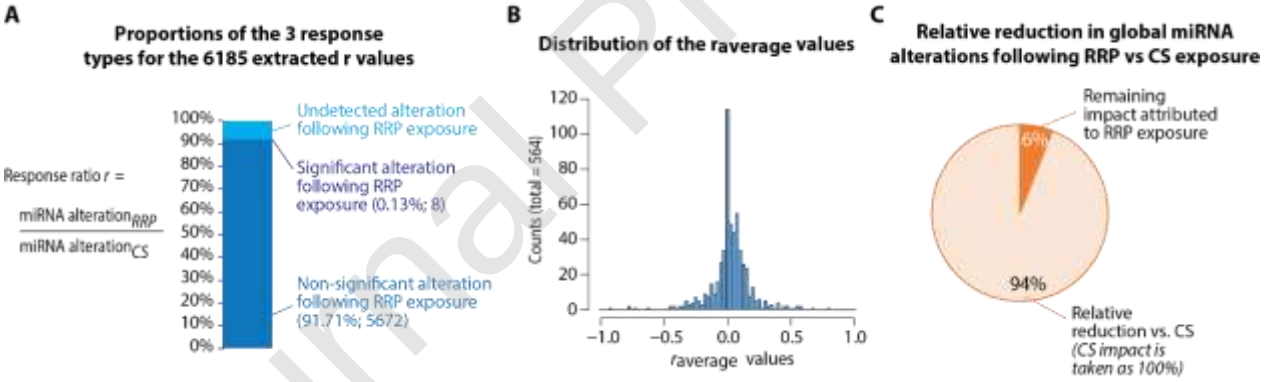


Figure 6. Evaluation of response ratios *r* for comparing the miRNA alterations following RRP exposure with those following CS exposure. (A) Proportions of the three RRP response types for the 6185 response ratio values extracted from the meta-analysis data, including data on 735 miRNAs and 73 consistently matching RRP–CS exposure treatment pairs. (B) Distribution of response ratio $r_{average}$ values obtained by averaging all available consistently matching RRP–CS exposure treatment pairs for individual miRNAs. (C) Global relative reduction in miRNA alterations observed in response to RRP aerosol exposure compared with CS. The CS-induced alteration was set to 100% and is represented by the full circle; hence, the relative reduction of the RRP exposure response is simply given by the complementary of the RRP-induced alterations relative to the CS-induced alterations. CS, 3R4F cigarette smoke and its total particulate matter fraction; RRP, reduced risk products.

Discussion

In this study, we conducted a meta-analysis of a miRNA dataset from 12 *in vitro* studies that compared the effects of exposure to different RRPs (e-vapor products, HTPs, and extracts from Swedish snus products) with those of exposure to CS in organotypic cultures from the aerodigestive tract. These cell culture models retain the three-dimensional structure of the native epithelia; they are cultured at the air–liquid interface, allowing direct exposure to CS and aerosols on the apical side, as it occurs in humans (Grego et al., 2016, Knudsen et al., 2015). These cultures can also be exposed apically to liquid solutions, a setup fit for assessing exposure to snus extracts (Zanetti et al., 2019).

Because of the complexity of the datasets from the 12 studies, which included multiple exposure types, tissue types, product doses, and post-exposure measurement time points, understanding the patterns of alterations in miRNA expression is not straightforward. We opted to use the t-SNE approach, an unsupervised exploratory reduction method (Van der Maaten and Hinton, 2008), to identify expression patterns across the 12 datasets. We found that the distinguishable t-SNE clusters corresponded roughly to the different tissue types in case of CS exposure. More precisely, the t-SNE results showed that the miRNA alterations in gingival and buccal cultures following CS exposure were clustered closely. Despite the differing doses and modes of administration used in these studies – buccal cultures were exposed acutely to whole CS (Zanetti et al., 2016, Zanetti et al., 2018), and gingival cultures were exposed to whole CS or its TPM fraction for 3 days (Zanetti et al., 2017, Zanetti et al., 2019) – the analysis revealed that the miRNA patterns of expression were similar although not identical. Although buccal cultures are non-cornified and gingival cultures are cornified, both culture models comprise keratinocytes that form a stratified squamous epithelium. Interestingly, the profiles of secreted inflammatory

mediators following CS exposure from buccal and gingival epithelial cultures were also comparable but not identical. For example, CS exposure in buccal cultures significantly increased the secretion of interleukin (IL) 1β and matrix metalloproteinase 1 (MMP-1) (Zanetti et al., 2016), while that in gingival cultures did not increase IL-1β secretion markedly, even though it did increase the secretion of MMP-1 (Zanetti et al., 2017).

