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RESEARCH ARTICLE

# Exhaled breath condensate contains extracellular vesicles (EVs) that carry miRNA cargos of lung tissue origin that can be selectively purified and analyzed

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## Abstract

Lung diseases, including lung cancer, are rising causes of global mortality. Despite novel imaging technologies and the development of biomarker assays, the detection of lung cancer remains a significant challenge. However, the lung communicates directly with the external environment and releases aerosolized droplets during normal tidal respiration, which can be collected, stored and analzsed as exhaled breath condensate (EBC). A few studies have suggested that EBC contains extracellular vesicles (EVs) whose microRNA (miRNA) cargos may be useful for evaluating different lung conditions, but the cellular origin of these EVs remains unknown. In this study, we used nanoparticle tracking, transmission electron microscopy, Western blot analyses and super resolution nanoimaging (ONi) to detect and validate the identity of exhaled EVs (exh-EVs). Using our customizable antibody-purification assay, EV-CATCHER, we initially determined that exh-EVs can be selectively enriched from EBC using antibodies against three tetraspanins (CD9, CD63 and CD81). Using ONi we also revealed that some exh-EVs harbour lung-specific proteins expressed in bronchiolar Clara cells (Clara Cell Secretory Protein [CCSP]) and Alveolar Type II cells (Surfactant protein C [SFTPC]). When conducting miRNA next generation sequencing (NGS) of airway samples collected at five different anatomic levels (i.e., mouth rinse, mouth wash, bronchial brush, bronchoalveolar lavage [BAL] and EBC) from 18 subjects, we determined that miRNA profiles of exh-EVs clustered closely to those of BAL EVs but not to those of other airway samples. When comparing the miRNA profiles of EVs purified from matched BAL and EBC samples with our three tetraspanins EV-CATCHER assay, we captured significant miRNA expression differences associated with smoking, asthma and lung tumor status of our subjects, which were also reproducibly detected in EVs selectively purified with our anti-CCSP/SFTPC EV-CATCHER assay from the same samples, but that confirmed their lung tissue origin. Our findings underscore that enriching exh-EV subpopulations from EBC allows non-invasive sampling of EVs produced by lung tissues.

**KEYWORDS**
biomarker, EV-CATCHER, exhaled breath condensate, extracellular vesicles, lung cancer, microRNA, next generation sequencing, non-invasive

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## 1 | INTRODUCTION

Pulmonary disease is ranked as the third-leading cause of mortality worldwide (GBD 2019 Chronic Respiratory Diseases Collaborators, 2023). Chronic obstructive pulmonary disease (COPD) and asthma, respectively claimed the lives of 3.3 and 0.4 million people worldwide in 2019 (Safiri et al., 2022; Safiri et al., 2022). Lung cancer alone has steadily been ranked as the second leading cause of cancer incidence (2.1 million cases per year) and mortality (1.8 million deaths per year) in both men and women worldwide (Barta et al., 2019; Siegel et al., 2021; Sung et al., 2021). It is estimated that the number of people diagnosed with lung cancer and those who die from it will nearly double by 2040 (Ferlay et al., 2021; Nicholson et al., 2022). However, contrary to other pulmonary diseases, early-stage lung cancer presents with common and non-specific respiratory symptoms, which can delay its detection and contribute to poor outcomes. Although there is a direct correlation between smoking and the development of lung tumors, only a fraction of high-risk subjects actively engage in early screening programs (Blandin Knight et al., 2017; Haddad et al., 2020). Additionally, even though smoking is a major known risk factor, other environmental and genetic factors, which remain largely unknown, make early identification of individuals who will develop lung cancer difficult. Currently, it is estimated that only ~15% of new lung cancer cases are diagnosed at an early and curable stage (Kanwal et al., 2017; Shankar et al., 2019; Yu et al., 2021). Thus, there is an urgent need for the development of non-invasive, rapid, cost-effective and highly sensitive early detection strategies to improve disease outcome.

Although computed tomography (CT) scan is widely used to detect lung abnormalities, it cannot accurately distinguish between benign and malignant lung nodules, and for a complete clinical diagnosis invasive biopsies are required (Billatos et al., 2018; Corner et al., 2005; Van Hal & Diab Garcia, 2021). As a non-inasive alternative approach, it has been proposed that targeted molecular testing may achieve a greater sensitivity than current clinical imaging modalities (Blandin Knight et al., 2017). Therefore, several molecular assays have been evaluated for the ultra-sensitive detection of circulating tumor material, including circulating tumour cells (CTCs), circulating tumor DNA (ctDNA), deregulated circulating transcripts (circular RNAs, mRNAs, microRNAs [miRNAs], long non-coding RNAs) and more recently circulating biomarkers (DNA, RNAs, proteins) stably packaged within circulating tumor extracellular vesicles (Dama et al., 2021; de Fraipont et al., 2019; Haranguș et al., 2019; Zhong et al., 2021). Although these technological advances may help decrease the invasiveness of testing, the sensitivity of blood-based biomarkers remains limited to the detection of advanced lung cancer.

Considering that the lung communicates directly with the external environment, different types of airway-derived biofluids (i.e., saliva, sputum, bronchial brushing, bronchoalveolar lavage [BAL], etc.) have been experimentally evaluated for their potential to provide meaningful biomarkers originating from the lung with hope to help develop more accurate lung cancer detection strategies (Anglim et al., 2008; Boots et al., 2015; Chen et al., 2021; Engel et al., 2004; Horváth et al., 2005; Matthiesen, 2020; Mutlu et al., 2021; Papineni & Rosenthal, 1997; Rahimpour et al., 2018; Safiri et al., 2022; Schmidt et al., 2016). Although bronchoscopy with BAL is the most direct approach for sampling the biofluid that lines the lower respiratory tract where lung tumors reside, the invasive nature of this procedure has precluded its implementation as a screening approach and has ignited a search for less intrusive airway sampling methods. Of these the collection of exhaled breath has gained a lot of interest because it is minimally invasive and can simply be carried out during normal tidal respiration (Wallace & Pleil, 2018; Winters et al., 2017). Biochemical analyses of exhaled breath have demonstrated that it contains two distinct biological fractions, which can be separately collected and analysed: volatile organic compounds (VOCs) which are gases diffused from the circulation, and non-volatile organic compounds (non-VOCs), which are aerosolized compounds originating from the respiratory tract (Hunt, 2007). Non-VOCs can easily be condensed into a biofluid also known as exhaled breath condensate (EBC), which is of interest in molecular biology because it contains metabolites (e.g., nitrite, urea, amino acid) and macromolecules (i.e., proteins, RNA, DNA) that can be precisely quantified even after long term storage whereas VOCs can only be analysed during subject monitoring (Boots et al., 2015; Mazzatenta et al., 2021; Rahimpour et al., 2018).

The collection of EBC can be performed non-invasively using FDA-registered commercial devices (Horváth et al., 2005; Hunt, 2007; Papineni & Rosenthal, 1997). It is thought that the macromolecules contained in EBC become aerosolized from the biofluid that lines epithelial lung cells due to the velocity of air turbulence caused by normal respiration inside the lungs (Hunt, 2007). Early analyses of EBC content have revealed the presence of surfactant proteins, which have suggested that some of the exhaled macromolecules may originate in deep lung alveoli, the respiratory units responsible for gas exchanges between the circulatory system and the external environment (Powers & Dhamoon, 2023). The terminal bronchioles are the smallest airway passages in direct contact with the alveoli where different types of epithelial cells reside, including the non-ciliated secretory lung-specific Clara cells (Crystal et al., 2008). These cells are particularly interesting because they uniquely express the Clara Cell Specific Protein (CCSP) in the lung tissue and secrete it in large quantities to protect the respiratory tract against oxidative stress and inflammation (Chang et al., 2000). Contrarily to the terminal bronchioles, the alveoli are only composed of two types of cells, the alveolar type I cells that are involved in gas exchange and the alveolar type II cells that are progenitors of type I cells but that produce all pulmonary surfactant, whose purpose is to reduce the air-liquid surface tension to enhance gas exchange and to actively contribute to the immune defense of the lungs (Whitsett et al., 2010). The surfactant Protein C (SFTPC) is one of these molecules and it is uniquely produced by alveolar type II cells (Krygier et al., 2022; Leibel et al., 2019). Although the exact

cellular origin of lung cancer initiating cells remains unknown, both Clara cells and alveolar type II cells have been experimentally implicated in the initiation of lung adenocarcinoma (Dermer, 1982; Jackson et al., 2001; Xu et al., 2012).

To date, several molecular studies conducted on EBC have been focused on its DNA and RNA content with genomic DNA methylation profiling (Xiao et al., 2014), genomic DNA mutational profiling (Youssef et al., 2017), mitochondrial DNA mutational profiling (Yang Ai et al., 2013), the profiling of uniquely deregulated mRNAs (Mehta et al., 2016) and more recently the profiling of microRNAs (miRNAs) to evaluate the detection of lung cancer, including a study that we recently conducted (Pérez-Sánchez et al., 2021; Rai et al., 2023; Shi et al., 2023; Xie et al., 2020). MiRNAs are of particular interest because their deregulated expression is directly associated with the initiation and development of lung cancers (Xiao et al., 2014; Yang Ai et al., 2013; Youssef et al., 2017), and they have shown promise for prognostic evaluation of lung cancers (Acunzo et al., 2015; O'Brien et al., 2018; Santos et al., 2020), and their quantitation in biofluids shows promise for prognostic evaluation of lung tumors (Asakura et al., 2020; Yanaihara et al., 2006; Zhu et al., 2021). However, considering that intact exhaled miRNAs can be detected in EBC (Shi et al., 2023), we and others have hypothesized that they may be packaged and protected within exhaled Extracellular Vesicles (exh-EVs) (Cherchi et al., 2023; Dobhal et al., 2020; Lucchetti et al., 2021; Purghè et al., 2021; Sinha et al., 2013). Studies have shown that the molecular cargos of EVs are protected from degradation by a robust double-layered membrane, which harbors generic (i.e., common to many cell types) and cell-specific surface proteins and receptors acquired from their cell-of-origin (Sheta et al., 2023; van Niel et al., 2018). EVs are produced by all human cells and due to their small size (~30 to 120 nm in diameter) they can diffuse into tissues and circulate in any biofluid (Sheta et al., 2023; van Niel et al., 2018). Particularly, recent studies have demonstrated that EVs released by tumor cells contain a variety of pre-packaged functional biomolecules including miRNAs, whose local and long-distance delivery to target cells enables intercellular communication (Mills et al., 2019; O'Brien et al., 2020). Functional studies of EVs produced and released in different biofluids (i.e., blood, BAL and pleural lavage) by primary lung tumor cells (He et al., 2021) have demonstrated that their miRNA cargos, which are differentially packaged than those of normal cells, can modulate angiogenesis (Mao et al., 2020), cellular proliferation (He et al., 2019) and immune response of target cells (Fabbri et al., 2012) and also participate in the education of distal pre-metastatic lung niche cells to promote the uptake of CTCs (Zeng et al., 2018). Thus, it is currently proposed that the purification of tumor EVs from airway biofluids and the quantification of their miRNA cargos may enable non-invasive detection of unique signatures produced by tumor cells for non-invasive detection of lung cancer.

Considering that only a few studies have suggested that EBC may contain EVs (An et al., 2021; Dobhal et al., 2020; Lucchetti et al., 2021), our primary objective was to experimentally confirm the presence and to precisely validate the identity of EVs contained in human EBC. Upon purification and identification of exhaled EVs (exh-EVs), we then sought to determine whether they contained miRNAs of lung tissue origin and thus conducted small-RNA next generation sequencing (NGS) analyses of EBC and exh-EVs in comparison to other airway biofluids. Next, by leveraging our ultra-sensitive and customizable EV-CATCHER (Extracellular Vesicle Capture by AnTibody of CHoice and Enzymatic Release) purification assay (Mitchell et al., 2021) to selectively target different populations of exh-EVs, we evaluated the potential of uniquely selecting exh-EV miRNA cargos to distinguish subjects based on the condition of their respiratory tract.

## 2 | MATERIALS AND METHODS

### 2.1 | Clinical specimen collection

#### 2.1.1 | Subject recruitment and specimen collection at the Montefiore Medical Center (MMC)

A longstanding RTube™-based collection procedure was used at the Montefiore–Einstein Medical Center/Comprehensive Cancer Center for the collection of exhaled breath condensate (EBC), mouth rinse, buccal brushings, bronchial brushings and bronchoalveolar lavage (BAL) from an initial 18 subjects undergoing clinically indicated bronchoscopy, under IRB protocol (#2007-407). In person interviews were conducted, clinical data obtained and verified, and non-invasive EBC, mouth rinse and buccal brush specimens were collected before the clinically indicated bronchoscopic procedure. All EBC collections were performed using the RTube™ breath condensate collection devices (Respiratory Research Inc, Cat#2501). This single-use FDA-registered device is comprised of a coated plastic collection tube and a mouthpiece where during inhalation air enters the base of the mouthpiece where air can be inhaled (the air enters the mouthpiece through a filter at its base) and during exhalation the breath is directed upward into a polypropylene tube where exhaled droplets can be condensed. The collection tube contains a unique custom duckbill valve, which opens only during exhalation and seals during inhalation, thereby ensuring one-way condensation of EBC into the collection tube. Moreover, as the duckbill is in contact with the collection tube via a plastic O-ring, it can be plunged upward into the collection tube to pool the condensed EBC, thereby avoiding cumbersome centrifugation of the tube in order to collect the EBC content. It is also important to note that the design of the large "T" section of the mouthpiece ensures that saliva is separated from exhaled breath and does not enter the collection tube. During EBC collection, a chilled aluminium cylinder covered with a cloth sleeve is positioned over the collection tube. In this fashion, the chilled cylinder allows

for exhaled droplets to condense and deposit on the inner surface of the collection tube. We have observed that over the course of 10 min, a healthy subject breathing without excessive exertion or hyperventilation will generate an average of 1.5–2 mL of EBC biofluid. Mouth rinse was collected by 5 mL standard commercial EtOH-containing mouth rinse and buccal brushings were obtained by means of inner cheek swabs using sterile cytologic brushes. During the bronchoscopy procedure, research-devoted bronchoalveolar lavage was obtained by means of normal saline (NS) lavage of 40 mL input with ~10–20 mL return and endobronchial brushings were collected using sterile cytologic brushes by means of standard clinical procedures. Following the analyses on the first 18 subjects we expanded our cohort analyses to include matched EBC and BAL that were collected from an additional 51 subjects (i.e., a total of 69 EBC and BAL specimens were analyzed in this study) in the same manner as described above. Specimens were snap frozen on dry ice immediately upon collection in the pre-procedure and bronchoscopy suite. Longer term storage was maintained at −80°C.

