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ORIGINAL ARTICLE

Extracellular Vesicle-Encapsulated microRNAs as Novel Biomarkers of Lung Health

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Abstract

Rationale: Early detection of respiratory diseases is critical to facilitate delivery of disease-modifying interventions. Extracellular vesicle-enriched microRNAs (EV-miRNAs) may represent reliable markers of early lung injury.

Objectives: Evaluate associations of plasma EV-miRNAs with lung function.

Methods: The prospective NAS (Normative Aging Study) collected plasma EV-miRNA measurements from 1996–2015 and spirometry every 3–5 years through 2019. Associations of EV-miRNAs with baseline lung function were modeled using linear regression. To complement the individual miRNA approach, unsupervised machine learning was used to identify clusters of participants with distinct EV-miRNA profiles. Associations of EV-miRNA profiles with multivariate latent longitudinal lung function trajectories were modeled using log binomial regression. Biological functions of significant EV-miRNAs were explored using pathway analyses. Results were replicated in an independent sample of NAS participants and in the HEALS (Health Effects of Arsenic Longitudinal Study).

Measurements and Main Results: In the main cohort of 656 participants, 51 plasma EV-miRNAs were associated with baseline lung function (false discovery rate-adjusted P value < 0.05), 28 of which were replicated in the independent NAS sample and/or in the HEALS cohort. A subset of participants with distinct EV-miRNA expression patterns had increased risk of declining lung function over time, which was replicated in the independent NAS sample. Significant EV-miRNAs were shown in pathway analyses to target biological pathways that regulate respiratory cellular immunity, the lung inflammatory response, and airway structural integrity.

Conclusions: Plasma EV-miRNAs may represent a robust biomarker of subclinical lung injury and may facilitate early identification and treatment of patients at risk of developing overt lung disease.

Keywords: extracellular vesicles; microRNAs; lung function; spirometry

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Author Contributions: C.M.E. and H.W. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. P.S.V., T.R.B., P.D.H., A.V., and L.C.L. were responsible for generating the Normative Aging Study data. F.P. was responsible for generating the Health Effects of Arsenic Longitudinal Study data. C.M.E., H.W., S.G., P.S.V., A.A.L., D.S., J.S., and A.A.B. contributed to study design. C.M.E., S.G., J.S., A.A.B., and H.W. contributed to the analyses. C.M.E. and H.W. drafted the manuscript. All authors revised the manuscript and had final responsibility for the decision to submit for publication.

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At a Glance Commentary

Scientific Knowledge on the Subject: Extracellular vesicles (EVs) are nano-sized, membrane-bound vesicles that arise naturally from cells in the lung and contain biologically active cargo including EV-encapsulated microRNAs (EV-miRNAs). Emerging research suggests EV-miRNAs may provide a mechanistic link between inhaled exposures and the pathogenesis of chronic lung diseases.

What This Study Adds to the Field: This is the first extracellular vesicle-wide association study to evaluate associations of global plasma EV-miRNAs with lung function in humans. The study suggests that EV-miRNAs may represent a viable biomarker of subclinical lung injury and may help identify individuals at risk of developing lung function impairment.

Chronic respiratory diseases, including chronic obstructive pulmonary disease (COPD), affect over 540 million people worldwide and are leading causes of disability and death (1). The prevalence of chronic respiratory diseases continues to increase, which may be partially attributed to a rising global burden of air pollution and smoking (2). Spirometry remains the gold standard for diagnosing many respiratory diseases (3), but early pathophysiologic changes precede overt decrements in spirometric parameters (4, 5). Early detection of respiratory diseases can facilitate delivery of disease-modifying interventions that slow lung function decline (6–8). Thus, as the burden of respiratory diseases continues to rise, the need for robust markers of subclinical lung injury becomes increasingly urgent.

Circulating extracellular vesicles (EVs) may be accessible markers of early lung damage. EVs are nano-sized, membrane-bound vesicles that arise naturally from cells in the lung and other organs in the body (9). EVs facilitate intercellular communication by transferring biologically active cargo to neighboring cells. This cargo includes small, noncoding microRNAs (EV-miRNAs) that regulate mRNA stability and alter biological activity in recipient cells (10). EVs are released in response to inhaled exposures including cigarette smoke and particulate air pollution (11, 12). EVs modulate immunologic processes via their miRNA cargo (13) and trigger a pro-inflammatory signaling cascade that may contribute to airway remodeling (14). EV-miRNAs may therefore provide a mechanistic link between inhaled exposures and the pathogenesis of chronic lung diseases.

Emerging research suggests changes in blood EVs correlate with lung function decline and respiratory diseases. Cross-sectional profiling of plasma EVs in nonsmokers, smokers, and COPD patients showed differential EV concentration, phenotypic characteristics, and EV-miRNA expression in COPD patients compared with smokers and nonsmokers (15). In a prospective study of 48 patients with COPD, higher numbers of endothelial microparticles, a type of EVs, were predictive of accelerated lung function decline (16). However, while prior studies have suggested associations between plasma EVs and lung function impairment, the extent to which these relationships are independent of confounding factors such as smoking remains unknown. Further, no prior studies have examined the role of total EV-miRNAs in the context of lung function decline over time. We therefore aimed to evaluate associations of EV-miRNAs with lung function in two cohorts of older adults.

Methods

Study Design

The US Veterans Affairs NAS (Normative Aging Study) is a longitudinal cohort study based in Massachusetts that recruited 2,280 men aged 21–80 years (17, 18). Participants were enrolled from 1961 to 1970 and were free of known chronic medical conditions at the time of enrollment. Participants underwent comprehensive medical examinations every 3–5 years on a continuous rolling basis. The present study included 656 men in the main cohort and 80 men in the replication cohort, all of whom were among the study's original participants, who provided plasma for EV-miRNA measurements between 1996 and 2015. The study was approved by the Veterans Affairs Boston Healthcare System Institutional Review Board.

The HEALS (Health Effects of Arsenic Longitudinal Study) is a prospective cohort study in Araihazar, Bangladesh (19). A subset of participants ($N = 15$) who were free of chronic medical conditions were chosen for replication. The study was approved by the Ethical Committee of the Bangladesh Medical Research Council. All study procedures were approved by the Columbia University Institutional Review Board. All study participants provided consent at each visit.

EV-miRNA Profiling

Fasting venous blood was collected each visit and centrifuged to separate plasma. Plasma samples were immediately stored at −80°C and banked for use in future studies. Aliquots were thawed just before use in this study. EVs were isolated using a modified ultracentrifugation method (20, 21). This workflow was previously validated by visualizing EVs using transmission electron microscopy and immune-gold labeling with antibodies for CD-81 and CD-63 surface markers according to International Society for Extracellular Vesicles guidelines (12, 20, 22, 23).

In NAS, miRNAs were isolated using the Plasma/Serum Circulating and Exosomal RNA Purification Kit (Norgen Biotek) and sequenced using a previously validated protocol (24, 25). In HEALS, miRNAs were profiled using the TaqMan OpenArray™ system (ThermoFisher Scientific). Complete protocols for EV-miRNA sequencing are described in the online supplement.

Spirometry

Spirometry was performed using water-seal or portable spirometers in accordance with American Thoracic Society guidelines (26). Portable spirometers were validated in previous studies (27). Lung function

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parameters including the FEV_1, FVC, and the maximal midexpiratory flow (MMEF) were measured in both cohorts.