Furthermore, the t-SNE analysis revealed that the CS-induced miRNA alterations in the pseudostratified epithelial tissue types (nasal, bronchial, and small airway epithelia) were clustered together and distinct from the clusters observed in buccal and gingival cultures. Therefore, the miRNA alterations following CS exposure in these three culture types were markedly similar. This notion is aligned with the "field of injury" hypothesis, which proposes that exposure to inhaled insults elicits a common molecular response throughout the respiratory tract (Sridhar et al., 2008). However, our observation (*i.e.*, the clusters in the nasal, bronchial, and small airway tissues being distant from those in the buccal and gingival tissues) differed from the findings of Sridhar and colleagues (Sridhar et al., 2008). Sridhar and colleagues found that the high-level gene expression observed in nasal cells collected from human donors was similar to that in cells collected from the buccal mucosa following tobacco exposure. This difference could be attributed to the diverse datasets used in the two analyses: Our present meta-analysis assessed miRNA profiles in *in vitro* culture models, whereas Sridhar and colleagues compared mRNA datasets from nasal and buccal epithelial cells from biopsy samples. Furthermore, while our *in vitro* datasets might provide a larger dataset with greater sensitivity (*i.e.*, the doses were controlled), they reflect the results of a limited number of donors.

Another aspect to consider when comparing the miRNA responses in gingival and buccal cultures with those in small airway, bronchial, and nasal cultures response is the CS dose applied:

The gingival and buccal cultures were exposed to higher doses of CS than the small airway, bronchial and nasal cultures. This difference in exposure was related to the higher resistance of the oral cultures to CS. Therefore, with the responses in nasal, bronchial, and small airway cultures being lower than those in gingival and buccal cultures, the correlations of miRNA alterations among the nasal, bronchial, and small airway studies were less marked.

The next step of our meta-analysis focused on identifying the miRNAs underlying the alteration patterns by extracting the associations between miRNA responses and tissue types. This was done by determining whether a given miRNA was "responding" or not in a given tissue – that is, whether or not the miRNA being considered was exhibiting an absolute differential expression value > 0.5 and a corresponding p value < 0.05 in at least one exposure treatment involving this tissue. When applied to the five available tissue types, this binary "response status" enabled us to classify the 736 detected miRNAs into $2^5 = 32$ groups, each of them associated with a specific tissue response pattern.

We found 14 miRNAs with upregulated expression and 9 with downregulated expression in gingival cultures. Among these, the expression of miR-125b-5p, miR-30b-5p, and miR-200b was downregulated. The expression of miR-125b-5p has also been reported to be downregulated in inflamed gingival biopsy specimens (Ogata et al., 2014). This miRNA targets cytochrome P450 1A1, modulating the xenobiotic metabolism response; this finding is consistent with the notes in a previous publication (Burgess et al., 2015). Further, the expression level of mir-30b-5p in saliva has been reported to be lower in patients with severe periodontitis than in healthy individuals (Fujimori et al., 2019). In the present study, the expression of miR-200b, which has been reported to be upregulated in inflamed gingiva (Ogata et al., 2014), was found to be upregulated in *in vitro* gingival cultures following exposure to CS. The discrepancy could be

attributable to the samples (*i.e.*, the organotypic gingival culture models include only keratinocytes, whereas the biopsy samples might comprise various cell types, such as inflammatory cells and fibroblasts). Among the miRNAs with upregulated expression, miR-150-3p has also been shown to exhibit upregulated expression in periodontitis patients (Ogata et al., 2014).

The present analysis also identified 3 miRNAs with consistently altered expression following CS exposure in buccal, gingival, and bronchial cultures. The expression of two of these miRNAs (miR-149-3p and miR-328-5p) was upregulated and that of the third (miR-99a-5p) was downregulated. Upregulation of miR-149-3p expression following CS exposure has also been reported in bronchoscopy samples from asthma patients (Solberg et al., 2012), blood samples from smokers (Shen et al., 2017a), and THP-1 cells (Shen et al., 2017a). In contrast, Chan and colleagues reported that the expression of miR-149-3p is downregulated in gingival periapical lesions and linked to inflammation (Chan et al., 2013). Again, it is possible that the contribution of cell types other than keratinocytes might account for this discrepancy and that the molecular mechanisms activated in response to CS might differ from those triggered by a given disease or disorder. The downregulation of miR-99a-5p expression observed in our analysis was particularly interesting because the expression of miR-99a-5p has been reported to be downregulated more than that of other miRNAs in periodontal lesions (Pettiette et al., 2019) and gingiva from periodontitis patients (Ogata et al., 2014, Stoecklin-Wasmer et al., 2012). Moreover, miR-99a-5p expression is also downregulated in bronchial epithelial brushes from asthma patients when compared with the levels detected in samples from healthy subjects (Solberg et al., 2012). In an animal model, miR-99a-5p was hypothesized to regulate the initial phases of dermal wound-healing by regulating the protein kinase B (AKT)/mammalian target of rapamycin (mTOR)