#### 2.1.2 | Subject recruitment and specimen collection at Hackensack University Medical Center (HUMC)

EBC samples were collected from (i) six treatment naïve stage IV lung cancer patients receiving treatment at the John Theurer Cancer Center (JTCC) at Hackensack University Medical Center (HUMC) and (ii) 12 healthy volunteers under an IRB-approved protocol (Pro#2020-0258). EBC collection from lung cancer patients ($n = 6$) and healthy volunteers ($n = 12$) was performed using the RTube™ breath condensate collection device and the above-described procedure during a single 10-min period unless otherwise stated. EBC specimens were all processed within 1 h of collection and stored as 500 $\mu$L aliquots at −80°C.

### 2.2 | Spectradyne microfluidic resistive pulse sensing (MRPS)

The particle size distribution of exhaled EVs isolated from EBC was measured using MRPS on the Spectradyne nCS1 instrument (Spectradyne LLC, Signal Hill CA). The microfluidic system was initially primed with a solution of 0.2 $\mu$m filtered DPBS containing 1% Tween 20 (v/v). 2 $\mu$L of purified exh-EVs were loaded into TS-400 cartridges, which allow for the analysis of particles between 65 and 400 nm and instrument pressure and voltage parameters were determined automatically by the instrument software. Following each acquisition of data from > 10,000 particle detection events for each sample, data were combined into a single stats file, and using the nCS1 Data Viewer software and peak filters and background subtraction were applied, according to manufacturer recommendations. Peak filters set were (i) transit time < 60 $\mu$s, (ii) diameter > 65 nm and (iii) signal-to-noise ratio (S/N) > 10 for all samples. Additionally, combined stats files were analyzed for size distribution and particle concentration and peak-filtered CSD graphs were generated.

### 2.3 | Ultracentrifugation of EBC for concentration and isolation of exh-EVs

In order to initially characterize exhaled EVs (exh-EVs) contained within EBC, we collected 60 mL of EBC from each of two healthy donors, using the RTube™ breath condensate collection devices, with three 20-min collection periods per day for 5 consecutive days from each donor. After each collection period EBC was collected into individual Eppendorf tubes and stored at −80°C until the total EBC volume of 60 mL was achieved from each donor. Once collected the 60 mL of EBC obtained from each donor was defrosted and combined separately prior to downstream concentration and processing. In order to concentrate and purify all exh-EVs from the 60 mL EBC, the gold standard sequential centrifugation approach for EV isolation was used (van Niel et al., 2018). Briefly, centrifugation was performed at 300 × $g$ at 4°C for 5 min, followed by 10 min at 2000 × $g$ at 4°C to remove any potential larger cellular debris. The supernatant was then centrifuged at 10,000 × $g$ at 4°C for 30 min to remove any macrovesicles and after being transferred to a clean ultracentrifuge tube, the supernatant was centrifuged at 100,000 × $g$ for 90 min at 4°C. The resulting supernatant was discarded, and the exh-EV pellet was resuspended in 10 mL of sterile 1× PBS and again centrifuged at 100,000 × $g$ for 90 min at 4°C. The final resulting exh-EV pellet from each donor split into three for downstream EV-CATCHER isolations and exh-EV characterization experiments.

### 2.4 | Western blot analysis

Western blot analyses were conducted to characterize the presence of common EV protein markers present on exhaled EVs (exh-EVs) isolated from EBC. Isolated exh-EVs purified by means of ultracentrifugation, three-tetraspanine (tTSP) (CD9/CD63/CD81) EV-CATCHER and CCSP/SFTPC EV-CATCHER assays were separated on 4%−12% polyacrylamide precast mini-PROTEAN TGX gels (Bio-Rad, #4561086) by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). 5 $\mu$L of PageRuler™ Plus prestained Protein ladder (ThermoFisher, #26620) was loaded and used for gel orientation and

determination of molecular weights of separated proteins. 10 $\mu$g of each purified recombinant protein was loaded, and gels were run at 100 V for 90 min (Power Pac 300, Bio-Rad) in 1x Tris/Glycine/SDS buffer (Bio-Rad, #1610732). After proteins were separated, gels were UV activated on a Chemi-Doc™ MP (Bio-Rad) system to allow for the activation UV-dependent 2,2,2,-trichloroethanol (TCE) labelling of tryptophan residues in proteins and thus their stain-free visualization. Proteins were then transferred to 0.2 $\mu$m polyvinylidene fluoride (PVDF) membranes (Bio-Rad, #1704156) using a semidry electro-transfer system (TransBlot Turbo v1.02, Bio-Rad) for 30 min at 25 V. Proteins were visualized using the stain-free blot protocol provided on a Chemi-Doc™ MP (Bio-Rad) system to evaluate protein transfer and membranes were blocked using EveryBlot blocking buffer (Bio-Rad, #12010020) for 30 min. Membranes were incubated at 4°C O/N with TBS-T (1x TBS, pH 6.8, 0.1% Tween20) diluted anti-mouse primary antibodies targeted against Apolipoprotein B (Novus Biologicals, Cat#MAB4124), Albumin (Novus Biologicals, Cat#MAB1455), CD63 (Abcam, Cat#ab59479), or with anti-rabbit primary antibodies targeted against Apolipoprotein A1 (Novus Biologicals, Cat#MAB36641), CD81 (Abcam, #ab233692) and CD9 (Abcam, #ab263023). Membranes were washed with TBS-T (3 × 5 min) before incubation in anti-mouse IgG horseradish peroxidase conjugated secondary antibodies for 1 h, with gentle agitation at RT. Membranes were washed with TBS-T (3 × 5 min) before proteins were detected using SuperSignal™ West Femto Maximum Sensitivity Substrate (Pierce, #34095) and protein bands were visualised using ImageLab 4.0 software on a Chemi-Doc MP (Bio-Rad) imaging system.

## 2.5 | EV-CATCHER isolation of the exhaled extracellular vesicles

The isolation of exhaled EVs (exh-EVs) was performed using the EV-CATCHER isolation protocol described by Mitchell et al. (2021) using a combination of CD9/CD63/CD81 (i.e., triple tetraspanin [Ttsp] exh-EV isolation) or CCSP/SFTPC (i.e., lung specific exh-EV isolation) as the capture antibodies (van Niel et al., 2018). Briefly, equimolar amounts of 5′-Azide modified and 3′-Biotin modified oligonucleotides (Integrated DNA Technologies) were annealed in 1x RNA annealing buffer (60 mM KCl, 6 mM HEPES pH 7.5, 0.2 mM $MgCl_2$), prior to separation on a 15% non-denaturing polyacrylamide (PAGE) gel. The annealed double stranded (ds) DNA product was visualized on a blue light box with SYBR® Gold™ dye (ThermoFisher, #S11494), excised, crushed using a gel breaker tube (IST Engineering, #3388-100), resuspended in 400 mM NaCl and placed on a thermomixer set to 4°C and 1100 RPM overnight (O/N). The solution was filtered, and the dsDNA linker was purified using the QIAEX® II gel extraction kit (Qiagen, #20021) according to manufacturer instructions. Capture antibodies (1 mg/mL) used for exh-EV pulls, were activated using 5 $\mu$L of freshly prepared 4 mM DBCO-NHS ester (Lumiprobe, #94720) and incubated for 30 min at room temperature (RT) in the dark. Reactions were stopped by adding 2.5 $\mu$L of 1 M Tris-Cl (pH 8.0) at RT for 5 min in the dark. DBCO-activated antibodies were then desalted using Zeba desalting columns (ThermoFisher, #89882). and quantified on a Nanodrop 2000 instrument prior to the preparation of antibody-dsDNA (Ab-dsDNA) stock solutions (i.e., 100 $\mu$g of activated antibody conjugated to 50 $\mu$g of purified DNA linker. The Ab-dsDNA conjugates were then bound to streptavidin coated 96-well plates (Pierce, #15120) by incubating 1 $\mu$g of each Ab-dsDNA in 100 $\mu$L PBS per well (2 wells were prepared per sample). Solutions were carefully removed, and wells were washed three times with cold 1x PBS solution, prior to addition of RNase-A (12.5 $\mu$g/mL) treated samples (100 $\mu$L). Plates were sealed using microAMP optical adhesive film (Applied Biosystems, #4311971) and placed on a shaker at 300 RPM at 4°C, O/N. Samples were carefully removed, wells were washed three times with cold 1x PBS and 100 $\mu$L of freshly prepared uracil glycosylase (UNG) enzyme (ThermoFisher, #EN0362) in 1x PBS (1x UNG buffer [200 mM Tris-Cl (pH 8.0), 10 mM EDTA and 100 mM NaCl], with 1 unit of enzyme) was added to each well. Plates were incubated at 37°C for 2 h on a shaker at 300 RPM for UNG digestion of the dsDNA linker, and exh-EVs were collected for downstream analyses.

## 2.6 | Transmission electron microscopy

Transmission electron microscopy (TEM) of exhaled EVs (exh-EVs) purified by ultracentrifugation and the EV-CATCHER assay was performed at the analytical imaging facility at the Albert Einstein College of Medicine, Bronx, NY. Briefly, purified exh-EVs were fixed using 2% glutaraldehyde in phosphate buffer (Electron Microscopy Services, #6536-05) and stored at 4°C. 300 mesh formvar-coated grids were inverted onto 20 $\mu$L of fixed exh-EV suspensions for 2 min and wicked dry. Grids were then inverted onto 40 $\mu$L of 2% aqueous uranyl acetate for 1 min, and wicked dry. Samples were imaged on a JEOL JEM-1400+ transmission electron microscope (JEOL Ltd.; Tokyo, Japan) operating at an accelerating voltage of 80 kV. High-resolution TIFF images were acquired and saved using an AMT 16 MP digital camera system (Advanced Microscopy Techniques Corp.; Woburn, MA).

## 2.7 | ONi super resolution nanoimaging

Purified exhaled EVs (exh-EVs) were processed for imaging on the ONi super resolution Nanoimager using the ONi EV Profiler kit v2.0 according to manufacturer's instructions. Briefly, the surface of the assay capture chip was prepared by applying 5 $\mu$L of S3

buffer to each lane and incubated at room temperature for 10 min. 30 $\mu$L of W1 was then applied to each lane to remove excess S3, after which 10 $\mu$L of S4 buffer was slowly pipetted to each lane ensuring that no bubbles were introduced into lanes. After a 10-min incubation period at room temperature lanes were again washed by applying 30 $\mu$L of W1 buffer to each lane. EV capture was then performed by immediately applying 10 $\mu$L of ultracentrifuged, tTSP (CD9/CD63/CD81) EV-CATCHER, and CCSP/SFTPC EV-CATCHER purified exh-EVs and allowing binding to occur for 15 min. Lanes were then washed using 30 $\mu$L of W1 buffer and captured exh-EVs were fixed by adding 20 $\mu$L of F1 to each lane and incubating the chip at room temperature for 10-min. The staining of captured exh-EVs was performed by firstly preparing a three-antibody working solution comprising the CD63-Alexa Fluor™ 568, CD9-Alexa Fluor™ 488 and CCSP-Alexa Fluor™ 647 antibodies, or the CD63-Fluor™ 568, CD9-Alexa Fluor™ 488 and SFTPC-Alexa Fluor™ 647 antibodies, combined together in W1 buffer so that each antibody is at a dilution of 1:20. The final staining solution was prepared by combining 1 $\mu$L of the prepared working solution with 9 $\mu$L of N1 buffer for each lane, gently pipetting to mix the solution and applying 10 $\mu$L to each lane of the EV profiler chip and allowed to incubate for 50 min at room temperature in the dark. Immediately following antibody incubation, the lanes were washed with 30 $\mu$L of W1 buffer followed by a 20-min incubation with 20 $\mu$L of F1 buffer for 10 min. A final wash step was performed and BCubed™ dSTORM imaging buffer added to each well immediately before EV profiler chips were imaged. Image acquisition on the ONi super resolution Nanoimager was performed in the NimOS Light program with a 640 dichroic split using the following parameters: 640 nm laser set to 20%–30% laser power, the 560 nm laser at 35% laser power and the 473/488 nm laser set to 70% laser power. The number of runs (frames) for all laser lines was set to 1000 and all image analyses were performed using CODI software.

