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The Promise of Liquid Biopsies: Extracellular Vesicle microRNAs Open the Door to Future Study in Lung Disease

Chronic lung diseases such as chronic obstructive pulmonary disease (COPD), asthma, and interstitial lung disease develop in concert with long-term exposure to inhaled particulate matter and other acute injuries. Chronic lung diseases worsen with increased exposure to air pollution, particulate matter (e.g., particulate matter $\leqslant$2.5 μm in aerodynamic diameter and particulate matter $\leqslant$10 μm in aerodynamic diameter), and smoke (1). Therefore, the development of biomarkers that reflect early changes in the lung in response to injury may be a viable approach to advancing our knowledge of lung disease development.

Peripheral blood biomarkers such as proteins, mRNA, microRNAs (miRNA), and extracellular vesicles (EVs) have revolutionized translational studies in pulmonary diseases (2–6). The promise of identifying components of the peripheral blood as novel biomarkers of distal lung disease has yet to yield watershed therapeutic benefits despite the large body of research in this area. There are fundamental biologic limitations to drawing conclusions about circulating proteins or RNA signals (such as miRNAs) for distal organ-specific pathogenesis. However, EVs may offer important bridges over these gaps between the peripheral blood and distal organ injury, such as the lung (Figure 1).

EVs are nano-sized particles (150–250 nm) composed of phospholipid bilayers typically derived from the plasma membrane of the cell of origin. EVs are released by cells of diverse origin (structural and immune) into circulation, in which they function as peripheral messengers in cell–cell crosstalk (7). EV cargo contains nucleic acids, lipids, proteins, and mRNA and noncoding RNAs such as miRNAs that are important for mediating their paracrine effects. Given the central role of EVs in cell–cell communication, their activity in systems during health and disease offers potential as a biomarker for the early detection of lung injury. Previous studies have provided evidence to support the role of circulating EVs in the pathogenesis of pulmonary diseases such as COPD, asthma, pulmonary hypertension, acute lung injury and acute respiratory distress syndrome, and lung cancer (8).

In this issue of the *Journal*, Eckhardt and colleagues (pp. 50–59) identify an association between plasma-derived EV miRNAs and baseline lung function. In addition, they describe specific EV miRNA signatures associated with longitudinal lung function trajectories using two population-based cohorts (9). The authors leveraged the U.S. Veterans Affairs NAS (Normative Aging Study), which enrolled healthy male subjects and prospectively assessed health status, including serial pulmonary function testing and plasma collection, as their main cohort ($n = 656$; plasma samples from a later time point in NAS [$n = 401$] were also analyzed). The stored plasma samples were used to isolate EVs using a modified ultracentrifugation method, and total EV RNA was extracted using a kit-based approach followed by small RNA sequencing. These findings were subsequently validated using two independent replication cohorts, a subset of NAS participants ($n = 80$; EV miRNA profiling and analysis similar to the main cohort) and a prospective HEALS (Health Effects of Arsenic Longitudinal Study) cohort from Bangladesh ($n = 15$) (10).

The authors describe a novel analysis with EV miRNA data using linear regression models to compare associations between EV miRNAs with baseline lung function, referred to as EVWAS (EV miRNA-wide association study), analogous to genome-wide association studies. The initial analysis yielded 13 and 16 EV miRNAs associated with lower baseline FEV_1 and FVC, respectively. Interestingly, one EV miRNA was significantly associated with both lower FEV_1 and FVC (hsa-miR-24–3p). Recently, miR-24–3p was shown, in an analysis of lung tissue from subjects with COPD, to correlate with radiographic emphysema, and it is thought that miR-24–3p regulates DNA damage through Bcl-2-like protein 11 (BIM) activity and apoptosis, suggesting specific miRNAs can regulate cellular stress responses in the lung (11).