Covariates

Covariates were collected at every visit. Age, sex, and race/ethnicity were self-reported. Body mass index (BMI) was calculated based upon measured height and weight. Smoking histories were updated every visit. Participants reported their smoking start and stop dates and the average number of cigarettes smoked per day, which were used to calculate total smoking pack-years. Physical activity was assessed using a validated scale (28) and recorded as metabolic equivalent tasks (METs) per week.

Main Cohort and Replication

The main analyses were conducted in 656 NAS participants using baseline EV-miRNA data (Figure E1 in the online supplement). Parallel replication was performed in 80 independent NAS participants. In addition, analyses were repeated using EV-miRNAs measured at a second time point (median 6 years after baseline [interquartile range (IQR), 4–8]) in 401 of the original 656 participants. The parallel replication used independent samples and ensured observed associations were not a product of the randomly selected samples.

Results were replicated in HEALS, an external cohort that used a different miRNA assay. The orthogonal validation in HEALS was designed to show results are reproducible on other technological platforms and in diverse populations. Further details about replication are described in the online supplement.

Statistical Analysis

Using an approach similar to a genome-wide association study, we performed an EVWAS (EV-miRNA-wide association study). Linear regression models were fitted to test associations of EV-miRNAs with baseline lung function in each group. Models were adjusted for baseline age, BMI, smoking pack-years, and METs. All terms were modeled as linear because they displayed a linear association with spirometry data. Use of generalized additive models and cubic splines did not improve model fit. False discovery rate (FDR) correction was used to account for multiple comparisons (29).

In NAS, final estimates were pooled from 10 multiple imputed datasets, as five participants (0.8%) were missing baseline spirometry and 36 (5.5%) were missing baseline METs. In sensitivity analyses, participants with preexisting physician-diagnosed lung diseases including asthma, emphysema, and chronic bronchitis were excluded. To assess potential effect modification, models were stratified by smoking status.

DNA Intelligent Analysis (DIANA)-miRPath version 3.0 software (30) was used to explore the biological roles of EV-miRNAs that were positively versus negatively associated with lung function. To provide greater confidence in pathway analyses, only EV-miRNAs that replicated outside of the main cohort were included in the pathway analyses. Further, DIANA-miRPath analyses were restricted to EV-miRNAs with experimentally validated mRNA interactions from DIANA-TarBase (31, 32). Enriched non-cancer-related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were identified using an FDR threshold of <0.05.

Multivariate latent class growth modeling groups individuals who share similar longitudinal data patterns and was used to identify longitudinal lung function trajectories (33, 34). The measured FEV_1, FVC, FEV_1/FVC, and MMEF informed the latent classes. Multiple link functions and trajectory class numbers were evaluated. The optimal model was selected based upon multiple model fit indices including the Bayes Information Criteria, the posterior probability for each class (>70%), and the interpretability of identified trajectory shapes (35, 36). Associations of individual EV-miRNAs with lung function trajectories were modeled using log binomial regression. Models were adjusted for baseline age, BMI, smoking pack-years, and METs, and corrected for FDR. Modeling details are available in the online supplement.

To complement the individual EV-miRNA approach, unsupervised machine learning was used to evaluate associations of complex EV-miRNA profiles, rather than individual EV-miRNAs in isolation, with lung function trajectories. K-means clustering was applied to all plasma EV-miRNAs to partition participants into distinct and nonoverlapping clusters based

Table 1. Baseline Characteristics of Normative Aging Study Participants

	Main Analysis	Replication Group
N	656	80
Age, mean (SD)	73.2 (7.0)	74.1 (6.7)
BMI, mean (SD)	28.3 (4.0)	27.6 (4.6)
Metabolic equivalent of task (hours/week, median (IQR)	7.4 (2.5–19.6)	7.1 (1.5–16.9)
Self-reported smoking status, no. (%)		
Never	200 (30.5)	27 (33.8)
Former	431 (65.7)	49 (61.3)
Current	25 (3.8)	4 (5.0)
Pack-years in ever smokers, median (IQR)	12.0 (0.0–33.3)	10.0 (0.0–32.2)
Baseline lung function, mean (SD)		
FVC, L	3.3 (0.7)	3.4 (0.7)
FEV_1, L	2.5 (0.6)	2.5 (0.6)
FEV_1/FVC	74.9 (8.2)	74.3 (8.8)
MMEF, L/min	239.0 (109.9)	234.4 (108.1)
No. spirometry exams, median (IQR)	3.0 (1.0–4.0)	3.0 (1.0–7.0)
Years of spirometry follow-up, median (IQR)	6.0 (0.0–12.0)	6.0 (0.0–18.0)
Medical comorbidities, no. (%)		
Coronary artery disease*	202 (30.8)	22 (27.5)
Diabetes†	128 (19.5)	14 (17.5)
Hypertension‡	481 (73.3)	53 (66.3)
Lung disease§	61 (9.3)	4 (5.0)
Obesity‖	181 (27.6)	20 (25.0)

Definition of abbreviations: BMI = body mass index; IQR = interquartile range; L = liters; MMEF = maximal mid-expiratory flow; SD = standard deviation.
*Coronary Artery Disease: Myocardial infarction or angina pectoris based on Framingham Study criteria.
†Diabetes: Physician-diagnosed or fasting glucose > 126 mg/dL.
‡Hypertension: Blood pressure ⩾ 140/90 mm Hg.
§Lung Disease: Physician-diagnosed asthma, emphysema, or chronic bronchitis.
‖Obesity: BMI ⩾ 30.0 kg/m^2

on their EV-miRNA profiles. K-means clustering is an unsupervised machine learning algorithm that partitions data points into groups based on similarities in the data. Using several internal validation metrics and measures of stability as guides (37), varying cluster sizes were produced and analyzed to ensure the results were not specific to any chosen cluster size. Log-binomial regression was used to identify EV-miRNA profiles associated with lung function trajectory class, adjusting for age, BMI, smoking pack-years, and METs. To characterize the EV-miRNA profiles associated with lung function trajectories, least absolute shrinkage and selection operator (LASSO) regression was used to identify key EV-miRNA drivers of cluster formation. The optimal λ, which is a tuning parameter that determines the amount of coefficient shrinkage in a penalized regression, was chosen using leave-one-out cross-validation.

Statistical analyses were performed using *R* software (4.0.3) (38).

Results

Baseline Characteristics

The baseline characteristics of the cohorts are shown in Table 1. The NAS population was comprised of European-American participants, while the HEALS cohort was comprised of Bangladeshi participants (Table E1in the online supplement). The mean age in the main NAS cohort was 73.2 (7.0) years. Over 50% of participants were current or former smokers. In total, 473 (72%) of the main NAS participants had follow-up spirometry (Figure E2).

EV-miRNAs and Lung Function

In the main NAS cohort, 381 EV-miRNAs were detected in at least 70% of plasma samples. Of these, 13 EV-miRNAs were associated with lower baseline FEV_1 and 16 with lower FVC (Figure 1, Figure E3). The miRNA hsa-miR-24–3p was most strongly associated with lower lung function, such that each doubling in hsa-miR-24–3p was associated with a 134 (95% confidence interval [CI] 40–220) mL reduction in FEV_1 and a 191 (95% CI 80–300) mL decrement in FVC.

In comparison, 27 EV-miRNAs were associated with higher baseline FEV_1 and 28 with higher FVC (Figure 1, Figure E3). The miRNA hsa-miR-2110 was most strongly associated with higher lung function, such that a 130 (95% CI 70–190) mL increase in FEV_1 and a 165 (95% CI 90–240) mL increment in FVC were observed per doubling of hsa-miR-2110.

