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Cell-free nucleic acids in exhaled breath condensate: emerging non-invasive biomarkers for pulmonary disease and systemic RNA communication

Jane L. Zen* and George B. Hanna

Department of Surgery and Cancer, Imperial College London, Hammersmith Hospital, London, UK

* Corresponding author; E-mail: j.zen24@imperial.ac.uk.

Highlights:

- EBC nucleic acids enable non-invasive molecular profiling of lung disease.
- EV-associated RNAs improve stability and biomarker potential in EBC.
- EBC may reflect systemic extracellular RNA communication beyond the lung.

Abstract: Cell-free nucleic acids, particularly microRNAs (miRNAs), have emerged as promising diagnostic biomarkers for disease detection, including cancer, owing to their relative stability, disease-specific expression patterns, and functional involvement in disease pathogenesis. Exhaled breath condensate (EBC) is a non-invasively collected biofluid obtained through tidal breathing and has been shown to contain extracellular nucleic acids derived from the respiratory tract, present in both extracellular vesicle (EV)-associated and EV-free forms, and presents distinctive features that, in some contexts, offer practical advantages over other emerging breath-based diagnostic approaches. This narrative review synthesises previous advances in the characterisation and applications of cell free nucleic acids in EBC as potential diagnostic, prognostic, and monitoring biomarkers, with existing studies largely focused on lung cancers and other non-malignant respiratory pathologies, whilst addressing key methodological challenges and suggesting strategies for standardisation and improved translational utility. Importantly, it also examines the possibility of using EBC-derived nucleic acids to identify conditions in extra-pulmonary organs and broader systemic diseases, considering recent studies, whilst highlighting new research directions to be explored.

Keywords: Exhaled breath condensate; extracellular RNA; microRNA; extracellular vesicles; liquid biopsy; lung cancer; asthma; chronic obstructive pulmonary disease; cell-free nucleic acids; biomarkers

1. Introduction

In the era of precision medicine, the development of non-invasive biomarkers has become essential for early detection, accurate diagnosis, and disease monitoring. Cancer in particular remains a leading global health burden, with lung cancer alone accounting for approximately 2.5 million new cases and 1.8 million

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deaths annually, often being diagnosed at advanced stages where five-year survival rates hover below 30% [1]. Similarly, non-malignant respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF) affect hundreds of millions of people worldwide, contributing to substantial morbidity through progressive inflammation, tissue remodelling, and impaired pulmonary function. Conventional diagnostic approaches, including radiological imaging, endoscopy, invasive biopsies, and serum testing, often suffer from limitations in sensitivity, specificity, accessibility, and patient compliance.

Breath-based diagnostics have emerged as an attractive and biologically informative strategy. Simple gases such as nitric oxide, hydrogen peroxide (H_2O_2), carbon monoxide, and ammonia can reflect airway inflammation, oxidative stress, and metabolic dysfunction [2]. Volatile organic compounds (VOCs), a diverse group of carbon-based metabolites (50–300 Da), have also been widely investigated as metabolic signatures of respiratory and systemic disease and can be analysed using platforms such as gas chromatography–mass spectrometry [3]. Despite encouraging diagnostic performance, clinical translation of breath metabolomics remains limited by methodological variability and insufficient standardisation [4]. Exhaled breath condensate (EBC) is another promising non-invasive sampling approach obtained by cooling aerosolised droplets generated during tidal breathing, providing a repeatable "liquid biopsy" of the lung without the need for bronchoscopy or surgical sampling [5,6]. This condensed fraction captures aerosolised material originating from the airway lining fluid, enabling analysis of non-volatile biomolecules, including extracellular nucleic acids released from respiratory epithelial, tumour, immune, and microbial cells, often arising from cellular turnover, stress responses, apoptosis, and necrosis [6–10]—processes amplified under pathological conditions. These extracellular nucleic acids are frequently stabilised through association with protein complexes or encapsulation within extracellular vesicles (EVs), which protect them from degradation and preserve molecular signals reflective of pulmonary pathophysiology [11]. By sampling aerosolised material from the local lung microenvironment, EBC enables detection of molecular species that may be diluted or absent in systemic biofluids, improving specificity for respiratory conditions.