## 2.8 | RNA extractions

Small-RNA from exhaled EVs (exh-EVs) was isolated using the miRNeasy Serum/Plasma kit (Qiagen, Cat#217184) according to manufacturer's instructions with some modifications to improve total RNA yield. Briefly, QIAzol was added to 100 $\mu$L of exh-EVs, vortexed and incubated at RT for 3 min, after which chloroform was added to each sample. Samples were vortexed again and incubated at RT for 3 min. Samples were centrifuged at 12,000 × $g$, at 4°C for 15-min and the upper aqueous phase of each sample was carefully removed and transferred into new siliconised tubes, to which 1.7x volume of 100% ethanol and 2 $\mu$L of small RNA size markers (19 and 24 nt 1.5 ng each) were added per sample. Samples were incubated on ice for 40 min prior to column purification and then passed twice through RNeasy minElute columns, followed by a working solution of RPE wash buffer, and then ice cold 80% ethanol. Columns were spun to remove residual ethanol and total RNA was eluted with 50 $\mu$L of RNase-free water. Samples were then speed-vacuumed to 10 $\mu$L prior to small-RNA sequencing.

## 2.9 | Small-RNA cDNA library preparations

Small-RNA sequencing of RNA extracted from mouth-rinse, buccal brushings, exhaled breath condensates (EBC) and/or exhaled EVs (exh-EVs) purified from EBC , bronchial brushing, and bronchoalveolar lavage (BAL) and/or purified BAL EVs were performed using the cDNA library preparation protocol described by Loudig et al. (2017), with modifications for low input RNA from purified EVs (Sheta et al., 2023). In brief, total RNA from 18 samples, recovered from 100 $\mu$L biofluids or exh-EVs purified from whole EBC or BAL biofluids underwent individual ligations using truncated K227Q T4 RNA Ligase 2 with 18 different adenylated barcoded 3′ adapters in separate 1.5 mL Eppendorf tubes, overnight at 4°C. The following day, the ligations were heat deactivated, combined, precipitated on ice with 100% ethanol and centrifuged for 1 h at 14,000 rpm on ice at 4°C. The RNA was dried, resuspended, small-RNAs were separated on a 15% Urea-PAGE gel, excised and incubated in a 400 mM NaCl solution at 4°C on a thermomixer at 1100 RPM. The next day the RNA was precipitated in the presence of 100% ethanol, centrifuged, pelleted, resuspended, prior to being subjected to a second ligation with a 5′ adapter using T4 RNA Ligase 1 for 1 h at 37°C. The ligated product was separated on a 12% Urea-PAGE gel, ligated small-RNAs were excised, and incubated overnight in a 300 mM NaCl solution with 1 mL of 100 $\mu$M 3′ PCR primer, which was also the reverse-transcription primer, on a thermomixer at 1100 RPM at 4°C. The next day, the solution was filtered, precipitated with 100% ethanol, incubated on ice for 1 h, pelleted by centrifugation at 14,000 RPM for 1 h at 4°C, and a portion of this solution subjected to reverse transcription. A pilot PCR reaction was set up using the cDNA and the generation of amplicons was evaluated at cycles 10, 12, 14, 16, 18, 20 and 22, prior to analysis on a 2.5% agarose gel, and identification of the optimal PCR amplification cycle. A large-scale PCR reaction was set up using the cDNA for amplification, separation on a 2.5% agarose gel, isolation, and quantification on an Agilent Bioanalyzer. The small-RNA cDNA libraries were sequenced on an Illumina HiSeq-2500 sequencer, and the generated FASTQ files were processed for adapter trimming and small-RNA alignment to the hg-19 genome using the pipeline established in the Tuschl laboratory (Farazi et al., 2012). Read counts were normalized to total counts and subjected to statistical analyses (see below).

### 2.10 | Sequencing data analysis

The sequencing data analysis involved processing raw FASTQ files obtained from an Illumina HiSeq2500 sequencer. This processing was carried out using the RNAworld server, hosted at the Tuschl Laboratory, Rockefeller University. This processing encompassed tasks such as adapter trimming, read alignment, annotation and counting. Subsequently, miRNA analyses were executed using specialised Bioconductor packages within the R platform. To visualize the data, heat maps were generated from transformed counts utilising the "NMF" package, specifically the "aheatmap" function. Differential expression was conducted employing the "DESeq2" package. To filter out lowly-expressed miRNAs, the approach described by Chen et al. was implemented within the "edgeR" package. Notably, the differential expression models incorporated a batch variable (library) when necessary to address potential batch-related biases. To enhance the discriminative potential of miRNA profiles, a unique score, termed the "miRNA score" was computed a distinct score for each sample (Mazeh et al., 2018). This score was derived by aggregating the standardized levels ($z$-values) of upregulated miRNAs and the negative $z$-values of downregulated miRNAs, either considering all miRNAs or only those shown to be differentially expressed at a specified significance cutoff in the specified condition.

## 3 | RESULTS

### 3.1 | Exhaled breath condensates and study design

The RTube™ Exhaled Breath Condensate collection device, an FDA-registered single-use disposable handheld device (FDA#3004852415), was utilized for EBC collections from all subjects included in this study (Figure 1b). The detailed process of EBC collection, using the RTube™ Device, is detailed in Figure S1. Anatomically the terminal respiratory unit can be divided into the terminal bronchioles and the alveoli air sacs (Figure 1c, left). Terminal bronchioles are comprised of different epithelial cell types, including the non-ciliated bronchiolar secretory Clara cells, which uniquely express Clara cell specific protein (CCSP) in the lung; and respiratory alveoli that are only comprised of two cell types; the alveolar type II cells that uniquely produce surfactant proteins (i.e., A1/A2, B, D) and particularly Surfactant Protein C (SFTPC), and alveolar type I cells that are involved in gas exchange between the blood and the external environment (Figure 1c, right).

### 3.2 | Purification and validation of exhaled EVs (exh-EVs) in EBC

We first sought to determine whether EBC collected with the RTube™ device allowed for the condensation of exhaled EVs (exh-EVs). For this, we evaluated the purification of exh-EVs from the EBC collected from four healthy volunteer donors using our customized human anti-CD63 EV-CATCHER assay, which allows for the selective purification of EVs that harbour the CD63 tetraspanin from biofluids, as we previously described (Mitchell et al., 2021). After selectively evaluating the capture of CD63 positive exh-EVs from the four EBC samples (Figure 2a, green, red, light blue and purple plots), we analysed and counted the particle content of our immune-purified samples, and negative control (anti-CD63 antibody released without EVs after incubation with 1xPBS; Figure 2a, dark blue) on a Spectradyne nCS1 instrument using TS-400 or C400 cartridges (which targets nanoparticles ranging between 65 and 400 nm). Our analyses revealed the presence of nanoparticles, which fell within the expected size range of exh-EVs (65–150 nm). Next, following MISEV2018 guidelines, we sought to characterize the identity of exh-EVs purified from EBC collected from two healthy volunteers (60 mL EBC progressively collected per subject) following gold-standard ultracentrifugation (UC) and antibody-based targeted purification using a combination of three tetraspanin antibodies (anti-CD9, -CD63 and -CD81) using our triple tetraspanin (tTSP) EV-CATCHER assay. We conducted Western blot analyses of exh-EVs purified by UC and by our tTSP EV-CATCHER assay, which validated the presence of common EV tetraspanin protein markers (i.e., Figure 2b, see CD9, CD63 and CD81) with minimal to no visual detection of liposomal contaminating proteins, which include ApoA1 and ApoB, and no detection of albumin, a protein usually found as a high contaminant for EVs purified from blood but absent in our EBC samples (Figure 2b). Next, we conducted Transmission Electron Microscopy (TEM) analyses of exh-EVs purified both by UC and our tTSP EV-CATCHER assay from the EBC of our two volunteer donors. Our TEM data confirmed both the presence and morphology of EVs (i.e., nanoparticles with a cup shaped membrane) in EBC, with sizes ranging between 65 and 150 nm (Figure 2c, left and center panels), consistent with those described in the literature for EVs (Cizmar & Yuana, 2017).

Because previous reports had indicated that EBC contains the terminal bronchiole Clara cell CCSP protein and the Alveolar type II cell-derived SFTPC protein (Powers & Dhamoon, 2023), we sought to determine whether exh-EVs originating from the lung could be targeted using these two proteins as antigens to purify them from EBC. Thus, we customized our EV-CATCHER assay with anti-CCSP and anti-SFTPC antibodies and purified EVs from ultracentrifuged pellets from our two EBC samples (i.e., healthy volunteers). Our TEM analyses validated that our anti-CCSP/anti-SFTPC customised EV-CATCHER assay allowed

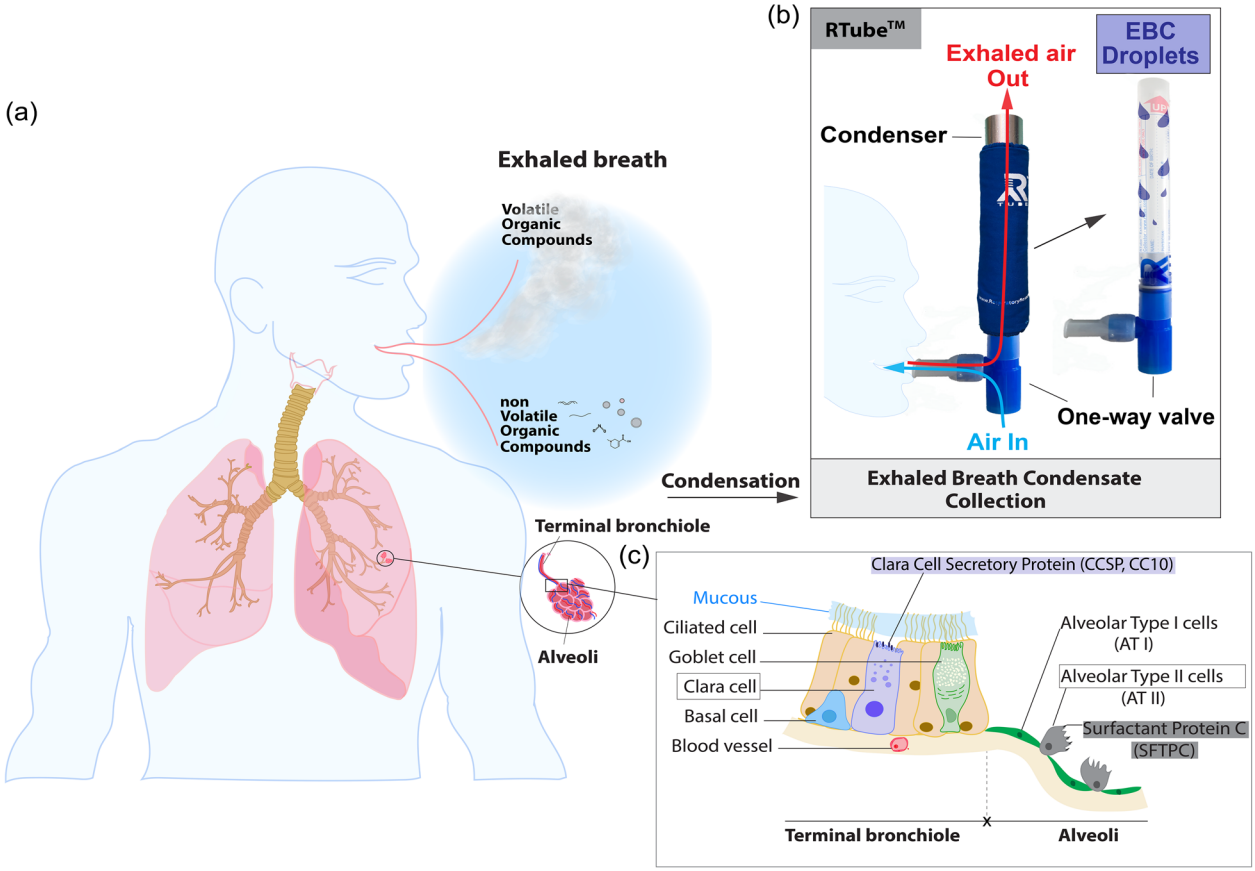



**FIGURE 1** The airway system and the non-invasive collection of exhaled breath condensates (EBC). (a) Volatile organic compounds (VOCs) and non-volatile organic compounds (non-VOCs) are exhaled out of the lungs during normal tidal respiration. Non-VOCs are enriched in metabolites and macromolecules by aerosolization of biofluids from the respiratory tract. (b) The collection of exhaled breath condensates using the disposable RTube™ device (FDA-registered). Prior to collection of EBC a pre-frozen metal cylinder, housed within an insulated cloth sleeve, is applied over the polypropylene section of the RTube™ collection device. The volunteer subject uses the mouthpiece of the device to inhale (see blue arrow for inhaled air) and exhale (see red arrow for exhaled air) during normal tidal respiratory cycles, for a period of 10-min. The exhaled air, which contains moisture, is progressively condensed into a biofluid onto the non-absorbent surface of the transparent polypropylene collection tube. Upon completion of the EBC collection, the transparent collection tube is disconnected, positioned onto a plunger where the lower gasket is pushed upward to gather the biofluid at the top of the tube. The EBC is then collected with a pipette and transferred into an Eppendorf for storage or analysis. (c) Architectural cellular representation of the terminal bronchiole and alveoli unit with its different epithelial cells. The terminal bronchiole is comprised of Ciliated, Goblet, Clara and Basal epithelial cells. Clara cells uniquely express the Clara Cell Secretory protein (CCSP). The alveoli are composed of Alveolar Epithelial Type I (ATI) and Alveolar Epithelial Type II cells (ATII). ATII cells uniquely express different surfactant proteins and particularly Surfactant Protein C, which is a membrane protein that undergoes cleavage before release into the biofluid lining the lungs.

for the purification of exh-EVs from EBC (Figure 2c right panels). Therefore, next we aimed to verify that these purified exh-EVs indeed harbored these secretory proteins on their membrane surface. To confirm the localization of two tetraspanins (CD9 and CD63; Figure 2d left and center panels) and the two distinct secretory proteins (CCSP and SFTPC), we employed super-resolution nanoimaging (ONi) (Figure 2d, right panels) to image these four proteins. We determined that CCSP, which is found in non-ciliated bronchiolar Clara cells, was located on the surface of exh-EVs purified by UC (Figure 2d, left panels), our tTSP EV-CATCHER assay (Figure 2d, center panels), and our anti-CCSP/SFTPC EV-CATCHER assays (Figure 2d, left panels). Furthermore, we determined that SFTPC, which is produced by Alveolar Type II cells, could also be detected on the surface of exh-EVs (Figure 2d, right panel). Evaluations of the complete fields of EV captures on the ONi revealed different distributions of triple positive CD9, CD63, CCSP and CD9, CD63, SFTPC, showing an apparent lower abundance of the latter within our two EBC samples (see Figure S2). As another important validation (see Figure S3) and to further confirm the origin of exh-EVs, we conducted nanoparticle analysis with a Spectradyne nCS1 instrument using the TS-400 cartridge (Figure S3a), TEM (Figure S3b) and ONi of EVs purified by ultracentrifugation from BAL of two subjects (Figure S3c). We confirmed the presence of CD9, CD63, CCSP and SFTPC on the surface membrane of BAL-EVs, which further validated that exh-EVs found in EBC originate from the biofluid that lines terminal bronchioles and alveoli.