KEGG (Kyoto-Encyclopedia of Genes and Genomes) pathway analysis for EV miRNAs that had associations with lung function highlighted pathways associated with metabolism, cellular processes, and novel canonical signaling. The KEGG pathways associated with these EV miRNAs yield hypothesis-generating insights into the role of miRNA signaling in lung health. Given the longitudinal nature of pulmonary function measurements in NAS, these authors were able to classify NAS participants into two distinct groups on the basis of their lung function trajectory: "stable" and "declining". There was a significant association between hsa-miR-532–5p and a higher risk of belonging to the "declining" trajectory. Interestingly, miR-532–5p has been implicated in the modulation of epithelial-to-mesenchymal transition regulating angiogenic pathways in a vascular injury model (12). Finally, using a machine learning approach to create clusters of the EV miRNA data, the authors found that cluster one showed a greater risk of "declining" lung function class membership. In addition, the least absolute shrinkage and selection operator regression model revealed key EV miRNA drivers (11 EV miRNAs: 8 upregulated and 3 downregulated) that were distinct in cluster one from other EV miRNAs (9) (Figure 1).

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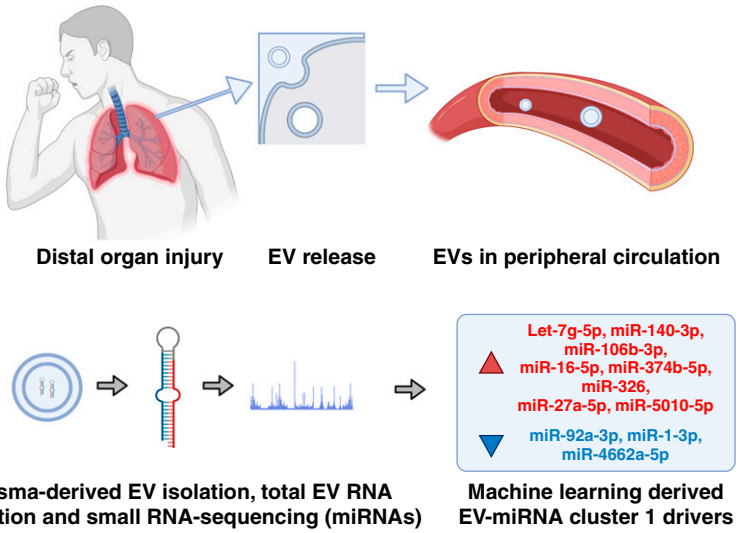

Figure 1. Summary schematic showing an overview of the proposed distal organ injury and subsequent extracellular vesicle (EV) release and isolation of EVs from the peripheral circulation. After being isolated from peripheral blood samples, EVs were used for RNA extraction and EV microRNA (miRNA) profiling by small RNA sequencing. Differentially expressed EV miRNAs were queried via various statistical methods to identify the association with lung function, which is used as a vital determinant of lung health.

The strength of the study is the prospective, population-based cohort design evaluating EV miRNAs with appropriate adjustments for well-defined confounders and determinants of lung health. It is important to highlight some important limitations of this study. The lack of detailed biochemical and phenotypic characterization of EVs, such as particle size and concentration, and a lack of EV-specific surface markers limit conclusions about cell type-specific EV associations. EV miRNA analysis used stored samples, which may have compromised the quality of EV miRNAs and could lead to missing signals. It remains unknown if EV miRNAs associated with lung function alter the expression of target genes that regulate key biological functions and cellular processes during health and disease. There could be other forms of noncoding RNAs (13), proteins, or lipids present in EV cargo that may serve as novel biomarkers and should be considered for future studies.