signaling pathway (Jin et al., 2013). Therefore, it could be speculated that miR-99a-5p is involved in structural changes induced by environmental factors (*e.g.*, tissue damage) in epithelial tissues.

The differential expression of miR-34b-5p was specific to CS-induced downregulation in bronchial and small airway cultures. This was interesting because the expression of miR-34b-5p has been reported to be strongly downregulated in bronchoscopy specimens from asthma patients in comparison with healthy subjects (Solberg et al., 2012) and in induced sputum from current smokers with COPD in comparison with that from never-smokers without airway limitation (Pottelberge et al., 2011). In bronchial cultures, specifically, we detected two miRNAs with downregulated expression (miR-100-5p and miR-92b-3p) and one miRNA with upregulated expression (miR-193b-3p) following CS exposure. Keller and colleagues reported that the blood levels of miR-92b-3p differ significantly between COPD patients who were current smokers and those who were former smokers (Keller et al., 2018).

Three miRNAs – miR-146a-5p, miR-30c-5p, and miR-132-3p – with altered expression following CS have been linked to inflammation. We found that the downregulation of miR-146a-5p expression was highly specific to small airway cultures in our analysis. An inverse correlation between miR-146a levels and inflammation in alveolar epithelial cells has been reported (Perry et al., 2008); additionally, in a previous study, the expression of miR-146a-5p was downregulated in fibroblasts from COPD patients and involved in epithelial–fibroblast communication, supporting its role in proinflammatory signaling (Osei et al., 2017). Furthermore, the expression of miR-30c-5p, which was downregulated following CS exposure in gingival, bronchial, and small airway cultures in the present analysis, has also been found to be downregulated in the lungs of COPD patients (Shen et al., 2017b) and in bronchoscopy specimens from asthma patients (Solberg et al.,

2012). The expression of this miRNA was also reported to be downregulated in gingival biopsy specimens from patients with periodontitis (Pettiette et al., 2019). Finally, in the present analysis, the expression of miR-132-3p was upregulated upon CS exposure, and this upregulation was specific for buccal, small airway, nasal, and bronchial cultures. miR-132-3p is an evolutionarily conserved miRNA (Haviv et al., 2018); we have previously shown that it might be involved in inflammatory response in buccal cultures (Zanetti et al., 2018) and in the downregulation of *MMP9* expression. miR-132-3p might be involved in the progression from inflammation to wound-healing processes in keratinocytes (Liu et al., 2015), and its expression has been reported to be increased in human bronchial brushes exposed to allergens, suggesting that it modulates the inflammatory response (Rider et al., 2016). Overexpression of miR-132-3p has been shown to inhibit lipopolysaccharide-mediated inflammation in alveolar macrophages by targeting acetylcholinesterase (Liu et al., 2015). Overall, these findings help identify miR-132-3p as a potential marker of inflammation and indicate that its expression might be altered in various tissues in response to CS exposure.

Other miRNAs of interest whose expression was modulated by CS were miR-141-3p (specific for small airway, buccal, and gingival tissues), miR-27b-3p (bronchial and gingival tissues), and let-7g-5p (small airway and gingival tissues). The expression of all three miRNAs has been reported to be downregulated in bronchoscopy specimens from asthma patients (Solberg et al., 2012). We observed a similar regulation of miRNAs in bronchoscopy specimens from asthma patients (Solberg et al., 2012) and in the lung organotypic cultures (bronchial and small airway) exposed to CS in our study. CS exposure might modify the inflammatory responses associated with asthma and might be a cause of adult-onset asthma (Thomson et al., 2004). It has also been shown that the risk of adult-onset asthma in smokers and former smokers is higher than

in non-smokers (Piipari et al., 2004). More recently, a study suggested that cigarette smoking could initiate alterations in small airway lung functions before onset of asthma (Jaakkola et al., 2019). Therefore, organotypic lung epithelial cultures exposed to CS might be a promising avenue for studying early molecular mechanisms in the pathogenesis of asthma. We provide in Table 2, the list of miRNAs that were discovered in the present meta-analysis to be altered following exposure to CS.