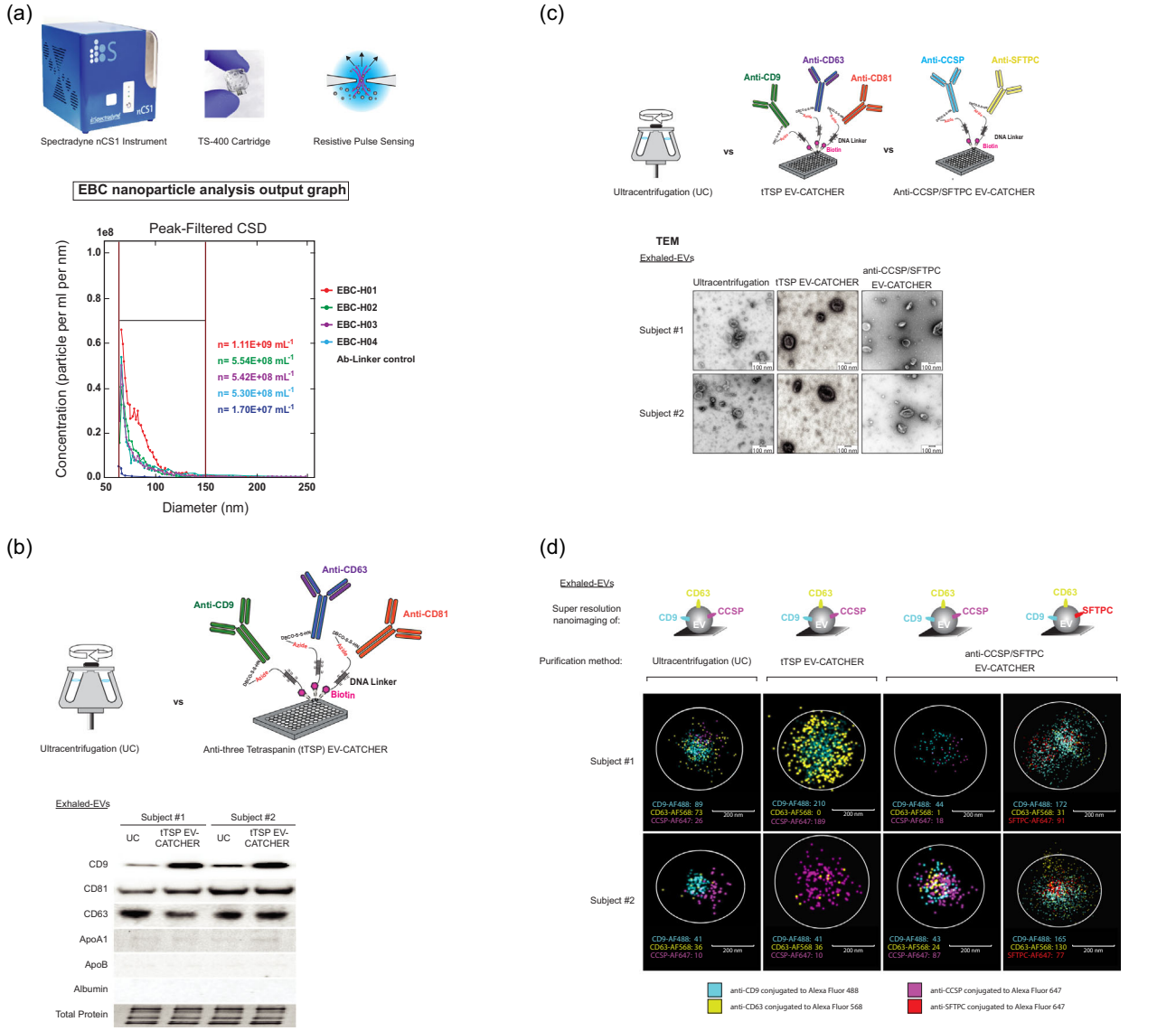



**FIGURE 2** Identification and validation of exhaled Extracellular Vesicles (exh-EVs). (a) Image of the Spectradyne nCS1 nanoparticle analyser, C400 cartridges used to quantify nanoparticles between 65 and 400 nm, and the schematic of the Resistive Pulse Sensing mechanical process employed to count and size nanoparticles. EBC samples collected with the RTube™ device from four healthy subjects (See green, red, light blue and purple plots) were subjected to the human anti-CD63 EV-CATCHER assay and were analyzed on a Spectradyne nCS1 nanoparticle analysis instrument. The size of the nanoparticles detected in these purified samples is displayed on the x-axis of the plot (nanometres), and their number on the y-axis of the plot (particle per millilitre per nanometre). The dark blue plot displays a negative control of the EV-CATCHER assay performed using 1xPBS to account for any signal obtained from the digested Ab-DNA. (b) Schematics of ultracentrifugation (UC) and the tTSP (CD9/CD63/CD81) EV-CATCHER assay, used to purify extracellular vesicles (EVs) from the EBC used to purify exh-EVs. (c) Transmission Electron Microscopy (TEM) images of exh-EVs purified from the EBC from our two controls. (d) Schematic of the different exh-EV proteins detected by our fluorescent antibodies. Representative ONi super resolution nanoimaging images of exh-EVs purified from the EBC of our two healthy controls by UC, tTSP (CD9/CD63/CD81) EV-CATCHER and the CCSP/SFTPC EV-CATCHER assays. Antibodies targeted against CD9-AlexaFluor™ 488 (cyan), CD63-AlexaFluor™ 568 (yellow) and either Clara Cell Secretory Protein (CCSP)-AlexaFluor™ 647 (purple) or the Surfactant Protein C (SFTPC)-AlexaFluor™ 647 (red) allowed for detection of their surface expression of immobilized exh-EVs.

## 3.3 | Evaluating the lung tissue EV surrogacy of exh-EVs by miRNA NGS analyses

We sought to determine whether the microRNA (miRNA) cargos of purified exh-EVs could be utilised as a surrogate measure for those of lung tissue EVs. Thus, for these experiments we collected biofluids from five different anatomic airway levels: mouth rinse, buccal brushing, bronchial brushing, bronchoalveolar lavage (BAL) and EBC from 18 subjects (Table 1) selected from a cohort of patients evaluated at the Montefiore Medical Center, Bronx NY, and undergoing bronchoscopy for clinical respiratory reasons. We performed small-RNA extractions from whole biofluids obtained from mouth rinse (200 $\mu$L), EBC (200 $\mu$L), BAL (200 $\mu$L), brushes obtained from buccal brush (200 $\mu$L) and bronchial brushing (200 $\mu$L), and from EVs purified using our

**TABLE 1** Demographics and clinical data on subjects ($n = 18$) recruited from the Montefiore Medical Center (MMC), which are included for analysis of their 5 level of airway samples (i.e., mouth rinse, buccal brush, Bronchial brush, Bronchoalveolar lavage [BAL] and exhaled breath condensates [EBC] samples) in this study.

| Subject ID | Age | Gender | Smoking Status | Pack Years | Quit Years | Underlying Lung Disease | Tumor Diagnosis | Tumor Stage |
|---|---|---|---|---|---|---|---|---|
| **677** | 63 | M | Current | 46 | 0 | None | Benign | – |
| **783** | 43 | F | Never | 0 | 0 | Asthma | Mets/Other | – |
| **1118** | 55 | F | Never | 0 | 0 | COPD | Benign | – |
| **1124** | 68 | F | Never | 0 | 0 | None | Mets/Other | – |
| **1283** | 28 | M | Never | 0 | 0 | None | Benign | – |
| **1350** | 67 | M | Current | 30 | 16 | COPD | Squamous | IV |
| **1353** | 58 | M | Never | 0 | 0 | None | Benign | – |
| **1354** | 59 | M | Current | 23.5 | 0 | COPD | Adeno | IIIA |
| **1355** | 53 | M | Never | 0 | 0 | None | Mets/Other | – |
| **1357** | 61 | M | Current | 30 | 0 | None | NSCLC | IIIB |
| **1360** | 74 | F | Current | 59 | 0 | Asthma, COPD | Small Cell | L |
| **1362** | 61 | F | Never | 0 | 0 | None | Benign | – |
| **1364** | 72 | M | Current | 116 | 0 | None | Squamous | IIA |
| **1371** | 49 | M | Current | 18 | 0 | Bronchiectasis | Benign | – |
| **1433** | 67 | M | Current | 12.25 | 0 | COPD | Squamous | III |
| **1435** | 78 | F | Former | 58 | 1 | COPD | Squamous | IIIA |
| **1436** | 66 | F | Never | 0 | 0 | None | Benign | – |
| **1439** | 72 | F | Current | 29.5 | 0 | None | Adeno | IIIB |

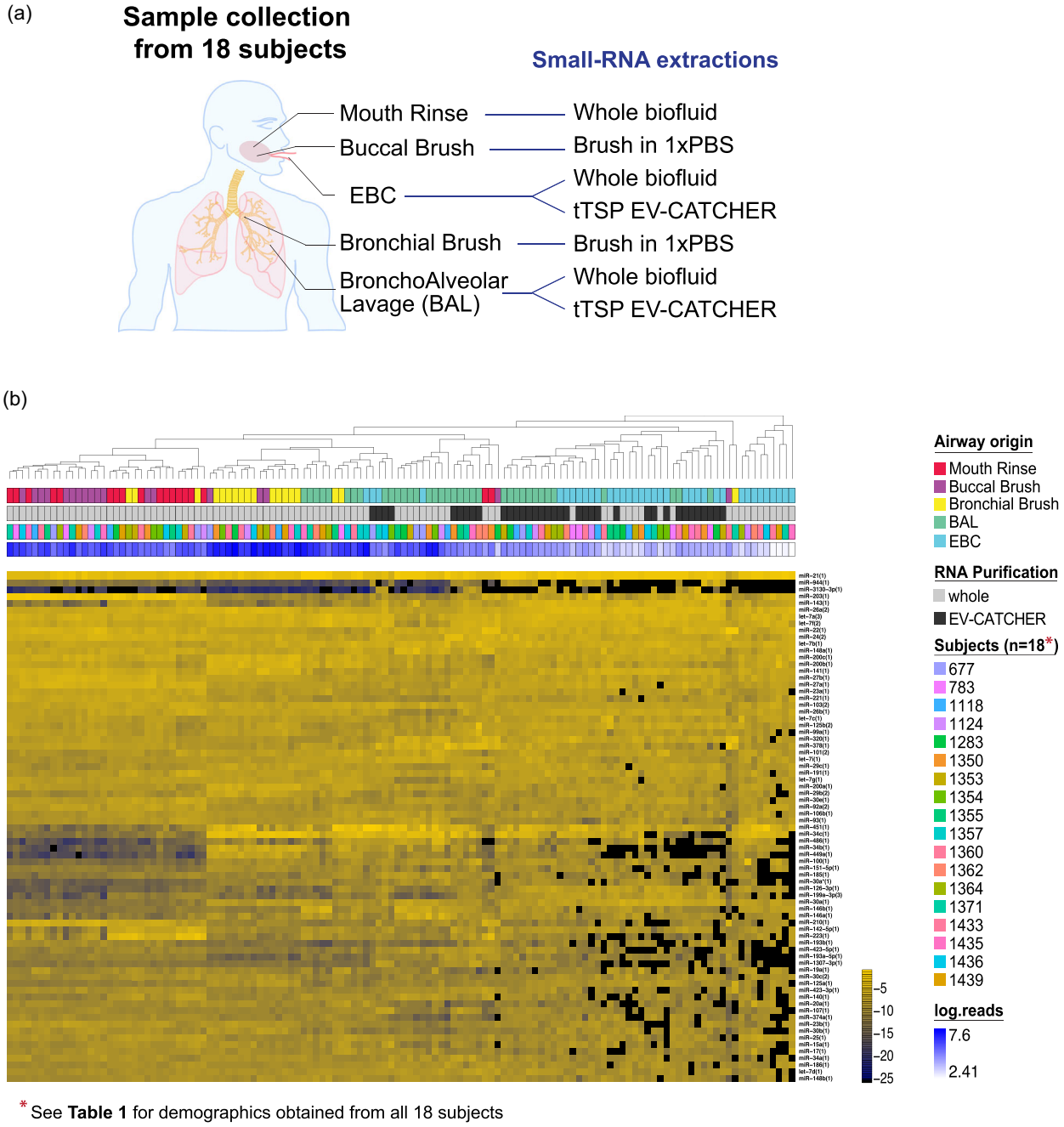
*Note*: On the right of the table are details on the samples that were collected and evaluated (i.e., mouth wash [MW], buccal brush [BB], bronchial brush [BrB], bronchoalveolar lavage [BAL] and exhaled breath condensates [EBC]) in small-RNA NGS libraries 1 through 7.