Results were similar when participants with preexisting physician-diagnosed lung diseases ($N = 61$) were excluded from the analyses (Figure E5) and in analyses stratified by smoking status (Figure E6). EV-miRNAs that were associated with lung function were not associated with other chronic conditions including hypertension, diabetes, and coronary artery disease (Figure E7).

In total, 23 of the 51 EV-miRNAs associated with lung function in the main analysis were persistently associated with

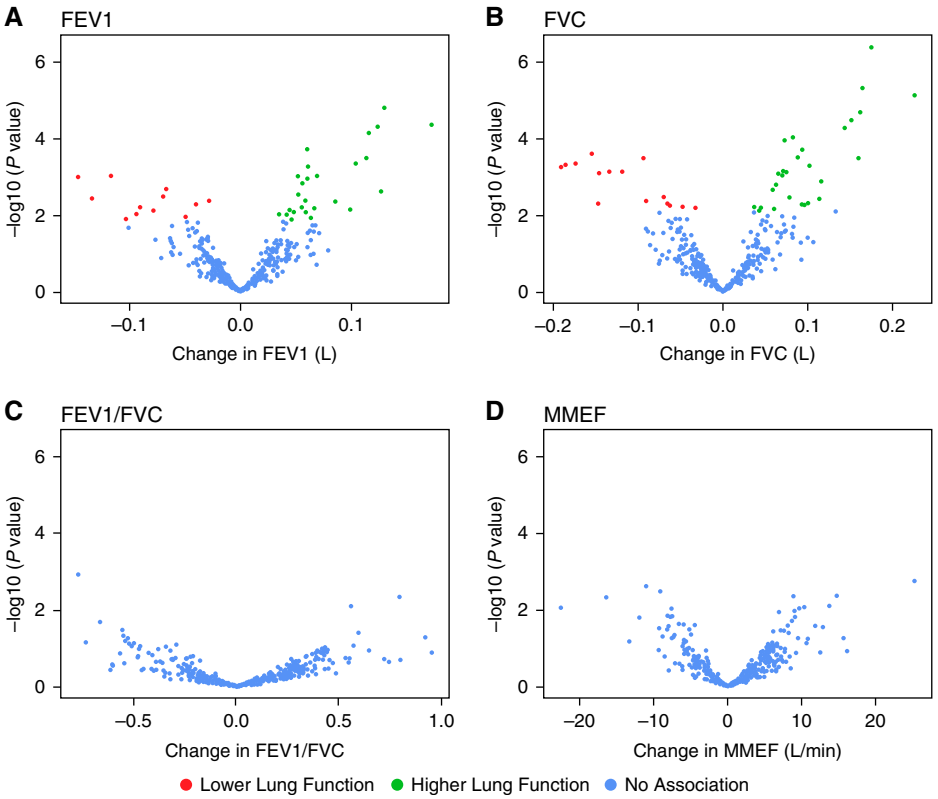

Figure 1. Volcano plots showing associations of plasma extracellular vesicle-encapsulated microRNAs (EV-miRNAs) with baseline lung function. Each point in the plot represents an effect estimate as fold change (*x*-axis) and –log10 (*P* value) (*y*-axis) for each individual miRNA. Red points represent EV-miRNAs associated with lower baseline lung function and green points are EV-miRNAs associated with higher baseline lung function after correction for false discovery rate. Models were adjusted for baseline age, body mass index, smoking pack-years, and metabolic equivalents of task. To see a version of Figure 1 that labels the differentially expressed EV-miRNAs, please refer to Figure E3. L = liters; MMEF = maximal mid-expiratory flow.

lung function at a second time point in the original NAS participants. Due to limited sensitivity of the HEALS assay, only a fraction of the EV-miRNAs detected in NAS were detected in over 33% of HEALS participants. Nonetheless, across the parallel replication and external validation groups, 13 EV-miRNAs were consistently associated with lower FEV_1 and 13 with lower FVC (Tables E2 and E3). Twelve overlapping EV-miRNAs were associated with both lower FEV_1 and FVC (Figure E4). In comparison, 12 EV-miRNAs were consistently associated with higher FEV_1 and 11 with higher FVC (Tables E2 and E3); 10 overlapping EV-miRNAs were associated with both higher FEV_1 and FVC (Figure E4).

Pathway Analysis of EV-miRNAs

A pathway analysis was performed for the EV-miRNAs that were positively associated with lung function in the main and replication cohorts. Significant KEGG pathways included fatty acid biosynthesis/metabolism, steroid biosynthesis, adherens junctions, lysine degradation, protein processing in the endoplasmic reticulum (ER), cell cycle, and the hippo, transforming growth factor-β (TGF-β), and thyroid hormone signaling pathways (FDR-adjusted P value < 0.05) (Figure 2A, Table E4).

A separate pathway analysis was performed for the replicable EV-miRNAs that were negatively associated with lung function. Except for steroid biosynthesis and ER protein processing, the same pathways were enriched, in addition to five new pathways including bacterial invasion of epithelial cells, extracellular matrix (ECM)-receptor interaction, and the FoxO, mTOR, and p53 signaling pathways (FDR-adjusted P value < 0.05) (Figure 2B; Table E5). Ten EV-miRNAs did not have experimentally validated mRNA targets (Tables E4 and E5).

EV-miRNAs and Latent Lung Function Trajectories

Among 656 NAS participants contributing 4,730 person-years of follow-up, the multivariate latent class growth model yielded two distinct lung function trajectory classes (Figure 3). Participants in the "Stable" trajectory class ($N = 141$) had steady lung function over time, while participants in the "Declining" trajectory class ($N = 332$) had decreasing lung function. The miRNA hsa-miR-532–5p was associated with higher risk of belonging to the "Declining" trajectory class (Relative Risk [RR] 1.12, 95% CI 1.05–1.19), while hsa-miR-193b-5p was associated with lower risk of belonging to the "Declining" trajectory class (RR 0.94, 95% CI 0.91–0.97).

In the parallel replication cohort, "Stable" and "Declining" trajectory classes were again identified. The hsa-miR-193–5p was persistently associated with lower risk of belonging to the "Declining" trajectory class (RR 0.57, 95% CI 0.37–0.89).

Machine Learning-Generated EV-miRNA Profiles

To comprehensively capture complex patterns of EV-miRNAs and identify specific profiles that precede lung function decline, unsupervised K-means clustering was applied to partition participants based on the similarity of their EV-miRNA profiles.