Table 2. List of miRNAs regulated by CS

miRNA	*In vitro* response					*In vivo* response	Potential activity	References
	Small airway	Bronchial	Nasal	Buccal	Gingival			
let-7g-5p	down	-	-	-	down	Downregulated in bronchoscopy samples from asthma patients	Unknown	(Solberg et al., 2012)
miR-27b-3p	-	down	-	-	down	Downregulated in bronchoscopy samples from asthma patients	Unknown	(Solberg et al., 2012)
miR-30b-5p	-		-	-	down	Lower in the saliva of severe periodontitis patients than in healthy individuals	Unknown	(Fujimori et al., 2019)
miR−30c−5p	down	down	-	-	down	Downregulated in the lungs of COPD patients, in bronchoscopy samples from asthma patients, and in gingival biopsy samples from patients with periodontitis	Unknown	(Shen et al., 2017b, Solberg et al., 2012, Pettiette et al., 2019)
miR−34b−5p	down	down	-	-	-	Downregulated in bronchoscopy samples from asthma patients and in induced sputum from current-smoker patients with COPD compared with never-smokers without airway limitation	Unknown	(Solberg et al., 2012, Van Pottelberge et al., 2011)
miR-92b-3p	-	down	-	-	-	Significantly different in the blood of COPD patients who were current smokers and in	Unknown	(Keller et al., 2018)

						the blood of COPD patients who were former smokers		
miR-99a-5p	-	down	-	down	down	Downregulated in periodontal lesions and gingiva from periodontitis patients and in bronchial epithelial brushes from asthma patients	Involved in structural changes (*e.g.*, tissue damage) in epithelial tissues	(Pettiette et al., 2019, Ogata et al., 2014, Stoecklin-Wasmer et al., 2012, Solberg et al., 2012, Jin et al., 2013)
miR-125b-5p	-	-	-	-	down	Downregulated in inflamed gingival biopsy samples	Modulation of the xenobiotic metabolism response	(Ogata et al., 2014, Burgess et al., 2015, Zanetti et al., 2018, Zanetti et al., 2019)
miR-132-3p	up	up	up	up	-	Increased in human bronchial brushes exposed to allergens	Marker of inflammation; progression from inflammation to wound-healing processes in keratinocytes	(Liu et al., 2015, Rider et al., 2016, Zanetti et al., 2018)
miR-141-3p	down	-	-	down	down	Downregulated in bronchoscopy samples from asthma patients	Unknown	(Solberg et al., 2012)

miR-146a-5p	down					Inversely correlated with the levels of inflammatory mediators in blood samples from patients with COPD. Downregulated in fibroblasts of COPD patients. Inversely correlated with the level of miR-146a and inflammation in alveolar epithelial cells	Involved in epithelial–fibroblast communication, supporting its role in proinflammatory signaling	(Chen et al., 2018, Osei et al., 2017, Perry et al., 2008)
miR-149-3p		up		up	up	Upregulated in bronchoscopy samples of asthma patients. Downregulated in gingival periapical lesions	Involved in inflammatory response	(Solberg et al., 2012, Chan et al., 2013)
miR-150-3p	-	-	-	-	up	Upregulated in periodontitis patients	Unknown	(Ogata et al., 2014)
miR-200b					down	Upregulated in inflamed gingiva (the discrepancy could be attributable to the sample type: organotypic gingival cultures comprise only keratinocytes, whereas biopsy samples include different cell types, such as inflammatory cells and fibroblasts)	Unknown	(Ogata et al., 2014)

"-" indicates no specific association with the culture type. "Down" indicates downregulated expression following exposure. "Up" indicates upregulated expression following exposure. ALI, air–liquid interface; CS, cigarette smoke; COPD, chronic obstructive pulmonary disease.

Considering the increasing availability of RRPs such as e-vapor products, HTPs, and Swedish snus, a systematic assessment of how exposure to these products might alter miRNA expression is still lacking. Only a few publications to date have assessed miRNA expression in response to e-vapor product exposure (Solleti et al., 2017, Song et al., 2019, Iskandar et al., 2019), while no study, except those reported in this meta-analysis (Table 1), has investigated the effects of aerosols from HTPs and Swedish snus on miRNA expression profiles.