Amongst extracellular nucleic acids, regulatory non-coding RNAs are of particular interest. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression post transcriptionally and represent especially promising biomarkers due to their stability, disease-specific dysregulation, and roles in key pathological processes like oncogenesis, inflammation, fibrosis, and immune modulation [12–15]. Tumour- or inflammation-derived exosomal miRNAs in EBC have shown particular promise across malignancies, especially lung cancer, where signatures like upregulation of miR-21 and miR-155 correlate with tumour subtype, stage, and prognosis, as well as in non-malignant conditions, where differential miRNA profiles can distinguish asthma phenotypes, COPD severity, or IPF progression from healthy states or overlapping syndromes [16]. Emerging evidence also extends to other RNAs, including long non-coding RNAs (lncRNA), messenger RNA (mRNA), and DNA detected in EBC. Whilst non-coding RNAs represent the primary focus of this review, other nucleic acid classes, including cell-free DNA (cfDNA) and mitochondrial DNA (mtDNA), provide complementary information on genomic or mitochondrial damage, oxidative stress, and microbial dynamics within the respiratory tract.

This review focuses on regulatory ncRNAs in EBC, particularly miRNAs and lncRNAs, as diagnostic, prognostic, and disease-monitoring biomarkers for respiratory diseases, whilst considering cfDNA, mtDNA, and coding transcripts in a complementary contextual framework. We examine their biological origins,

analytical methodologies utilised for their detection (e.g., next-generation sequencing (NGS), quantitative PCR (qPCR)), standardisation hurdles, and translational prospects. Moreover, given that miRNAs can undergo cross-organ transfer through EVs, especially under pathophysiologic conditions, we also explore the emerging possibility that EBC-derived nucleic acids may capture molecular signals arising from extra-pulmonary diseases, in light of studies published over the past decade. Beyond serving as a window into local airway pathology, emerging data on extracellular RNA trafficking suggest that EBC may also encode echoes of systemic injury along axes like the liver–lung and gut–lung interfaces, a concept explored in later sections.

2. Cell-free nucleic acids detected in EBC

2.1. Molecular composition of EBC and detection of nucleic acids

Exhaled breath contains a complex array of biological constituents, including volatile components such as gases and VOCs and non-volatile molecules such as proteins, lipids, and nucleic acids (Figure 1). Because EBC forms through cooling aerosolised droplets from the respiratory tract, it provides access to non-volatile molecular species not detectable by conventional gas-phase breath analysis. These include markers of oxidative stress (H_2O_2, isoprostanes, aldehydes) [17–20], inflammatory mediators such as cytokines, chemokines, and eicosanoids, as well as proteins, enzymatic factors, ions, and electrolytes [17,21–23]. Amongst these constituents, cell-free nucleic acids—particularly regulatory non-coding RNAs—have attracted growing interest as non-invasive biomarkers because they can be sampled repeatedly and reflect local airway pathophysiology.