These results open the door to many important follow-up studies and approaches. For instance, these findings should be extended to cohorts with pulmonary disease, including preclinical disease states, to explore the usefulness of these EV miRNA signals in pathologic states. Isolation and systematic characterization of EVs from diverse biological fluids will provide deeper insight into distal organ signaling. These data highlight the complex networks that maintain lung health and the complexity of perturbations of that network associated with lung injury. Untargeted analysis of EV cargo from various biological sources offers the potential to map the complex signaling network of lung disease and offers hope for early biomarkers of lung disease. ■

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Risk Stratifying Interstitial Lung Abnormalities to Guide Early Diagnosis of Interstitial Lung Diseases

Delayed diagnosis is common in interstitial lung diseases (ILDs) and is associated with decreased quality of life and a poor prognosis (1). Early diagnosis and initiation of appropriate management could improve patient outcomes (2–5). Studies in idiopathic pulmonary fibrosis (IPF) demonstrated that antifibrotic therapies also slow down disease progression in patients with more preserved lung function (3, 5).

Interstitial lung abnormalities (ILAs) found incidentally on computed tomography (CT) performed for other purposes, such as lung cancer screening or diagnostic cardiac CT, may facilitate early diagnosis of ILD, allowing for early treatment and removal of triggers that drive ILD progression. However, ILAs are relatively frequent, especially in older subjects (6). Systematic evaluation of population-based and lung cancer–screening cohorts showed a prevalence of ILAs of 4–9% in (former) smokers and 2–7% in never-smokers (7). With increasing use of CT scans, clinicians are confronted with the question, "what to do with this person with ILAs?" Risk stratification of ILAs is urgently needed to differentiate two subsets: *1*) ILAs with a high likelihood of progression to clinically relevant ILD; versus *2*) ILAs that pose no such risk and do not need further evaluation and follow-up.

In this issue of the *Journal*, Rose and colleagues (pp. 60–68) add another piece of evidence to the puzzle of risk stratification of ILAs (8). They investigated whether combining CT data and pulmonary function (spirometry and D_{LCO}) could identify subjects with suspected ILD, associated with worse outcomes, within participants with ILAs in the Genetic Epidemiology of Chronic Obstructive Pulmonary Disease (COPDGene) cohort, a U.S.-based multicenter prospective cohort study of (current and former) smokers (9). People with known lung diseases other than COPD or asthma were excluded. CT scans were assessed for percentage of emphysema and ILAs, defined per criteria of the Fleischner Society (10). CT scans showing ILAs were scored on the presence of definite fibrosis. Importantly, D_{LCO} was corrected for the percentage of emphysema on CT. For suspected ILD, the authors used the following definition: presence of ILAs and at least one of the following three criteria: *1*) definite fibrosis on CT; *2*) post-bronchodilator FVC $<$ 80% predicted; or *3*) D_{LCO} $<$ 70% predicted after adjustment for emphysema.

Ten percent of participants (443 out of 4,360) had ILAs. Of those with ILAs, 239 (54%) met the criteria of suspected ILD; within this subset, 16% had definite fibrosis on CT, 57% had an FVC $<$ 80%, and 67% had a D_{LCO} $<$ 80% after adjustment for emphysema. The majority (62%) of participants with suspected ILD met only one criterium, 35% met two criteria, and 3% met all three criteria. Participants with suspected ILD were more likely to be of self-identified Black or African American race and had a higher pack-year smoking history. Compared with the ILA group, subjects with suspected ILD were more likely to have worse clinical endpoints (including quality of life, 6-minute-walk test, and respiratory exacerbations). Mortality rates were higher in the suspected ILD than in the ILA group (15% vs. 6%).

This study has several strengths, including the large, multicenter, prospective cohort design with longitudinal data collection. The authors take the important step of splitting ILAs in two separate subgroups with vastly different outcomes: ILAs versus suspected ILD. Although ILA is a CT-defined entity, suspected ILD is defined by a combination of radiological and physiological abnormalities and could be seen as a potential early phase in the evolutionary continuum of ILD (11). However, suspected ILD and even definite ILD is not a diagnosis but merely an umbrella term warranting further investigations, ideally in an experienced ILD center with a multidisciplinary team discussion (11, 12). Although it feels intuitively correct to refer subjects with suspected ILD for further work-up and follow-up, it is too early to conclude that people with ILAs without suspected ILD can be safely discharged from follow-up.

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