human tTSP EV-CATCHER assay (anti-CD9/CD63/CD81) from matched EBC (200 $\mu$L) and BAL (200 $\mu$L) samples, as detailed in Figure 3a. We then conducted small-RNA sequencing experiments of the different RNA samples and obtained miRNA expression profiles for the 18 subjects across all five anatomic airway levels. The miRNA data were analyzed, revealing that the miRNA profiles of samples obtained from the mouth and buccal brush clustered together (Figure 3b heatmap, left), the miRNA profiles from bronchial brushing and whole BAL clustered together (Figure 3b heatmap, center), and the miRNA profiles of EVs purified from BAL and exh-EVs purified from EBC clustered together (Figure 3b, right). These results indicated that the miRNA contents of EBC and exh-EVs more closely recapitulated those of BAL-derived EVs that originate in the deep lung.

As we observed that miRNA profiles of whole EBC generally displayed a lower number of miRNA reads than miRNA profiles obtained from the other biofluids (Figure 3b, see log.reads color gradient on heatmap), we evaluated the number of miRNA reads from each of the five different airway samples in Figure 3c (upper panel). We observed that BAL, bronchial brushing, buccal brushing and mouth rinse all provided the largest number of miRNA reads, possibly due to larger RNA inputs from different biological sources (i.e., cell debris and EVs,). Then, we observed that NGS data from whole EBC samples contained 100- to 1000-fold fewer miRNA read counts compared to the other four airway levels (Figure 3c, upper panel). Because both BAL and EBC were analyzed as both whole biofluid fractions and EV fractions (i.e., purified using our tTSP EV-CATCHER), we compared the total number of miRNA reads between the whole biofluids the purified EV fractions (Figure 3 lower panels). As anticipated for BAL, small-RNA sequencing of the whole biofluid generated over a 100-fold higher number of miRNA reads than RNA purified from EVs isolated from BAL EVs (Figure 3c, bottom left panel). However, to our surprise, we determined that for EBC the opposite was observed, with an average of 10-fold increase in the number of miRNAs reads for RNA purified from exh-EVs than for RNA purified from whole EBC (Figure 3c, bottom right panel). These miRNA analyses indicate that the targeted isolation of exh-EV from EBC provides greater signal-to-noise ratios, and thus enriches miRNA reads for NGS analyses.

## 3.4 | Increasing miRNA read depth of exh-EVs by targeting terminal bronchiole and alveoli proteins prior to miRNA NGS analyses

As depicted in Figure 2, a population of exh-EVs contained within EBC harbors deep lung tissue-specific proteins, such as CCSP and SFTPC. Because small-RNA sequencing of exh-EVs purified with our tTSP EV-CATCHER assay from whole EBC enhanced the sequencing depth of miRNAs in small-RNA NGS libraries, we proceeded to investigate whether purifying exh-EVs of deep lung origin (i.e., positive for CSSP and/or SFTPC), would further improve the number of miRNA reads. For this purpose, we expanded our analyses from 18 subjects (Table 1) to 69 subjects (see demographic and clinical details on 51 additional subjects in Table S1) and thus compared the total number of miRNA reads obtained from RNA extracted from matched BAL and




* See **Table 1** for demographics obtained from all 18 subjects

**FIGURE 3** Comparative microRNA expression analyses of samples collected from five different anatomic airway levels from the same 18 subjects. (a) Schematic representation for the collection and analysis of 5 levels of airway specimens including mouth rinse, mouth brushing, bronchial brushing, bronchoalveolar lavage (BAL) and EBC from the same 18 subjects. Small-RNA collections were preformed from whole mouth rinse, from the 1xPBS solution for the brush used for buccal brushing, and the 1xPBS solution for the brush used for bronchial brushing. For EBC and BAL, small-RNA was extracted from whole biofluids, and from exh-EVs purified from both biofluids with our tTSP EVCATCHER. (b) Heat map analysis of small-RNA expression profiles obtained by next generation sequencing (NGS) of the RNA extracted from the 5 levels of airway sampling. The four rows of rectangles above the heatmap indicate: 1st row for airway origin (mouth rinse [red], buccal brushing [purple], bronchial brushing [yellow], BAL [green] and EBC [blue]), 2nd row for the type of selection (whole biofluid or tTSP EV-CATCHER purification of exh-EVs), 3rd row for the donors ID (from 18 subjects) and 4th row for the level of miRNA expression ($log_2$ of the total miRNA reads). (c) The top box plot displays the total miRNA read distribution for all 18 donors (NGS small-RNA libraries 1–7; See Table 1) for the 5 airway levels of collection (mouth rinse, buccal brush, bronchial brush, bronchoalveolar lavage (BAL) and EBC). The bottom left box plot displays the miRNA distribution for all 18 donors for small-RNA extracted and sequenced from whole BAL (left) and BAL EVs (right) purified with the tTSP EV-CATCHER assay from the same BAL samples. The bottom right box plot displays the miRNA distribution for all 18 donors for small-RNA extracted and sequenced from whole EBC and exh-EVs purified with the tTSP EV-CATCHER assay from the same EBC samples.

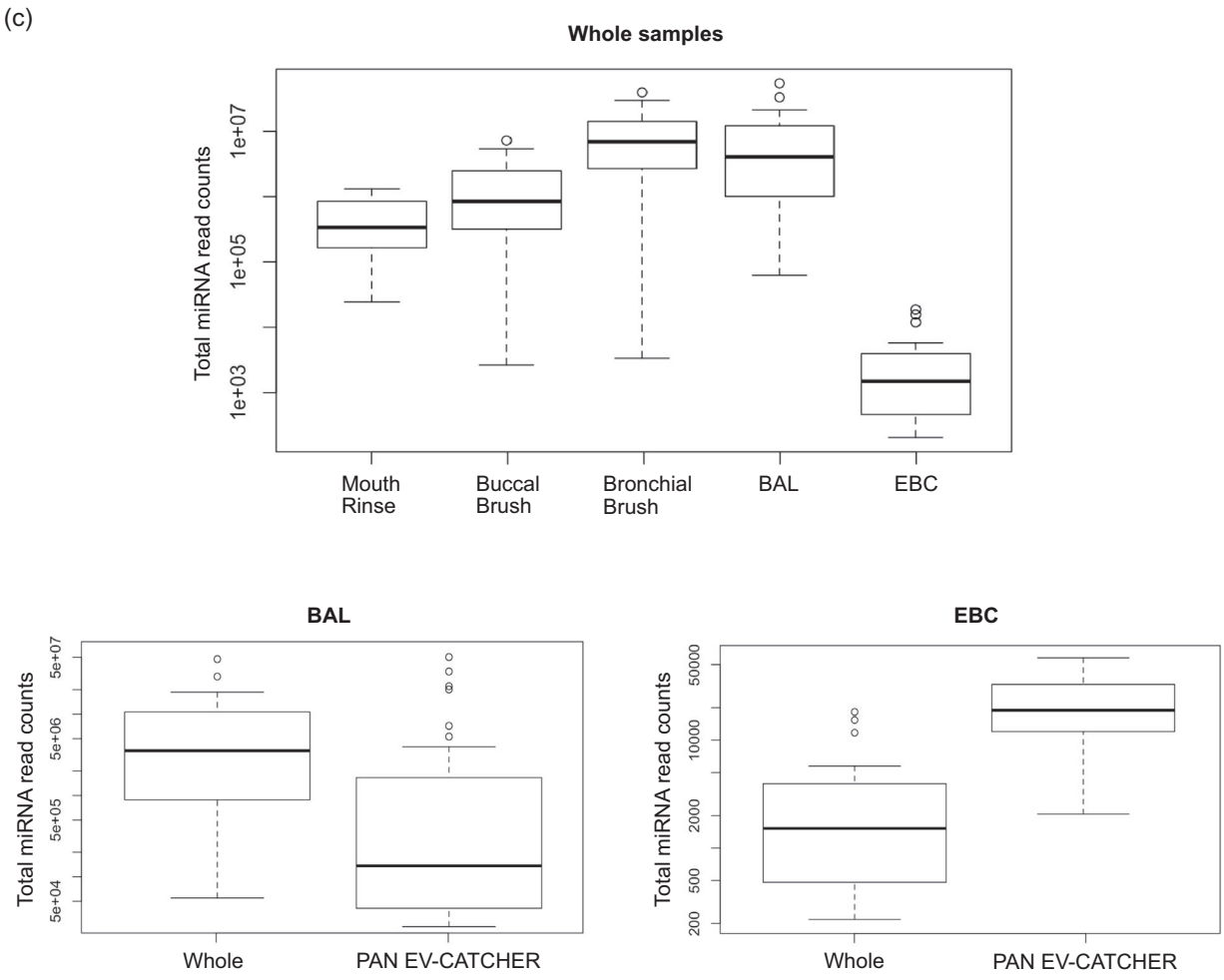



**FIGURE 3** Continued

EBC samples for all our small-RNA libraries (see sample distribution in different libraries included in this analysis detailed in Figure 4a).

As displayed on Figure 4b, the total miRNA reads for whole BAL, BAL-EVs purified with our tTSP-EV-CATCHER or our anti-CCSP/SFTPC EV-CATCHER were overall within the same orders of magnitude, but generally higher for whole BAL samples (Figure 4b). However, when we compared the total number of miRNA reads from whole EBC, exh-EVs purified using our tTSP EV-CATCHER assay, and exh-EVs purified with our anti-CCSP/SFTPC EV-CATCHER assay, we observed a steady increase in the number of miRNA reads for samples evaluated from the same subjects with all three methods (Figure 4b, left, middle and right, see samples from libraries 12–17 that underwent the three purification methods, light blue). We generally noted that the highest increase in the number of miRNA reads was for exh-EVs purified with our anti-CCSP/SFTPC EV-CATCHER assay, close to 100-fold when compared to whole EBC (Figure 4b, left, middle and right panels for libraries 8–11 [yellow] and 18–21 [green]).

In order to understand the reason for differences in the number of miRNA reads, we investigated the species content of the small-RNAs captured and sequenced from whole EBC, exh-EVs purified with our tTSP EV-CATCHER assay and exh-EVs purified with our CCSP/SFTPC EV-CATCHER assay for small-RNA libraries 12–17 (see Table S1), which included samples from 18 different subjects (Figure 4c). Our analyses revealed that exh-EVs purified with the CCSP/SFTPC EV-CATCHER assay contained an increased number of miRNAs, scRNAs, snoRNAs, scaRNAs, mt-tRNA and snRNA precursors, when compared to whole EBC and exh-EVs purified with the tTSP EV-CATCHER assay. Importantly, we noted that rRNAs, mRNAs, tRNAs, piRNAs, tRNA-rm, lincRNA and other non-coding RNAs (ncRNAs) were more abundant in the small-RNA libraries prepared using RNA extracted from whole EBC and from exh-EVs purified with the tTSP EV-CATCHER assay. These experiments validated that exh-EVs, which harbor lung tissue terminal bronchiole and alveoli secretory protein CCSP and SFTPC were enriched with miRNA transcripts when compared to whole EBC.