The evaluation of the response ratio *r* between the RRP- and CS-exposure treatments essentially involved comparison of two miRNA responses with very different magnitudes: a high response magnitude for the CS-exposure treatments and a low, almost undetectable, response magnitude for the consistently matching RRP-exposure treatments. This difference was expected because of the design of the products – that is, these RRPs contain reduced levels of harmful chemicals relative to CS (Peitsch et al., 2018). Performing such comparisons in the framework of a meta-analysis including more than 200 exposure conditions required a rigorous approach leveraging the structure of the rich input data and extracting reliable results from it. In all 12 of the included studies, the RRP aerosols/extracts were comparable to CS in terms of nicotine concentration. The biological effects of RRPs and CS were compared on the basis of similar nicotine concentrations because the RRPs are designed to have significantly lower levels of harmful and potentially harmful constituents than CS, while still delivering satisfying levels of nicotine (Smith et al., 2016). It has been shown that many smokers switching to alternative nicotine products adapt product use behaviors (puffing frequency, intensity, etc.) to achieve a nicotine intake similar to that in cigarette smoking (Stratton et al., 2001).

In the above analysis, we focused on the 6185 cases with reliable *r* values (*i.e.*, those miRNAs and consistently matching RRP–CS exposure treatment pairs that exhibited significant

alterations following the CS-exposure treatment). They contained a subset of 8 cases in which the miRNAs were also significantly altered following RRP exposure. A deeper analysis showed, in general, sporadic expression changes following RRP exposure (Supplementary Figure S2). Among these 8 cases, we found that a subset of miRNAs comprising the paralogs miR-320a/b/c were altered in small airway epithelial cultures, but only at the 24-h measurement time point following exposure to P4M3 e-vapor (Supplementary Figure S2). These cases essentially corresponded to the cluster (*d*) identified in Figure 4, demonstrating the sensitivity of the t-SNE technique. We concluded from the meta-analysis data that the significant miRNA alterations following RRP exposure could not be associated with more than one tissue type, RRP treatment, or post-exposure time (*i.e.*, these responses did not display any recurrent pattern).

Finally, we determined that the overall relative reduction in miRNA response in RRP-exposed cultures relative to CS-exposed cultures was 94%. This result is in the range of the previously described reduction in miRNA expression following CHTP 1.2 aerosol exposure relative to CS exposure (84–100%) (Zanetti et al., 2018, Iskandar et al., 2018). In these two earlier studies, it was also shown that the miRNA-based relative reduction values for CHTP 1.2 aerosol exposure were close to those obtained for explicit measures of the biological impact of exposure, such as the perturbation amplitudes of network models describing cellular stress or inflammation. Therefore, the results of this meta-analysis support the attribution of miRNA alterations as another reliable measure of reduction in relative effect in the case of consistently matching CS- and RRP-exposure treatments.

One of the limitations of the present study is the difficulty in directly using miRNA expression as a biomarker of exposure *in vivo*. Biofluids are reasonably preferred for biomarker sourcing because they can be obtained through minimally invasive means. Future studies could

be conducted on the expression of miRNAs in the basolateral medium of cultures. In addition, the cultures used in the *in vitro* studies represent only a subpopulation of epithelial cells from the aerodigestive tract; immune cells and fibroblasts were not integrated into the models. Therefore, the alterations in miRNA expression account only for the response of keratinocytes (for buccal and gingival cultures) and basal, ciliated, and goblet cells (for small airway, nasal, and bronchial cultures). Moreover, the buccal and gingival cultures in these studies were derived from one donor while the bronchial, nasal, and small airway cultures were derived from various donors.

Conclusions

We found that human organotypic epithelial cultures from the aerodigestive tract retain the capacity to express miRNAs in response to CS or its TPM fraction. A subset of miRNAs were tissue-specific, but, in some cases, the expression was shared among tissues, even of differing organs (*i.e.*, the mouth and lungs); this finding highlights miRNA patterns that had not been observed yet in humans. The observed changes were of particular interest in the context of the pathogenesis of periodontal diseases and asthma, making CS-exposed oral and lung epithelial cultures a promising avenue for research on early molecular events in the pathogenesis of periodontal and respiratory diseases. We identified key miRNAs (*e.g.*, miR-125b-5p, miR-132-3p, miR-99a-5p, and 146a-5p) that could potentially serve as biomarkers of CS exposure in human aerodigestive epithelial tissues. The meta-analysis also revealed that exposure to HTPs, e-vapor products, or Swedish snus exerted much smaller changes in miRNA expression than exposure to CS. Finally, no specific miRNA expression response pattern to HTPs, e-vapor products, or Swedish snus was identified.

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Declaration of interest

All authors are employees of Philip Morris International.

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