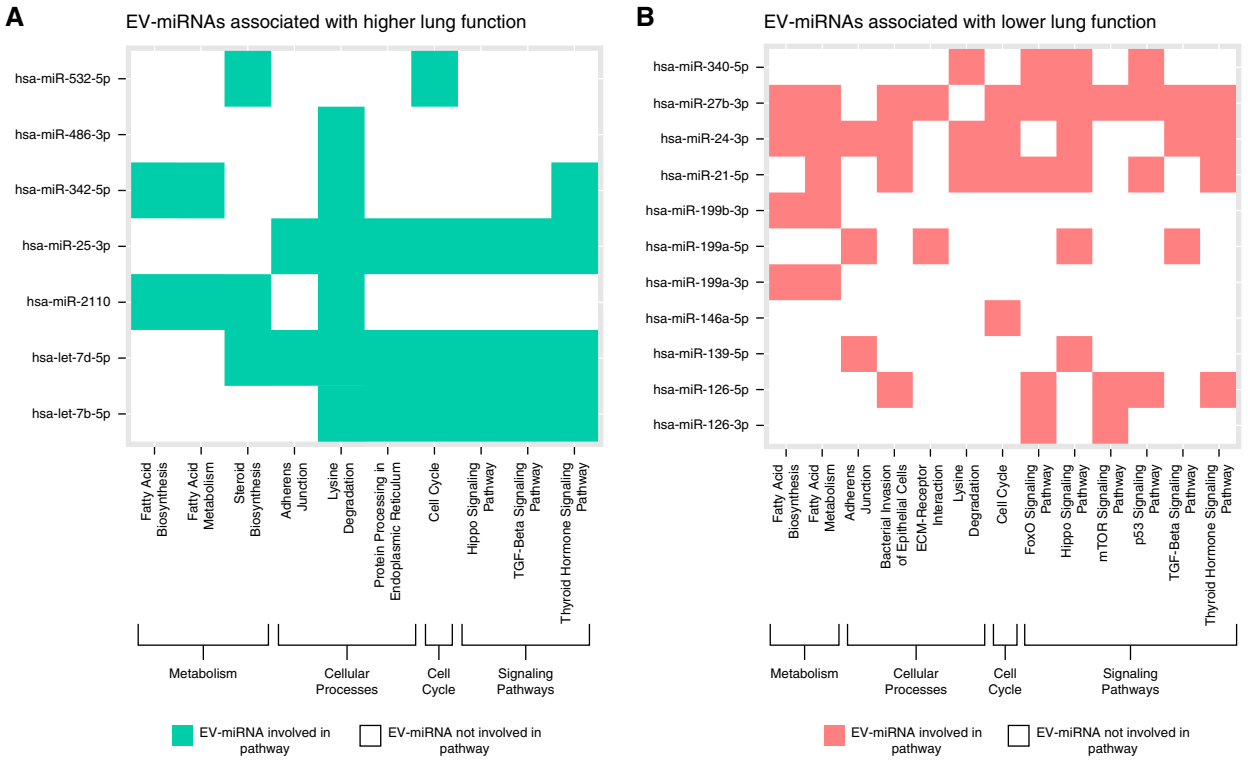

Figure 2. Biological pathways targeted by candidate extracellular vesicle-encapsulated microRNAs (EV-miRNAs) that were (*A*) positively associated with baseline lung function and (*B*) negatively associated with baseline lung function. (A) shows biological pathways targeted by the EV-miRNAs that were positively associated with baseline lung function. (B) shows biological pathways targeted by the EV-miRNAs that were negatively associated with baseline lung function. The green boxes in (*A*) and red boxes in (*B*) indicate KEGG pathways that were enriched for candidate EV-miRNAs (FDR-corrected P value < 0.05). ECM = extracellular matrix; FDR = false discovery rate; hsa = homosapiens and is the accepted convention to identify human microRNAs; KEGG = Kyoto Encyclopedia of Genes and Genomes; TGF = transforming growth factor.

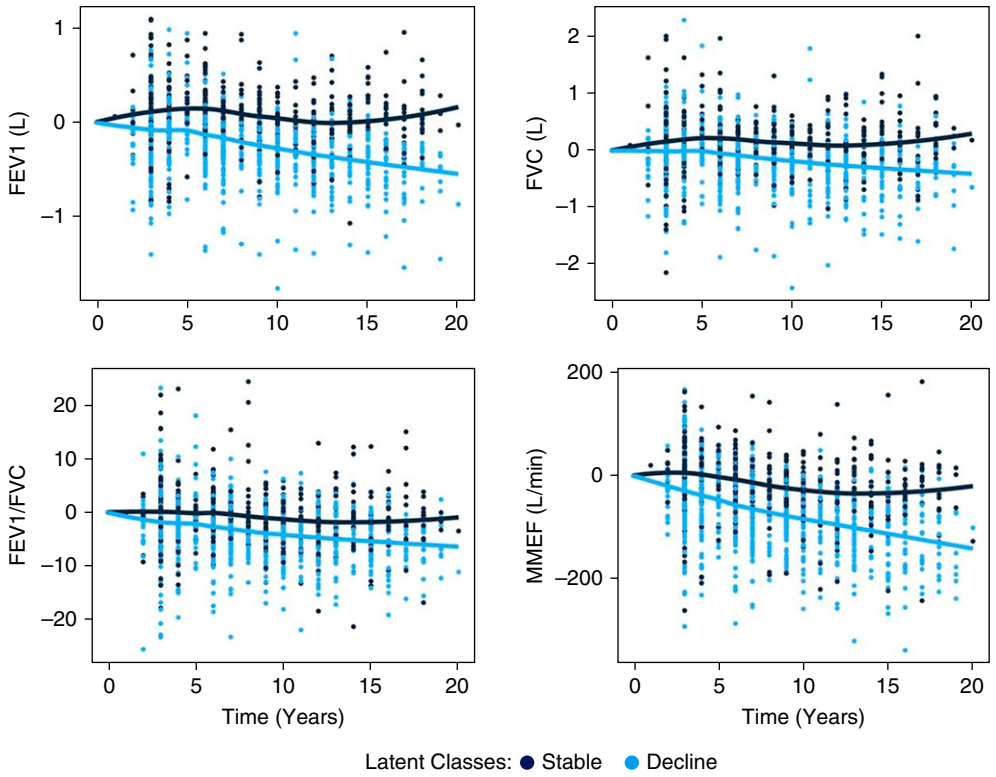

Figure 3. Lung function trajectory classes derived through multivariate latent class growth modeling. The multivariate latent class growth model yielded two distinct lung function trajectory classes. Participants in the "Stable" trajectory class ($N = 141$) had steady lung function over time, while participants in the "Declining" trajectory class ($N = 332$) had decreasing lung function. L = liters; MMEF = maximal mid-expiratory flow.