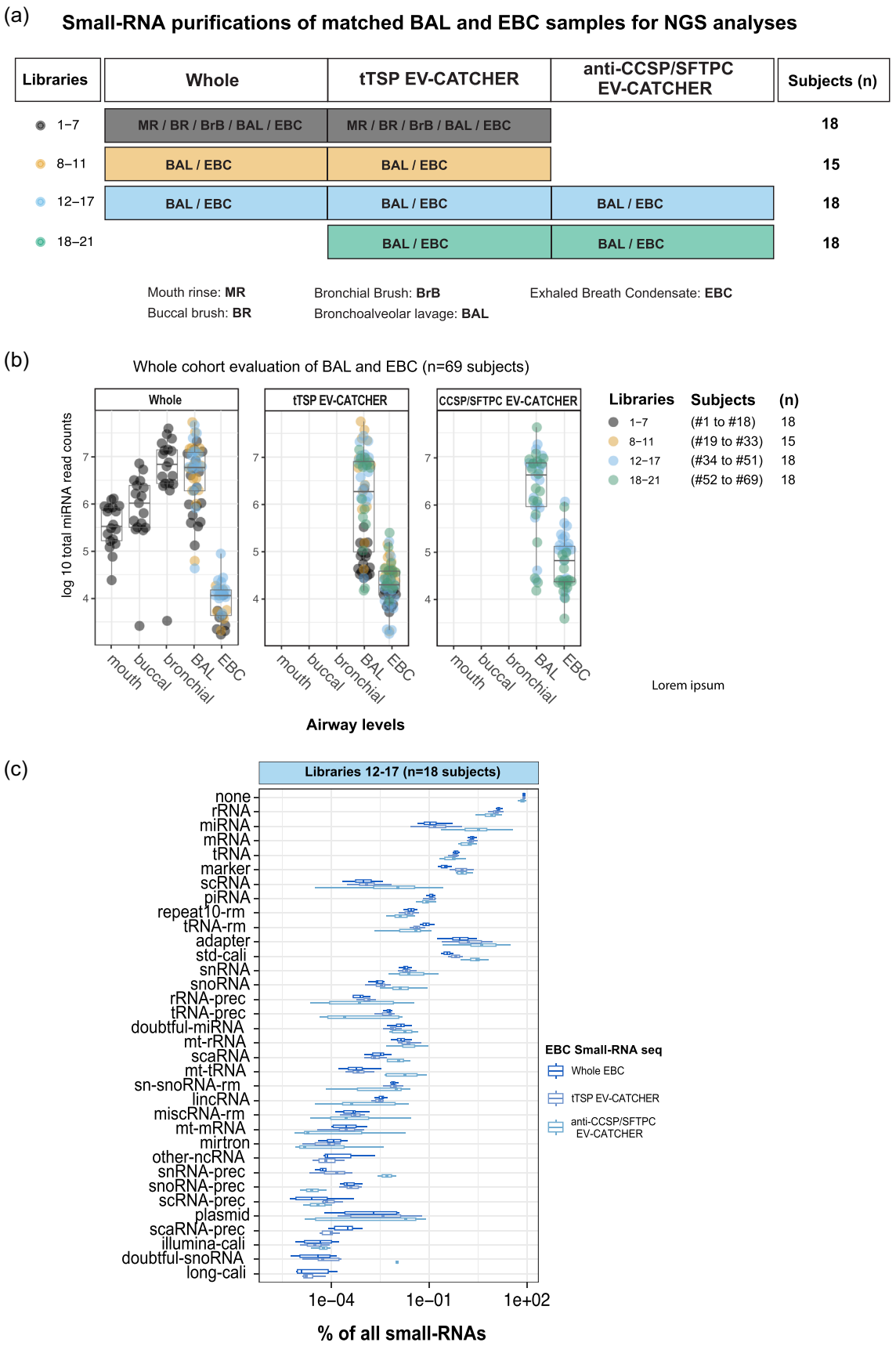



**FIGURE 4** Comparison of total miRNA reads and small-RNA species distribution based on airway level and purification methods. (a) Diagram displaying the small-RNA NGS scheme for specimens collected from 69 subjects (see Table 1 [$n = 18$] and Table S1 [$n = 51$]). (b) For libraries 1–7, miRNA expression profiling was conducted on five different levels of airway (i.e., Mouth rinse (MR), Buccal brush (BB), Bronchial brush (BrB), Bronchoalveolar lavage (BAL) and EBC) and EVs isolated using the tTSP EV-CATCHER assay from BAL and EBC, from the same 18 subjects (see Table 1, subjects 1–18). Small-RNA

*(Continues)*

**FIGURE 4** (Continued)
libraries 8–11 (See Table S1, $n = 15$; subjects 19–33) were prepared to analyze the miRNA expression profiles of subject-matched BAL (whole and tTSP EV-CATCHER purified EVs) and EBC (whole and tTSP EV-CATCHER purified EVs) samples. Small-RNA libraries 12–17 (See Table S1, $n = 18$; subjects 34–51) were prepared to analyse the miRNA expression profiles of subject-matched BAL (whole, tTSP EV-CATCHER and anti-CCSP/SFTPC EV-CATCHER purified EVs), and EBC (whole, tTSP EV-CATCHER and anti-CCSP/SFTPC EV-CATCHER purified EVs) samples. Small-RNA libraries 18–21 (See Table S1, $n = 18$; subjects 52–69) were prepared using small-RNA extracted from EVs purified with the tTSP EV-CATCHER and the anti-CCSP/SFTPC EV-CATCHER assays from subject-matched BAL and EBC samples. (b) Read distribution and expression differences for small-RNAs extracted and sequenced for libraries 12–17, from whole EBC (dark blue box plots), exh-EVs purified with the tTSP EV-CATCHER assay (medium blue box plots) and exh-EVs purified with the anti-CCSP/SFTPC EV-CATCHER assay (light blue box plots) from the same 18 EBC samples (See Table S1, $n = 18$; subjects 34–51).

## 3.5 | Evaluating miRNA surrogacy of exhaled EVs for BAL EVs for subjects classified by smoking, asthma and lung cancer status

Next, we examined whether the miRNA expression profiles of exh-EVs were representative of those BAL-derived EVs when evaluating the smoking, asthma or lung cancer status of the subjects with available and analyzed matched BAL and EBC samples.

Considering that smoking permanently affects the lung epithelium of a subject by altering miRNA expression in lung epithelial cells and the EVs they secrete in BAL (Wu et al., 2019), we evaluated whether miRNA expression differences in BAL-EVs of never, former and current smokers could also globally be detected in exh-EVs purified from matched EBC samples. We focused our analyses on miRNA NGS data obtained from libraries 12–17 and 18–21 (see Table S1), which included: (i) subjects whose smoking status was annotated as never ($n = 13$), former ($n = 13$) and current smokers ($n = 10$); (ii) matched BAL and EBC samples from the same subjects and (iii) RNA samples extracted from whole biofluid, EVs purified with our tTSP and anti-CCSP/SFTPC EV-CATCHER assays from matched BAL and EBC samples (Figure 5a). As displayed in Figure 5b, we calculated the miRNA z-score for the 317 miRNAs consistently detected between matched whole BAL and whole EBC. Each individual miRNA's z-value was added or subtracted to obtain a z-score for each subject in each of the three smoking groups (i.e., never, former and current smokers). For whole BAL and whole EBC, we observed similar increasing miRNA expression trends between never and current smokers, but the corresponding $p$-values did not reach significance (Figure 5b, upper and lower left panels, with $p$-values of 0.57 for whole BAL and 0.097 for whole EBC). However, when calculating the z-score for miRNAs sequenced from EVs captured with our tTSP EV-CATCHER assay from matched BAL and EBC samples, we observed a similar and significant miRNA expression increasing trend between never and current smokers (Figure 5b, upper and lower middle panels, with $p$-values of 0.028 for BAL and 0.015 for EBC). Finally, when comparing the z-scores of miRNAs sequenced from EVs purified with our anti-CCSP/SFTPC EV-CATCHER assay from matched BAL and EBC samples, we observed an even greater significant miRNA increasing expression trend between never and current smokers (Figure 5b, upper and lower right panels, with $p$-values of 0.0037 for BAL and 0.0076 for EBC). These results indicated that miRNA expression changes detected in BAL could also be detected in matched EBC samples, but that greater sensitivity may be achieved when analyzing the miRNA profiles of EVs selectively purified with our anti-CCSP/SFTPC EV-CATCHER assay.

Similarly, we evaluated whether miRNA expression differences detectable in BAL EVs of subjects diagnosed with asthma, compared to subjects without asthma, could also be detected in exh-EVs. Since our analyses repeatedly demonstrated that we achieved increased miRNA reads (Figure 4b,c) and more significant miRNA expression differences (Figure 5b) by evaluating EVs rather than whole biofluids (i.e., BAL or EBC), we concentrated these analyses on EVs purified with our tTSP and anti-CCSP/SFTPC EV-CATCHER assays from matched EBC and BAL samples of control subjects (i.e., without asthma) and patients diagnosed with asthma (Figure 5c). We determined that significant miRNA expression differences could not be detected in EVs purified from BAL ($p < 0.95$) or exh-EVs purified from matched EBC samples ($p < 0.29$) using our tTSP EV-CATCHER assay between control subjects without asthma and patients diagnosed with asthma (samples analysed in libraries 8–21, **See** Table S1). However, when globally evaluating miRNA z-score differences between EVs purified from BAL samples purified from control subjects and asthma patients, we observed a statistical significance ($p < 0.041$), which was similarly observed with the analysis of the same miRNAs in exh-EVs purified from matched EBC samples ($p < 0.019$) with our anti-CCSP/SFTPC EV-CATCHER assay from control subjects with asthma and subjects diagnosed with asthma (samples analysed in libraries 12–21, See Table S1). Finally, we examined whether significant miRNA expression differences (i.e., miRNA z-score) could similarly be detected between EVs purified from matched BAL and EBC samples, between subjects with benign lung lesions and patients with confirmed lung tumors (Figure 5d, samples analysed in libraries 12–17 and 18–21, **See** Table S1). Our analyses identified miRNA z-scores displaying that significantly differentially expressed miRNAs detected in EVs purified from BAL ($p < 0.022$) could also be detected in exh-EVs purified from matched EBC samples with our tTSP EV-CATCHER assay, between patients with benign lung lesions and patients with confirmed lung tumors (Figure 5d). We similarly detected more significant expression differences for the selected miRNAs between the two groups, for EVs purified from BAL ($p < 0.02$) and exh-EVs purified from matched EBC samples ($p < 0.0094$) with our anti-CCSP/SFTPC EV-CATCHER assay. Altogether these analyses support the conclusion that miRNA expression differences detected in BAL EVs based on the smoking, asthma and lung tumor status of subjects can also be