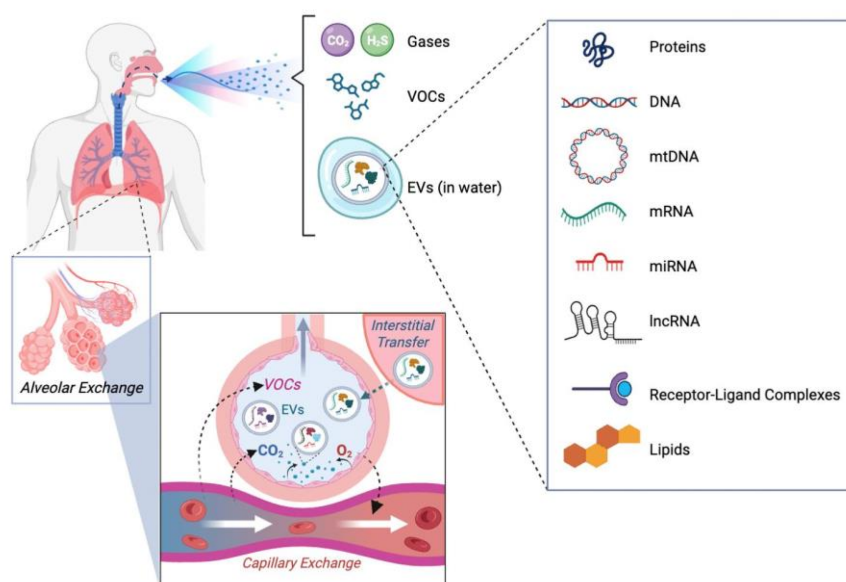

Figure 1. Molecular fractions in exhaled breath. Exhaled breath contains multiple biologically informative fractions, including gases and VOCs, as well as non-volatile biomolecules carried in aerosolized water droplets and EVs (often in the droplets). These components, which include nucleic acids, proteins, and lipids, originate from airway lining fluid (ALF) and resident and infiltrating cells throughout the respiratory tract. In the distal lung, circulating molecules can cross the alveolar-capillary interface, whilst locally released signals from alveolar epithelial, interstitial, and immune cells also contribute, enabling both systemic and lung-derived molecules to be recovered during breath collection. Figure was produced with Biorender.

Accumulating evidence shows that EBC contains multiple classes of extracellular nucleic acids originating from both host tissues and airway microbiota [24–28]. These include miRNAs [29–33], lncRNAs [24,34,35], mRNA [15,35,36], DNA [37–41] and smaller nucleic acid fragments from bacteria, viruses, and fungi colonising or infecting the airway [7–11]. They can be detected with high sensitivity using analytical approaches such as quantitative real-time PCR (qRT-PCR), probe-based microarrays, and NGS, enabling both targeted detection and unbiased discovery of novel RNA species in low-input condensate samples [42,43]. Across available studies that fractionated EBC into EV-enriched and EV-depleted components, a substantial proportion of detectable miRNA has been recovered in both fractions, with EV-enriched samples often showing higher per-volume miRNA concentrations and improved RNase resistance, whereas EV-depleted supernatants still contain measurable Argonaute-bound miRNAs and other cell-free nucleic acids (cfNAs) [12,15,18,24]. Given this and the central role of EVs in stabilising and trafficking regulatory RNAs, this review places greater emphasis on EV-associated miRNAs, whilst recognising that EV-free cfNAs represent a complementary and biologically relevant compartment.

A brief systematic survey in Medline resulted in 69 eligible experimental studies reporting the detection of extracellular nucleic acids in human EBC (Figure 2). Human DNA (26 studies) and RNA (20) were the most frequently investigated and reported nucleic acids. These were followed by viral RNA, bacterial DNA, bacterial RNA, and viral DNA, with 1 study describing fungal DNA (Figure 2A). Amongst human RNA species (Figure 2B), miRNA was by far the most examined, accounting for 80% of studies, whereas mRNA and lncRNA were less reported (each 5.0%).

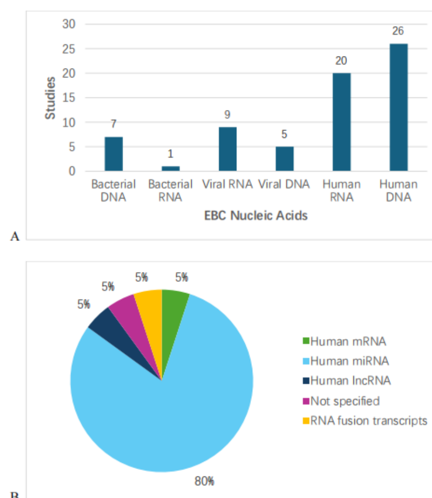

Figure 2. Distribution of reported detections of cell-free nucleic acids in human EBC. Distribution of reported detections of cell-free nucleic acids in EBC-based diagnostic studies. Data are based on a literature survey of studies indexed in PubMed up to 3 March 2026 using the search terms (“exhaled breath condensate” OR “breath condensate”) AND (miRNA OR microRNA OR RNA OR DNA OR mRNA OR lncRNA OR “small RNA*” OR “non-coding RNA*” OR “Piwi RNA” OR “cell-free DNA” OR cfDNA OR cfdna OR “extracellular RNA” OR “extracellular DNA” OR “nucleic acid” OR exosome OR “extracellular vesicle*”)*. A total of 158 records were identified, of which 63 studies met the inclusion criteria and were analysed. **(A)** Amongst identified nucleic acid types, human DNA (26) and human RNA (20) were most frequently detected, followed by viral RNA (9), bacterial DNA (7), viral DNA (5), fungal DNA (1). Some studies reported multiple nucleic acid categories and were therefore counted across more than one group; **(B)** Amongst human RNA studies, human miRNA (80%) was the most studied and reported in EBC.