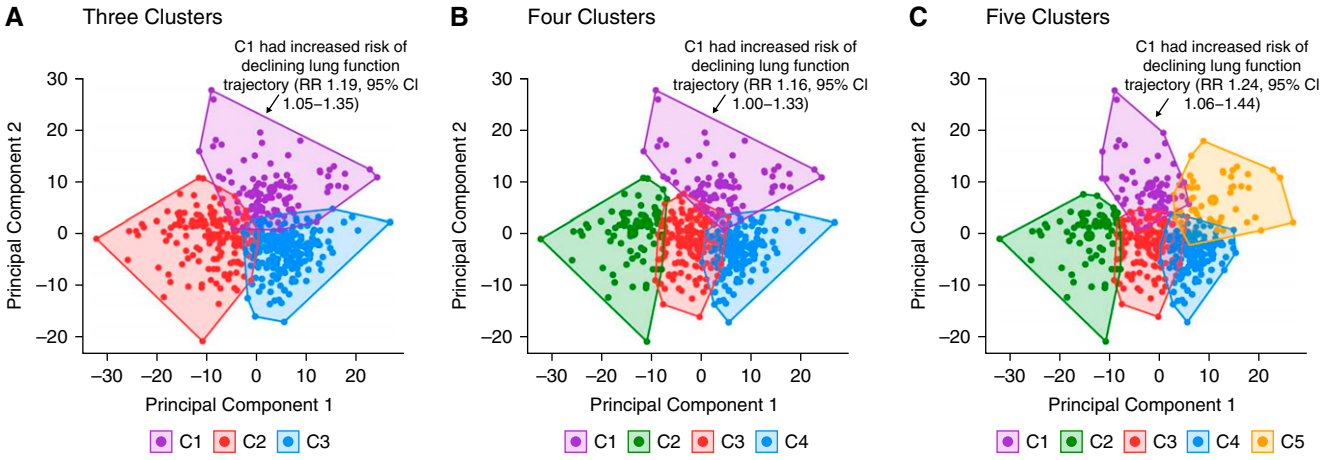

Figure 4. Participant clusters derived through similarities in extracellular vesicle-encapsulated microRNA (EV-miRNA) profiles had differential risk of belonging to the declining lung function trajectory class. Participants were divided into three (*A*), four (*B*), or five (*C*) distinct and non-overlapping clusters based on similarities in their EV-miRNA profiles. Each panel shows a principal component analysis (PCA) plot. Each point in each PCA plot represents one participant. The PCA plots show the two directions along which EV-miRNA data have the largest spread and the colors denote each K-means cluster of participants based upon their EV-miRNA profiles. The red cluster is the reference group in each figure. The RRs in each panel show the association of cluster 1 membership with lung function trajectory class ("Stable" [reference] versus "Declining") adjusted for baseline age, body mass index, smoking pack-years, and metabolic equivalents of task. A positive risk ratio means participants in the cluster have a higher risk of being in the "Declining lung function" trajectory class compared with the reference group. There was one cluster (purple, C1) that was consistently associated with the "Declining" lung function trajectory. C1 = cluster 1; C2 = cluster 3; C3 = cluster 3; C4 = cluster 4; C5 = cluster 5; CI = confidence interval; RR = relative risk.

Internal validation metrics (37) suggested the optimal number of EV-miRNA-based participant clusters ranged from 3–5 (Figure 4). When participants were divided into three clusters, one cluster (C1) had significantly higher risk of belonging to the "Declining" lung function class (RR 1.19, 95% CI 1.05–1.35) (Figures 4 and E8). Similarly, when participants were partitioned into four and five clusters, the same C1 persisted and its participants had consistently higher risk of belonging to the "Declining" lung function class (four clusters: RR 1.16, 95% CI 1.00–1.33; five clusters: RR 1.24, 95% CI 1.06–1.44) (Figures 4 and E8). When non-C1 clusters were pooled into one reference group, C1 participants had persistently higher risk of belonging to the "Declining" lung function class (Figure E9). In the parallel replication cohort, we again identified a subset of participants with higher risk of belonging to the "Declining" lung function trajectory based solely on EV-miRNA profiles (Figure E10). Together, these results demonstrate that a specific EV-miRNA profile clearly distinct from the others (Figure 5A) was associated with future lung function changes.

We additionally tested whether EV-miRNA-based clusters were associated with lung function trajectories independent of baseline lung function. Adding cluster groups to models with baseline lung function as predictors and trajectory class membership as the outcome significantly improved model fit (all likelihood ratio tests < 0.05). This finding demonstrates that EV-miRNA-based participant clusters contributed information independent of and in addition to baseline lung function measurements.

Identification of EV-miRNA Drivers of Cluster Formation

LASSO regression was applied to identify key EV-miRNA drivers that separated C1 from other EV-miRNA profiles. After harmonizing the results across models for three, four and five clusters, LASSO regression identified 11 EV-miRNAs that consistently differentiated the C1 EV-miRNA profile from other profiles (Figure 5B). Eight EV-miRNAs were highly expressed while three EV-miRNAs had low expression among C1 participants.

Pathway Analysis of EV-miRNAs with Differential Expression in C1 Profile

A pathway analysis was performed for the 11 EV-miRNAs that were differentially expressed in the C1 profile. Thirteen mRNA targets were identified, 12 of which overlapped with targets of the EV-miRNAs that were associated with baseline lung function (Figure 5C). RNA transport was additionally enriched for the C1 profile (FDR-adjusted P value < 0.05) (Table E6). Together, these results show that this distinctive EV-miRNA profile, characterized