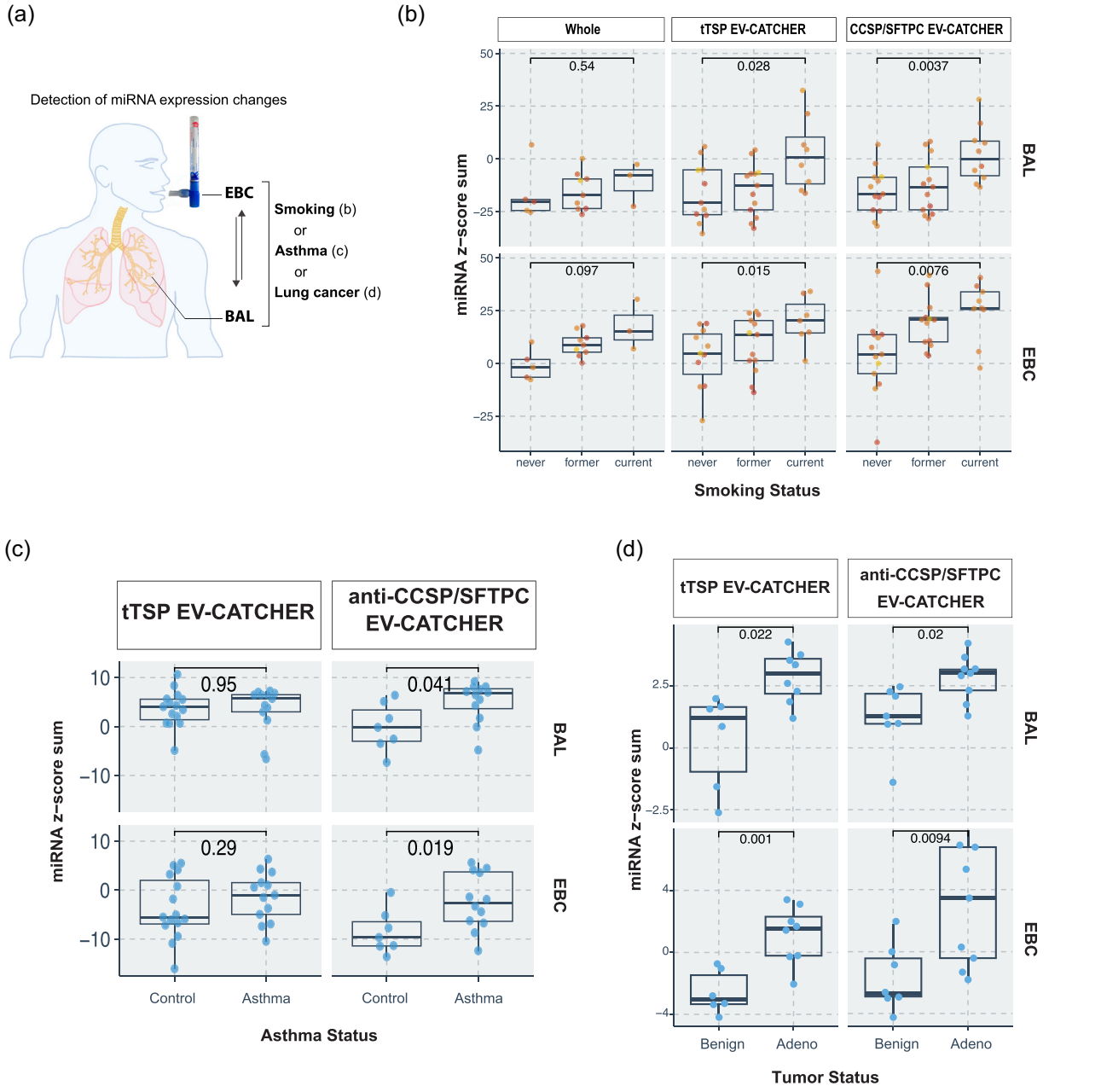



**FIGURE 5** Insights from miRNA expression analyses of exh-EVs as a surrogate measure for BAL EVs, highlighting distinctions in subjects based on smoking, asthma, or tumor status. (a) The schematic illustrates the careful selection of matched BAL and EBC samples from subjects, based on their smoking, asthma and tumor status, for subsequent miRNA NGS analyses. (b) Among the top 317 miRNAs consistently detected in BAL and EBC (libraries 12–17 and 18–21; Table S1), with known smoking statuses (never [$n = 13$], former [$n = 13$], current [$n = 10$]), box plot comparative analyses showcase miRNA z-scores. Calculations were performed on matched BAL and EBC samples for small-RNA analyses encompassing whole BAL and whole EBC (top and bottom left panels), BAL EVs and exh-EVs purified with the tTSP EV-CATCHER assay (top and bottom center panels), and BAL EVs and exh-EVs purified with the anti-CCSP/SFTPC EV-CATCHER assay (top and bottom right panels). The miRNA z-scores amalgamate the top upregulated miRNAs ($n = 8$) and top downregulated miRNAs ($n = 7$). *p*-values for miRNA-derived z-scores were determined using pairwise *t*-tests between never and current smokers, presented in each plot. (c) Box plot representation of miRNA z-scores for matched BAL and EBC specimens (subjects #52–69; libraries 1–21; Table 1) with EVs purified using the tTSP and anti-CCSP/SFTPC EV-CATCHER assays. This analysis contrasts control subjects without asthma ($n = 15$) with subjects having asthma ($n = 13$). MiRNA z-scores reflect a combination of the top upregulated miRNAs ($n = 4$) and top downregulated miRNAs ($n = 7$), commonly identified in RNA purified from EVs obtained through the tTSP EV-CATCHER assay for matched BAL and EBC samples. *p*-values were calculated using pairwise *t*-tests. (d) Box plot representation of miRNA z-scores for matched BAL and EBC specimens (subjects #52–69; libraries 12–21; Table S1) with EVs purified using the tTSP and anti-CCSP/SFTPC EV-CATCHER assays. These comparative analyses focus on BAL and EBC samples from seven individuals with benign lung lesions and nine patients diagnosed with lung adenocarcinoma (selected from libraries 12–21; Table S1). MiRNA z-scores are computed by summing the z-values of the two most upregulated (miR-126-3p, miR-339-3p) and two most downregulated miRNAs (let-7c, miR-184) in samples from patients with adenocarcinoma compared to those without cancer. *p*-values were calculated using pairwise *t*-tests.

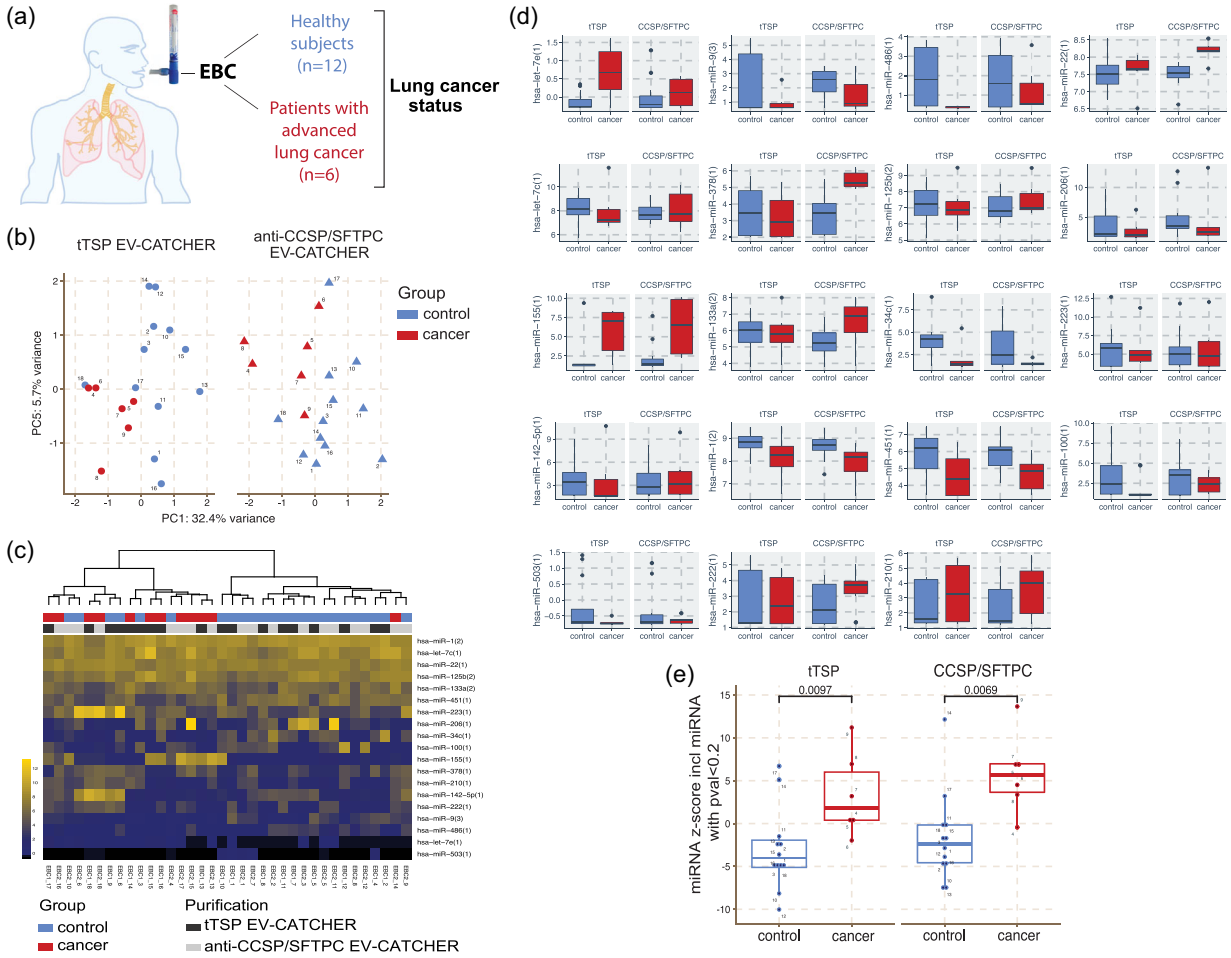



**FIGURE 6** MiRNA expression comparative analyses between exh-EVs purified with the tTSP and the anti-CCSP/SFTPC EV-CATCHER assays from a new set of EBC-only samples. (a) The human figure displays that EBC-only samples were collected from 18 subjects for evaluation of their tumor status using our two different exh-EV purification methods prior to miRNA NGS analyses. (b) Principal Component Analysis (PCA) plots that display the similarities in global miRNA expression between the 12 control (Healthy) and the 6 cancer (Lung cancer) subjects for exh-EVs that were purified from their EBC with the tTSP EV-CATCHER assay and the anti-CCSP/SFTPC EV-CATCHER assay. (c) Heat map analysis of the top 19 most differentially expressed miRNAs between the 12 controls (Healthy) and the 6 cancer (Lung cancer) subjects for exh-EVs that were purified from EBC using with the tTSP EV-CATCHER assay or the anti-CCSP/SFTPC EV-CATCHER assay. (d) Box plot analyses of the top 19 most differentially expressed miRNAs between the 12 controls (Healthy) and the 6 cancer (Lung cancer) subjects, displaying significant $\log_2$ fold expression differences, as detected by small-RNA NGS analyses. (e) Box plot analyses of the z-score obtained from the top 14 (out of 19) most differentially expressed miRNAs quantified by small-RNA NGS analyses between the 12 controls (healthy) and the 6 cancer (lung cancer) subjects for exh-EVs purified with the tTSP EV-CATCHER assay (left) or the anti-CCSP/SFTPC EV-CATCHER assay (right). The *p*-values identified between healthy controls and patients diagnosed with advanced lung cancer are displayed in the two panels.

detected in exh-EVs purified from matched EBC samples, and that BAL EVs and exh-EVs purified with our anti-CCSP/SFTPC EV-CATCHER assay contain miRNAs with greater discriminatory power.

## 3.6 | Evaluating the miRNA expression profiles of exh-EVs purified with our tTSP and anti-CCSP/SFTPC EV-CATCHER assays based on lung cancer status

As we demonstrated the surrogacy of EBC exh-EVs for BAL EVs, and conducted all of our analyses on EBC samples collected at the Montefiore Medical Center, Bronx NY (MMC; 69 subjects from Table 1 and Table S1), we sought to evaluate the robustness of our EV-CATCHER assays for targeted purification of exh-EVs from EBC samples collected (also with the RTube™ device, Figure 6a) at a different medical center, the Hackensack University Medical Center, Hackensack NJ (HUMC). As displayed in Table 2, for these analyses, we selected EBC samples that were collected from a group of non-smoking young healthy adults ($n = 12$), and from a small group of patients diagnosed with advanced bronchogenic carcinoma ($n = 6$; adenocarcinoma [NSCLC, $n = 4$], squamous cell carcinoma [SCLC; $n = 1$] and large cell carcinoma [a rare type of NSCLC; $n = 1$]). We selectively chose to analyse EBC samples from subjects at different ends of the health spectrum (i.e., healthy controls vs. lung cancer

**TABLE 2** Demographics and clinical data on subjects ($n = 16$) who were recruited at the Hackensack University Medical Center (HUMC) for sole collection of EBC.

| Controls | Gender | Age | Cases | Gender | Age | Cancer | Stage |
|---|---|---|---|---|---|---|---|
| 1 | F | 38 | 1 | M | 70 | Sq.CLC | Stage IV |
| 2 | F | 26 | 2 | M | 55 | Lg.CLC | Stage IV B |
| 3 | M | 34 | 3 | F | 75 | Adeno. | Stage IV A |
| 4 | F | 31 | 4 | F | 57 | Adeno. | Stage IV |
| 5 | F | 31 | 5 | M | 84 | Adeno. | Stage IV A |
| 6 | F | 25 | 6 | M | 81 | Adeno. | Stage IV |
| 7 | F | 44 | – | | | | |
| 8 | F | 30 | – | | | | |
| 9 | M | 50 | – | | | | |
| 10 | F | 39 | – | | | | |
| 11 | M | 19 | – | | | | |
| 12 | F | 35 | – | | | | |

*Note*: The subjects include 12 healthy non-smoking adults and 6 patients diagnosed with stage IV squamous cell lung carcinoma ($n = 1$, SCLC), large cell lung carcinoma ($n = 1$, a rare form of NSCLC) and non-small cell lung carcinoma ($n = 4$, NSCLC).

patients) to ensure that differentially expressed miRNAs could be detectable. As shown in Figure 6b, Principal Component Analysis (PCA) plots revealed apparent differential miRNA expression between our two groups (control [healthy] vs. cancer), for exh-EVs purified by both our tTSP and anti-CCSP/SFTPC EV-CATCHER assays. When evaluating the top 19 differentially expressed exh-EV miRNAs ($p < 0.05$) commonly identified by both EV-CATCHER purification assays (Figure 6c), we observed that EBC samples from healthy controls formed a distinct heat map cluster, whilst EBC samples from patients diagnosed with advanced lung cancer predominantly belonged to another heat map cluster. Next, when comparing the differential expression of these 19 miRNAs in individual box plots (Figure 6d), between the two groups (healthy controls vs. lung cancer patients) and for each purification method (tTSP vs. anti-CCSP/SFTPC EV-CATCHER assays), we determined that these miRNAs generally displayed the same differential expression trends, albeit with varying levels of significance. Specifically, we found that let-7e and miR-155 were more upregulated, whilst miR-9, miR-486, miR-34c, miR-206, miR-100 and miR-503 were more downregulated in exh-EVs purified with our tTSP EV-CATCHER assay than exh-EVs purified with our anti-CCSP/SFTPC EV-CATCHER assay (Figure 6d). Comparatively, we found that miR-22, miR-378, miR-125b, miR-133a, miR-222 and miR-210 were more upregulated, and miR-206, miR-34c, miR-1 and miR-451 more downregulated in exh-EVs purified with our anti-CCSP/SFTPC than our tTSP EV-CATCHER assays (Figure 6d). When we computed a group-related z-score of the top 14 (out of 19) most differentially expressed miRNAs, we observed that the miRNA analysis of exh-EVs purified with the anti-CCSP/SFTPC EV-CATCHER assay offered a slightly superior discriminatory power ($p$-value of $< 0.0069$) than that of exh-EVs purified with the tTSP EV-CATCHER assay (with a $p$-value of $< 0.0097$).

Altogether, these small-RNA NGS analyses confirmed the robustness of our EV-CATCHER assay and suggest that tissue-specific enrichment of exh-EVs from EBC may help identify miRNAs whose deregulated expression correlates with unique lung pathologies.

## 4 | DISCUSSION

In this study we evaluated and confirmed the presence of extracellular vesicles (EVs) in exhaled breath condensates (EBC). We analyzed their miRNA profiles and found a high degree of correlation with those purified from bronchoalveolar lavages (BAL). Using super resolution nanoimaging we demonstrated that exh-EVs harbor unique surface proteins of terminal bronchiole and alveoli origin (i.e., Clara Cell Specific Protein [CCSP] and Surfactant Protein C [SFTPC]), which we then leveraged for the enrichment of exh-EVs from EBC using our customizable EV-CATCHER assay. Our comparative miRNA expression analyses confirmed the surrogacy of exh-EVs purified from EBC for those similarly purified from matched BAL samples. Using the anti-CCSP/SFTPC EV-CATCHER assay, we further validated the lung tissue origin of exh-EVs and that subjects may be distinguished by analysis of exh-EVs, based on their lung pathologies.