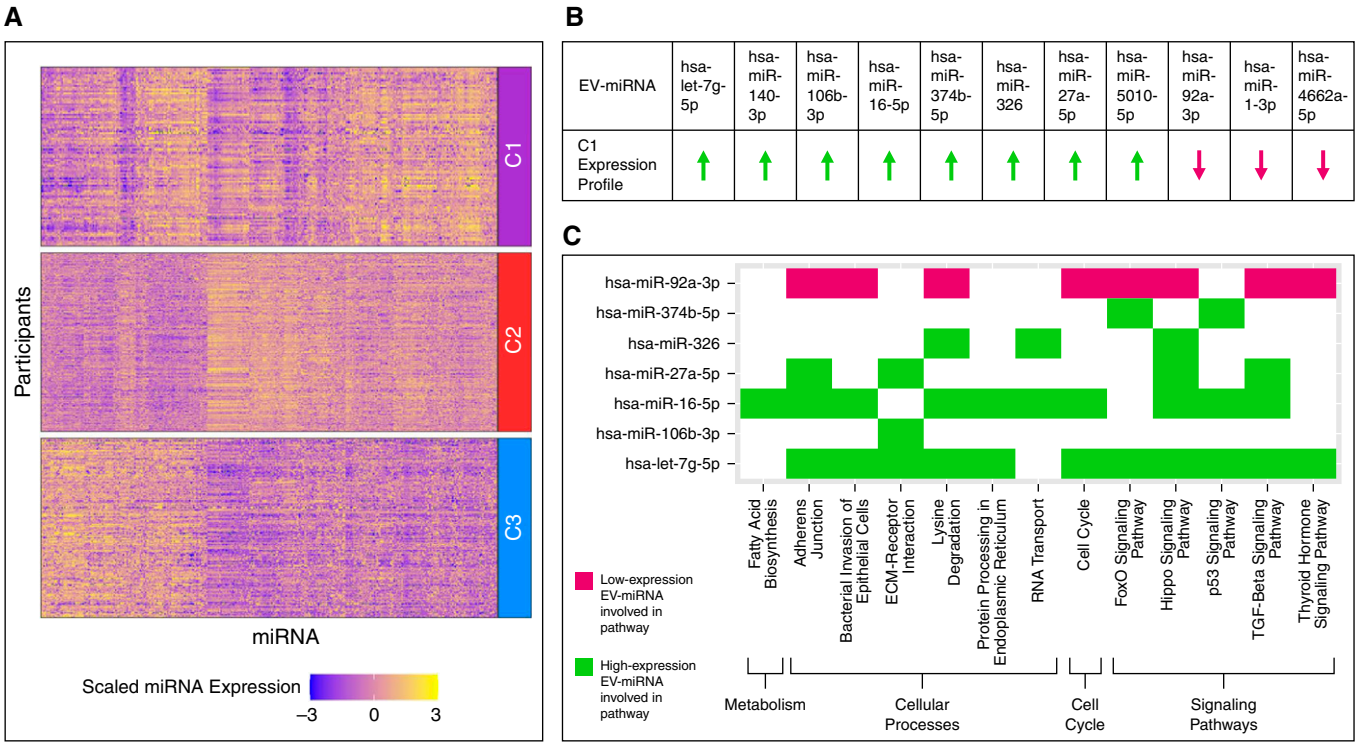

Figure 5. Evaluating differential extracellular vesicle-encapsulated microRNA (EV-miRNA) expression in Cluster One participants and identifying biological pathways targeted by key EV-miRNAs. (*A*) The heatmaps show EV-miRNA expression among participants in clusters 1, 2 and 3. Each row represents a single participant and each column represents a single miRNA. The expression values are represented by the color scale, such that the intensity increases from relatively low miRNA abundance (blue) to relatively high miRNA abundance (yellow). (*B*) A pathway analysis identified 13 biological pathways impacted by differentially expressed EV-miRNAs in cluster 1. The green boxes indicate KEGG pathways that were associated with highly expressed EV-miRNAs while the red boxes indicate KEGG pathways that were associated with EV-miRNAs with low expression. (*C*) LASSO regression identified eight individual EV-miRNAs that were highly expressed among Cluster 1 participants (green arrows) and three individual EV-miRNAs with low expression among C1 participants (red arrows). C1 = cluster 1; C2 = cluster 3; C3 = cluster 3; ECM = extracellular matrix; hsa = homosapiens and is the accepted convention to identify human microRNAs; TGF = transforming growth factor; KEGG = Kyoto Encyclopedia of Genes and Genomes; LASSO = least absolute shrinkage and selection operator.

by changes in 11 EV-miRNAs enriched in pathways related to lung tissue architecture and immunity, is associated with future lung function decline.

Discussion

In a large prospective study of older adults, plasma EV-miRNAs were associated with current and prospective lung function. Distinctive profiles made from a constellation of EV-miRNAs were significantly associated with future lung function decline. Observed associations of EV-miRNAs with lung function persisted in both parallel replication and external validation cohorts. Candidate EV-miRNAs interacted with mRNA targets that were enriched in cellular metabolism, airway structural integrity, and signal transduction pathways, which are highly relevant for respiratory cellular immunity and the lung inflammatory response (39, 40). EV-miRNAs may represent robust biomarkers of subclinical lung damage, and may help identify individuals at risk of developing symptomatic lung disease.

This study introduces the concept of the EVWAS, a study of global EV-miRNAs using an untargeted approach. To our knowledge, this is the first EVWAS to evaluate associations of plasma EV-miRNAs with lung function in humans. Across the main cohort, a parallel replication cohort, and an external validation cohort, 13 EV-miRNAs were positively associated with baseline FEV_1 and FVC, and 14 were negatively associated with baseline lung function, suggesting a strong association of EV-miRNAs with flow rates and vital capacity. Many of the differentially expressed EV-miRNAs were novel EV-miRNAs with respect to biological impact on lung function, including hsa-miR-2110, hsa-miR-24–3p, and hsa-miR-193–5p. However, our study also recapitulated findings from prior targeted studies of plasma EV-miRNAs. Hsa-let-7d-5p was positively associated with baseline lung function, which is consistent with existing literature showing hsa-let-7d-5p exerts a protective effect on lung function and mitigates the impact of inhaled environmental insults (41). In comparison, hsa-miR-21–5p was negatively associated with FVC, which is consistent with prior research showing hsa-miR-21–5p was correlated with FVC decline among patients with pulmonary fibrosis (42). The observed relationship between EV-miRNAs and lung function was not explained by the presence of comorbid medical conditions, and there was no strong evidence for effect modification by smoking.

Our results broaden the scope of limited existing research on the role of EVs in lung disease. Previous cross-sectional studies identified differential EV concentration, EV surface protein expression, and EV-miRNA expression among COPD patients compared with smokers and nonsmokers (15, 43, 44). One prior longitudinal study found that higher numbers of endothelial microparticles predicted accelerated FEV_1 decline in COPD patients after 1 year of follow-up (16). The present study expands upon these findings by evaluating the contribution of easily accessible plasma EV-miRNAs using an untargeted and unprecedented approach, and may highlight novel therapeutic targets for improving respiratory health.

While individual EV-miRNAs have clear associations with lung health, our results also suggest EV-miRNAs may function in biological networks that impact lung physiology. When participants were partitioned into EV-miRNA-based clusters to flexibly capture complex expression patterns and interplay between EV-miRNAs, one EV-miRNA profile was consistently associated with greater risk of future lung function decline. This EV-miRNA profile was associated with declining lung function to greater extent than other blood-based markers including C-reactive protein levels and blood leukocyte concentrations (45, 46). Combined with the observation that only two individual EV-miRNAs were prominently associated with lung function trajectory, the evidence suggests that a network of EV-miRNAs may act in concert to reflect and potentially generate future lung function impairment. Future studies should explore whether specific combinations of EV-miRNAs can maximize the predictive value for lung function decline and incident lung diseases.

In addition to identifying the potential utility of EV-miRNAs as clinical biomarkers, our results elucidated the biological pathways through which circulating EV-miRNAs may impact respiratory health. EV-miRNAs that were positively associated with baseline lung function modulate protein processing in the ER, which regulates protein folding, transport and degradation. Surfactant proteins are synthesized and processed in the ER and physiologic protein processing maintains cellular proteostasis in the lungs. Thus, conserved protein processing may represent a key pathway through which these EV-miRNAs preserve lung health (47).

EV-miRNAs that were negatively associated with baseline lung function modulate multiple signaling pathways including the mTOR signaling pathway. In particular, hsa-miR-27b-3p modulates the *TSC1* (tuberous sclerosis complex 1) gene (30), which regulates activation of mTOR signaling (48). Activation of mTOR signaling drives senescence of regenerative cells in the lung and contributes to destruction of the lung parenchyma (49). Significant EV-miRNAs also modulate the FoxO signaling pathway. Hsa-miR-126–5p mediates *SIRT1* (sirtuin 1) gene expression (30), which regulates FoxO activity (50). FoxO signaling moderates the lung inflammatory response and antioxidant genes, and reduced FoxO expression generates lung inflammation and airspace enlargement (51). Significant EV-miRNAs also modulate the p53 signaling pathway. Specifically, hsa-miR-340–5p mediates *MDM2* (mouse double minute 2) gene expression (30), which is the primary regulator of p53 activity (52). In turn, p53 signaling can trigger epithelial cell apoptosis by activating pro-apoptotic genes, leading to epithelial damage and distortion of the lung parenchyma (53).

Aside from regulating key signaling pathways, EV-miRNAs that were negatively associated with lung function also impact susceptibility of epithelial cells to bacterial invasion. Bacterial invasion triggers production of pro-inflammatory cytokines (54), which recruit immune cells and contribute to chronic airway inflammation (55–57). Additionally, significant EV-miRNAs modulate the interaction between lung cells and the lung ECM, which consists of elastic and collagen fibers that maintain lung architecture (58). The ECM generates biochemical signals that direct cellular function, and alteration of the lung ECM-cell surface receptor interaction can induce airway remodeling and loss of lung elasticity (59, 60). Future research should further investigate how plasma EV-miRNA profiles impact gene expression levels.