Given the limited number of reports describing the detection and the characteristics of EVs in EBC to date, we initially sought to establish whether current molecular validation standards (i.e., MISEV2018 guidelines [Barta et al., 2019; Siegel et al., 2021;

Théry et al., 2018]) could be effectively used to confirm the presence of EVs in EBC samples. We used MRPS and TEM to characterise exhaled EVs (exh-EVs), and Western blot analyses to validate the presence of tetraspanins (i.e., CD63, CD9, CD81) on purified exh-EVs, which we combined with ONi super resolution nanoimaging analyses to confirm the surface localization of these proteins. Importantly, we determined that our EV purification methods (i.e., ultracentrifugation and EV-CATCHER assay) yielded no detectable levels of albumin and ApoB, and only trace amounts of ApoA1, all of which are frequently encountered as contaminants in blood samples, but not for EBC. Our quantitation also revealed that although exh-EVs were detectable and purifiable from EBC, they were in low abundance and thus required a significant concentration step for subsequent evaluations. However, when using established EV purification techniques, including ultracentrifugation followed by immuno-purification using our customizable non-magnetic bead-based EV-CATCHER assay, we could effectively isolate and enrich for exh-EVs from EBC.

Next, to evaluate the biological utility of exh-EVs for the non-invasive sampling of the lung, we focused our analyses on biological markers uniquely found to be expressed by terminal bronchiole and alveoli cells. Specifically, we selected bronchiolar Clara cells because they express a unique lung protein (i.e., Clara Cell Specific Protein [CCSP]) and because they have been implicated in lung cancer initiation (Lin et al., 2012; Rowbotham & Kim, 2014; Sainz de et al., 2021). CCSP is a 15.8-kDa homodimeric protein with a central hydrophobic region, which is uniquely secreted in large amounts by non-ciliated terminal bronchiolar Clara cells to protect the respiratory tract from stress (i.e., oxidative, pathogenic and inflammatory) (Broeckaert & Bernard, 2000; Hayashida et al., 2000). Importantly, Wang *et al.* determined that although it is a secreted protein, it can also be localized in the membrane of Clara cells (Wang et al., 2012; Wong et al., 2009), and they suggested that the central hydrophobic region of CCSP (Rokicki et al., 2016) may allow for it to be trapped within the cytoplasmic membrane during its secretion. Then, we selected Alveolar type II (ATII) cells because they have also been implicated in lung cancer initiation and because they uniquely secrete SFTPC, a 21 kDa transmembrane proprotein (proSPC) that is processed through a sequence of proteolytic cleavages into a 3.7 kDa secretory protein before it is released from the cytoplasmic membrane, which has previously been detected on the surface of ATII cells cultured in vitro (Dickens et al., 2022; Mulugeta & Beers, 2006; Nureki et al., 2018; Weaver & Whitsett, 1991). Additionally, a recent study by Choudhary et al. (2021) also revealed that EVs produced by ATII cells contained SFTPC. As both CCSP and SFTPC have been localized in the membrane of their respective secretory cells, we hypothesized that they may also be present on the surface of exh-EVs and potentially be used for immuno-purification of exh-EVs using our customizable EV-CATCHER assay. Using super resolution nanoimaging (ONi), we confirmed that CCSP (Rokicki et al., 2016) and SFTPC (Sitaraman et al., 2021) could be detected on the surface of exh-EVs. Our data were consistent between exh-EVs purified by ultracentrifugation alone or in combination with our EV-CATCHER assay customized with either tTSP (i.e., triple tetraspanin; CD9/CD63/CD81) or monoclonal antibodies directly targeting the CCSP and SFTPC proteins. To our knowledge, this is the first such demonstration, as to date no published studies have revealed the localization of these two terminal bronchiole and alveolar cell-specific secretory proteins on the surface of EBC-derived exh-EVs. Furthermore, our immuno-purification analyses, using our customised EV-CATCHER assay, revealed that by targeting these two cell-specific secretory proteins, we could enrich EVs of lung tissue origin, which could also be found in BAL (Lam et al., 2021; Mainardi et al., 2014; Sainz de et al., 2021; Sutherland & Berns, 2010). Biologically, the presence of secretory proteins on the surface of exh-EVs may suggest a function for binding a variety of particles, bacteria and or viruses, prior to being aerosolized, within the biofluid lining the lungs. This hypothesis would align with recent findings, where we and others determined that circulating EVs detected in serum after viral infection harbor surface proteins (i.e., ACE-2) that can neutralize viruses (i.e., SARS-CoV-2) and thus contribute to the hosts immune defense (El-Shennawy et al., 2022; Mitchell et al., 2021).

Next, to further validate the surrogacy of exh-EV miRNA cargos for EVs of lung tissue origin, we conducted comprehensive miRNA expression profiling analyses of airway specimens collected at five different anatomic levels (i.e., mouth rinse, buccal brush, bronchial brush, BAL and EBC) from 18 subjects recruited at the Montefiore Medical Center, Bronx NY (MMC). We also used matched BAL and EBC samples from another 51 subjects from our MMC cohort to further confirm the robustness of our findings. All our EBC samples were uniformly collected using the FDA registered RTube™ device, and our analyses produced the following results. First, we showed that the miRNA expression profiles obtained from whole EBC and exh-EVs purified with our tTSP EV-CATCHER assay clustered closely with those of whole BAL and BAL EVs (also purified with our tTSP EV-CATCHER assay), but not with those of other airway specimens. Second, miRNA NGS analyses of whole EBC, exh-EVs purified with our tTSP EV-CATCHER assay, and exh-EVs purified with our CCSP/SFTPC EV-CATCHER assay from the same subjects showed that enriching exh-EVs prior to conducting miRNA NGS analyses significantly increased the number of miRNA reads by up to 100-fold (i.e., when compared to miRNA NGS analyses of whole EBC). This increase in miRNA reads was correlated with a decrease in ribosomal RNAs, tRNAs and other non-coding RNAs, which were less represented in exh-EVs, particularly those harboring proteins (i.e., CCSP and SFTPC) of terminal bronchiole and alveoli cellular origin. Third, we demonstrated the potential of exh-EV miRNAs to serve as surrogates for those of BAL EVs in relation to smoking, asthma and lung cancer status. Consistent with reports that smoking is associated with permanent miRNA expression changes in epithelial lung cells and their secreted EVs (Gower et al., 2011; Osborne & Minna, 2022; Smith et al., 2006; Spira et al., 2004; Wu et al., 2019), we observed that miRNA expression differences that were significantly detected in BAL EVs (i.e., especially those purified with the anti-CCSP/SFTPC EV-CATCHER assay) between never and current smokers could similarly be detected in exh-EVs purified

from EBC using the same EV-CATCHER assays (i.e., tTSP and anti-CCSP/SFTPC EV-CATCHER). Similarly, miRNA analyses conducted on matched BAL EVs and EBC exh-EVs obtained from patients diagnosed with asthma or lung tumors (i.e., in two separate analyses) further determined that similar miRNA expression changes can be detected between BAL EVs and exh-EVs, with the greatest discriminatory power provided by EVs purified with the anti-CCSP/SFTPC EV-CATCHER assay. Although these analyses were conducted on small subject/patient groups and are thus too preliminary to identify any miRNA biomarkers associated with specific lung conditions (i.e., no validation of these findings), they indicate that the non-invasive collection of EBC and miRNA analysis of lung cell-specific exh-EVs has potential to correlate with clinical and diagnostic information and should be further investigated.

Finally, as we had conducted all our experimental analyses on EBC samples collected from the same medical center (i.e., the MMC), we sought to evaluate whether our optimized molecular assays (i.e., exh-EV purification and NGS analyses) could be utilized on a different set of EBC samples collected at another medical center (i.e., Hackensack University Medical Center), using the same RTube™ collection device. Because the collection of BAL is invasive, our additional analyses were conducted using EBC-only samples, which were obtained from healthy subjects ($n = 12$) and a small group of patients diagnosed with advanced (i.e., stage IV) lung cancer ($n = 6$). For these analyses, we compared the identification of differentially expressed miRNAs between exh-EVs purified with our tTSP EV-CATCHER assay and those purified with our CCSP/SFTPC EV-CATCHER assay for our two groups to evaluate the robustness of our small-RNA NGS detection. Our comparative analyses robustly identified similarly differentially expressed miRNAs between exh-EVs purified by both EV purification methods. Furthermore, as observed with miRNA analyses conducted using exh-EVs purified from EBC of non-smokers versus smokers, subjects without asthma versus patients with asthma, and patients with or without lung tumors from MMC, we found that exh-EVs enriched using our the anti-CCSP/SFTPC EV-CATCHER assay identified more significantly differentially expressed miRNAs than those purified with our tTSP EV-CATCHER assay. Although these experiments were not sufficiently powered to identify exh-EV miRNA biomarkers or miRNA signatures for the detection of lung cancer, and will require validation, we found that the top 3 differentially expressed miRNAs (i.e., miR-22, miR-155, miR-34c) detectable in exh-EVs purified by our two methods (i.e., tTSP and anti-CCSP/SFTPC EV-CATCHER assays) between healthy controls and patients diagnosed with stage IV lung cancer had previously been described for their involvement in inflammation (i.e., miR-155), the regulation of cellular proliferation (miR-155 [Liu et al., 2021]), epithelial-to-mesenchymal transition (miR-155, miR-34c and miR-22 [Xu & Shi, 2019; Yang et al., 2023; Zhang et al., 2017]), and cellular migration in lung cancers (miR-155 and miR-34c [Ren et al., 2020; Xue et al., 2016]). Interestingly, we observed that we could detect specific miRNAs (i.e., miR-378, miR-22), which were significantly and consistently more differentially expressed in exh-EVs purified with the anti-CCSP/SFTPC EV-CATCHER assay customized for selective purification of Clara and Alveolar type II cells exh-EVs from the bulk of exh-EVs in EBC. These findings are preliminary, and we anticipate that additional and properly powered studies will be needed and will need to include EBC specimens from healthy adult subjects, subjects with history of lung disease and/or smoking, and patients with different types of lung cancers and at different stages. Such study will need to be conducted to fully evaluate the potential of quantifying exh-EV miRNAs for non-invasive detection of lung cancer, especially in comparison to existing technologies (i.e., CT scan) and state-of-the-art molecular detection assays to evaluate the sensitivity and reproducibility of this non-invasive approach. Based on this study, however, our findings suggest that stratifying exh-EVs from EBC based on their cellular origin, by using our customized EV-CATCHER assay or other ultra-sensitive immuno-purification assays, may enable detection of precise miRNA expression signatures associated with pathological lung cellular changes, but it will require further investigation. As EBC contains EVs of lung tissue origin, the analysis of exh-EVs offers a unique opportunity for discovery of lung-only biomarkers, whereas EVs that are released from the lung and that circulate in blood may be difficult to purify, due to their mixing with other organ EVs potentially harboring surface proteins similarly expressed in other cell-types outside of the lung. For example, studies have showed that CCSP, although enriched in lung tumors, can also be detected in prostate and endometrial cancers (Cioppi et al., 2004; Patierno et al., 2002), which would preclude the sole purification of Clara cell EVs from blood and may provide false lung cancer positive signals in patients with other types of cancers. Nevertheless, in order to refine the selective purification of exh-EVs, especially those associated with cells involved in lung diseases, we propose that proteomic analyses of exh-EVs purified from subjects diagnosed with these specific lung diseases may help identify unique lung disease-related surface markers to further enhance enrichment of disease-related exh-EVs and improve the discriminatory power of their biomarkers for the detection of these lung diseases.

In summary, our analyses confirm the utility of our customizable EV-CATCHER assay for the selective purification of exh-EVs harboring surface proteins of terminal bronchiole and alveoli lung tissue origin from EBC. However, it is important to note that although the collection of EBC is non-invasive, generally rapid, productive (up to 2 mL of biofluid within 10 min) and inexpensive, it is currently not performed in standard practice. Unfortunately, there are currently no biobanks with annotated EBC samples from which specimens may be selected and analyzed. Instead, the collection of EBC has to be done prospectively, and it will require time to assemble large enough cohorts to power studies that will evaluate the potential of exh-EVs miRNAs for non-invasive detection of different lung diseases, particularly lung cancer. Ultimately, the accurate distinction between benign and malignant lung nodules, which often cannot be made by CT imaging and thus requires biopsy, is the benchmark of sensitivity that putative exh-EV miRNA lung cancer biomarkers will have to achieve.

## AUTHOR CONTRIBUTION

Olivier Loudig and Simon Spivack conceived and planned the experiments. Kaushal Parikh, Martin Gutierrez, Rashmi Unawane and Lisa Biamonte were responsible for patient identification, consenting and collection of EBC at Hackensack University Medical Center (HUMC). Simon Spivack, Miao Shi, Ali Sadoughi, Chirag Shah, Taha Siddiqui and Aham Okorozo were responsible for patient identification, consenting, collection and selection of the five different airway level samples at Montefiore-Einstein Medical Center/Comprehensive Cancer Center. Megan I. Mitchell and Olivier Loudig carried out all experiments. Iddo Z. Ben-Dov and Kenny Ye performed statistical analyses. Olivier Loudig and Megan I. Mitchell were responsible for writing the manuscript and preparing all figures. All authors provided critical feedback and helped shape the research and analysis of this manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this paper.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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