Strengths of the present work include the prospective population-based cohort design, evaluation of total EV-miRNAs, large sample size, and adjustment for well-defined confounders and determinants of lung health. However, the study has several

limitations. First, plasma samples were stored for several years before analysis, which may impact EV-miRNA integrity. However, standard methods of EV enrichment were implemented, and high-quality miRNA sequencing data was obtained. Furthermore, such misclassification should not introduce confounding to the results. Second, the NAS cohort represents a homogeneous group of European-Americans. However, we successfully replicated our results in Bangladeshi participants from the HEALS cohort, suggesting that the interplay between EV-miRNAs and lung function may represent a ubiquitous mechanism that is generalizable across demographic groups. Third, 28% of the cohort did not have follow-up spirometry measurements. Loss to follow-up stemmed primarily from mortality, nonresponse, and use of continuous rolling follow-up. Nonetheless, compared with participants with follow-up spirometry, those without follow-up reported older baseline age, higher smoking pack-years, and lower initial lung function (Table E7), suggesting any selective survivor bias would generate an underestimation of the association between EV-miRNAs and longitudinal lung function. Fourth, although it is preferable to have both pre- and postbronchodilator spirometry (61), postbronchodilator measurements were not available in these cohorts. However, prebronchodilator spirometry is highly correlated with postbronchodilator spirometry and remains strongly predictive of clinical outcomes in longitudinal cohort studies (62, 63). Fifth, enrichment for EV-miRNAs naturally captures a small number of other miRNA carriers including ribonucleoproteins (64). However, these miRNAs have also been implicated in contributing to chronic lung diseases and are not expected to substantively impact results (65). Finally, the interactions between EV-miRNAs and cellular processes need further investigation. Nonetheless, our results emphasize the translational importance of EV-miRNAs in modulating lung health.

Conclusions

In summary, 28 plasma EV-miRNAs were consistently associated with lung function across multiple cohorts. A cluster of participants with a distinct EV-miRNA profile experienced increased risk of accelerated lung function decline. Key EV-miRNAs may impact respiratory physiology through biological pathways that regulate respiratory cellular immunity, lung inflammation, and airway structural integrity. Together, these results suggest that EV-miRNAs may represent a viable biomarker of subclinical lung injury and may help identify individuals at risk of developing lung function impairment. ■

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References

1. Labaki WW, Han MK. Chronic respiratory diseases: a global view. *Lancet Respir Med* 2020;8:531–533.
2. GBD 2017 Risk Factor Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* 2018;392:1923–1994.
3. Vestbo J, Hurd SS, Agustí AG, Jones PW, Vogelmeier C, Anzueto A, *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Respir Crit Care Med* 2013;187:347–365.
4. Cosio M, Ghezzo H, Hogg JC, Corbin R, Loveland M, Dosman J, *et al.* The relations between structural changes in small airways and pulmonary-function tests. *N Engl J Med* 1978;298: 1277–1281.
5. Johns DP, Walters JA, Walters EH. Diagnosis and early detection of COPD using spirometry. *J Thorac Dis* 2014;6:1557–1569.
6. Anthonisen NR, Connett JE, Kiley JP, Altose MD, Bailey WC, Buist AS, *et al.* Effects of smoking intervention and the use of an inhaled anticholinergic bronchodilator on the rate of decline of FEV_1. The Lung Health Study. *JAMA* 1994;272:1497–1505.
7. Zhou Y, Zhong NS, Li X, Chen S, Zheng J, Zhao D, *et al.* Tiotropium in early-stage chronic obstructive pulmonary disease. *N Engl J Med* 2017; 377:923–935.
8. Martinez FJ, Han MK, Allinson JP, Barr RG, Boucher RC, Calverley PMA, *et al.* At the root: defining and halting progression of early chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2018;197: 1540–1551.
9. Bobrie A, Colombo M, Krumeich S, Raposo G, Théry C. Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. *J Extracell Vesicles* 2012;1:18397.
10. O'Brien K, Breyne K, Ughetto S, Laurent LC, Breakefield XO. RNA delivery by extracellular vesicles in mammalian cells and its applications. *Nat Rev Mol Cell Biol* 2020;21: 585–606.
11. Strulovici-Barel Y, Staudt MR, Krause A, Gordon C, Tilley AE, Harvey BG, *et al.* Persistence of circulating endothelial microparticles in COPD despite smoking cessation. *Thorax* 2016;71:1137–1144.
12. Rodosthenous RS, Coull BA, Lu Q, Vokonas PS, Schwartz JD, Baccarelli AA. Ambient particulate matter and microRNAs in extracellular vesicles: a pilot study of older individuals. *Part Fibre Toxicol* 2016;13:13.
13. Kwok ZH, Ni K, Jin Y. Extracellular vesicle associated non-coding RNAs in lung infections and injury. *Cells* 2021;10:965.
14. Wahlund CJE, Eklund A, Grunewald J, Gabrielsson S. Pulmonary extracellular vesicles as mediators of local and systemic inflammation. *Front Cell Dev Biol* 2017;5:39.
15. Sundar IK, Li D, Rahman I. Small RNA-sequence analysis of plasma-derived extracellular vesicle miRNAs in smokers and patients with chronic obstructive pulmonary disease as circulating biomarkers. *J Extracell Vesicles* 2019;8:1684816.
16. Takahashi T, Kobayashi S, Fujino N, Suzuki T, Ota C, Tando Y, *et al.* Annual FEV_1 changes and numbers of circulating endothelial microparticles in patients with COPD: a prospective study. *BMJ Open* 2014;4:e004571.
17. Bell BRC, Damon H. The Normative Aging Study: an interdisciplinary and longitudinal study of health and aging. *Int J Aging Hum Dev* 1972; 3:5–17.
18. Wang C, Just A, Heiss J, Coull BA, Hou L, Zheng Y, *et al.* Biomarkers of aging and lung function in the normative aging study. *Aging (Albany NY)* 2020;12:11942–11966.
19. Ahsan H, Chen Y, Parvez F, Argos M, Hussain AI, Momotaj H, *et al.* Health Effects of Arsenic Longitudinal Study (HEALS): description of a multidisciplinary epidemiologic investigation. *J Expo Sci Environ Epidemiol* 2006;16:191–205.
20. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* 2006; Chapter 3: Unit 3 22.
21. Bollati V, Angelici L, Rizzo G, Pergoli L, Rota F, Hoxha M, *et al.* Microvesicle-associated microRNA expression is altered upon particulate matter exposure in healthy workers and in A549 cells. *J Appl Toxicol* 2015;35:59–67.
22. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, *et al.* Proteomic comparison defines novel markers to characterize

heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci USA* 2016;113:E968–E977.
23. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7:1535750.
24. Laurent LC, Alexander RP. RNA isolation from human serum and plasma samples using the Norgen exosomal RNA purification mini kit. *Protocol Exchange* 2015.
25. Srinivasan S, Yeri A, Cheah PS, Chung A, Danielson K, De Hoff P, *et al.* Small RNA sequencing across diverse biofluids identifies optimal methods for exRNA isolation. *Cell* 2019;177:446–462.e16.
26. Graham BL, Steenbruggen I, Miller MR, Barjaktarevic IZ, Cooper BG, Hall GL, *et al.* Standardization of Spirometry 2019 Update. An official American Thoracic Society and European Respiratory Society technical statement. *Am J Respir Crit Care Med* 2019;200:e70–e88.
27. Pérez-Padilla R, Vázquez-García JC, Márquez MN, Jardim JR, Pertuzé J, Lisboa C, *et al.*; Latin American COPD Prevalence Study (PLATINO) Team. The long-term stability of portable spirometers used in a multinational study of the prevalence of chronic obstructive pulmonary disease. *Respir Care* 2006;51:1167–1171.
28. Paffenbarger RS Jr, Hyde RT, Wing AL, Hsieh CC. Physical activity, all-cause mortality, and longevity of college alumni. *N Engl J Med* 1986; 314:605–613.
29. Benjamini YHY. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 1995;57:289–300.
30. Vlachos IS, Zagganas K, Paraskevopoulou MD, Georgakilas G, Karagkouni D, Vergoulis T, *et al.* DIANA-miRPath v3.0: deciphering microRNA function with experimental support. *Nucleic Acids Res* 2015; 43:W460–W466.
31. Karagkouni D, Paraskevopoulou MD, Chatzopoulos S, Vlachos IS, Tastsoglou S, Kanellos I, *et al.* DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA-gene interactions. *Nucleic Acids Res* 2018;46:D239–D245.
32. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, *et al.* DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic Acids Res* 2015;43:D153–D159.
33. MacCallum RCK, Kim C, Malarkey WB, Kiecolt-Glaser JK. Studying multivariate change using multilevel models and latent curve models. *Multivariate Behav Res* 1997;32:215–253.
34. Berlin KS, Parra GR, Williams NA. An introduction to latent variable mixture modeling (part 2): longitudinal latent class growth analysis and growth mixture models. *J Pediatr Psychol* 2014;39:188–203.
35. Weller BE, Bowen NK, Faubert SJ. Latent class analysis: a guide to best practice. *J Black Psychol* 2020;46:287–311.
36. Kim SY. Determining the number of latent classes in single- and multi-phase growth mixture models. *Struct Equ Modeling* 2014;21:263–279.
37. Brock GPV, Datta S, Datta S. clValid: an R package for cluster validation. *J Stat Softw* 2008;25:1–22.
38. Team RCR. A language and environment for statistical computing 2018; 2022 [cited 2022 June 6; accessed 2021 Sep 1]. Available from: https://www.R-project.org.
39. Seiler F, Hellberg J, Lepper PM, Kamyschnikow A, Herr C, Bischoff M, *et al.* FOXO transcription factors regulate innate immune mechanisms in respiratory epithelial cells. *J Immunol* 2013;190:1603–1613.
40. Uddin MA, Barabutis N. P53 in the impaired lungs. *DNA Repair (Amst)* 2020;95:102952.
41. Gao Y, Sun J, Dong C, Zhao M, Hu Y, Jin F. Extracellular vesicles derived from adipose mesenchymal stem cells alleviate PM2.5-induced lung injury and pulmonary fibrosis. *Med Sci Monit* 2020;26:e922782.
42. Makiguchi T, Yamada M, Yoshioka Y, Sugiura H, Koarai A, Chiba S, *et al.* Serum extracellular vesicular miR-21-5p is a predictor of the prognosis in idiopathic pulmonary fibrosis. *Respir Res* 2016;17:110.
43. Takahashi T, Kobayashi S, Fujino N, Suzuki T, Ota C, He M, *et al.* Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility. *Thorax* 2012; 67:1067–1074.
44. Gordon C, Gudi K, Krause A, Sackrowitz R, Harvey BG, Strulovici-Barel Y, *et al.* Circulating endothelial microparticles as a measure of early lung destruction in cigarette smokers. *Am J Respir Crit Care Med* 2011;184: 224–232.
45. Bui DS, Agusti A, Walters H, Lodge C, Perret JL, Lowe A, *et al.* Lung function trajectory and biomarkers in the Tasmanian Longitudinal Health Study. *ERJ Open Res* 2021;7:00020-2021.
46. Zeig-Owens R, Singh A, Aldrich TK, Hall CB, Schwartz T, Webber MP, *et al.* Blood leukocyte concentrations, FEV_1 decline, and airflow limitation. A 15-year longitudinal study of World Trade Center-exposed firefighters. *Ann Am Thorac Soc* 2018;15:173–183.
47. Wei J, Rahman S, Ayaub EA, Dickhout JG, Ask K. Protein misfolding and endoplasmic reticulum stress in chronic lung disease. *Chest* 2013;143: 1098–1105.
48. Houssaini A, Breau M, Kebe K, Abid S, Marcos E, Lipskaia L, *et al.* mTOR pathway activation drives lung cell senescence and emphysema. *JCI Insight* 2018;3:e93203.
49. Barnes PJ, Baker J, Donnelly LE. Cellular senescence as a mechanism and target in chronic lung diseases. *Am J Respir Crit Care Med* 2019; 200:556–564.
50. Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, *et al.* Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. *Science* 2004;303:2011–2015.
51. Hwang JW, Rajendrasozhan S, Yao H, Chung S, Sundar IK, Huyck HL, *et al.* FOXO3 deficiency leads to increased susceptibility to cigarette smoke-induced inflammation, airspace enlargement, and chronic obstructive pulmonary disease. *J Immunol* 2011;187: 987–998.
52. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005;24:2899–2908.
53. Nakashima N, Kuwano K, Maeyama T, Hagimoto N, Yoshimi M, Hamada N, *et al.* The p53-Mdm2 association in epithelial cells in idiopathic pulmonary fibrosis and non-specific interstitial pneumonia. *J Clin Pathol* 2005;58:583–589.
54. Kunyanee C, Kamjumphol W, Taweechaisupapong S, Kanthawong S, Wongwajana S, Wongratanacheewin S, *et al.* Burkholderia pseudomallei biofilm promotes adhesion, internalization and stimulates proinflammatory cytokines in human epithelial A549 cells. *PLoS One* 2016;11:e0160741.
55. Parker D, Prince A. Innate immunity in the respiratory epithelium. *Am J Respir Cell Mol Biol* 2011;45:189–201.
56. Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. *Am J Respir Med* 2002;1:19–25.
57. Hoshino T, Kato S, Oka N, Imaoka H, Kinoshita T, Takei S, *et al.* Pulmonary inflammation and emphysema: role of the cytokines IL-18 and IL-13. *Am J Respir Crit Care Med* 2007;176:49–62.
58. Burgstaller G, Oehrle B, Gerckens M, White ES, Schiller HB, Eickelberg O. The instructive extracellular matrix of the lung: basic composition and alterations in chronic lung disease. *Eur Respir J* 2017; 50: 1601805.
59. Karakioulaki M, Papakonstantinou E, Stolz D. Extracellular matrix remodelling in COPD. *Eur Respir Rev* 2020;29: 190124.
60. Togo S, Holz O, Liu X, Sugiura H, Kamio K, Wang X, *et al.* Lung fibroblast repair functions in patients with chronic obstructive pulmonary disease are altered by multiple mechanisms. *Am J Respir Crit Care Med* 2008;178:248–260.
61. Johannessen A, Lehmann S, Omenaas ER, Eide GE, Bakke PS, Gulsvik A. Post-bronchodilator spirometry reference values in adults and implications for disease management. *Am J Respir Crit Care Med* 2006;173:1316–1325.
62. Mannino DM, Diaz-Guzman E, Buist S. Pre- and post-bronchodilator lung function as predictors of mortality in the Lung Health Study. *Respir Res* 2011;12:136.
63. Kato B, Gulsvik A, Vollmer W, Janson C, Studnika M, Buist S, *et al.* Can spirometric norms be set using pre- or post- bronchodilator test results in older people? *Respir Res* 2012;13:102.
64. Driedonks TAP, Nolte-'t Hoen ENM. Circulating Y-RNAs in extracellular vesicles and ribonucleoprotein complexes; implications for the immune system. *Front Immunol* 2019;9:3164.
65. Omote N, Sauler M. Non-coding RNAs as regulators of cellular senescence in idiopathic pulmonary fibrosis and chronic obstructive pulmonary disease. *Front Med (Lausanne)* 2020;7:603047.