2.2. Regulatory non-coding RNAs and coding transcripts

As mentioned previously, miRNAs are the most extensively studied small regulatory molecules in EBC due to their short length, relative stability, and central roles in post-transcriptional gene regulation. Several miRNAs have been detected in EBC, including miR-155, associated with inflammatory responses, and miR-21 or members of the let-7 family, which are linked to lung cancer and pulmonary fibrosis [13,44,45]. Oncogenic miRNAs such as miR-21 promote tumour progression by repressing tumour-suppressor genes including phosphatase and tensin homolog (PTEN) and programmed cell death 4 (PDCD4), whereas tumour-suppressive miRNAs such as miR-486 are frequently downregulated in lung cancer [46,47]. Members of the miR-17-92 cluster similarly enhance cellular proliferation by inhibiting regulators such as PTEN and hypoxia-inducible factor 1α (HIF-1α) [48]. These disease-linked miRNAs form the primary focus of subsequent sections, where their diagnostic and prognostic utility in specific respiratory disorders is discussed.

Compared with longer RNAs, miRNAs are particularly well-suited to detection in the low-input, RNase-rich environment of EBC because their short length and characteristic secondary structures, together with protection within Argonaute complexes and EVs, confer relative resistance to extracellular degradation [12–15]. Intact mRNA is less frequently detected in breath because longer transcripts are more susceptible to RNase-mediated degradation in airway secretions. Nevertheless, several studies have identified specific mRNA transcripts in EBC, indicating that targeted gene expression readouts can be recovered from breath-derived samples. Detected transcripts include surfactant proteins A and D (SP-A, SP-D), markers of alveolar integrity, and inflammatory cytokines like IL-6 and TNF-α [49,50]. In addition to coding transcripts, lncRNAs have recently been detected in EBC and are emerging as promising but still under-studied biomarkers. Several lncRNAs have been used to distinguish lung cancer patients from healthy individuals [15,19,34]. Vardarli *et al.* reported that lncRNAs metastasis-associated lung cancer transcript 1 (MALAT1), HOX antisense intergenic RNA (HOTAIR), plasmacytoma variant translocation 1 (PVT1), nuclear paraspeckle assembly transcript 1 (NEAT1), Antisense Noncoding RNA in the INK4 Locus (ANRIL), and RTK signaling antagonist 4-intronic transcript 1 (SPRY4-IT1) detected in EBC may serve as potential non-invasive biomarkers for non-small cell lung cancer (NSCLC) [34], illustrating that EBC can capture both oncogenic and tumour-suppressive lncRNA signatures.

Many mRNAs contain miRNA response elements (MREs) and can compete with other transcripts—including lncRNAs and circular RNAs (circRNAs)—for miRNA binding, forming competing endogenous RNA (ceRNA) networks that regulate miRNA availability and downstream gene expression [51,52]. In these networks, changes in the abundance of one RNA species can influence multiple coding and non-coding transcripts sharing common miRNA pathways. Although such interactions have not yet been directly characterised in EBC, co-detection of miRNAs, lncRNAs, and disease-associated mRNAs in breath condensate suggests that EBC-derived RNA signatures may reflect underlying regulatory networks in the lung. To date, however, these ceRNA interactions have not been directly mapped within EBC-derived RNA populations, and current inferences are extrapolated from tissue and cell-based studies rather than breath-specific datasets.

By contrast, cfDNA fragments in EBC, although highly informative for mutations and methylation, are less directly coupled to post-transcriptional regulatory networks and can be more susceptible to

dilution and sampling variability than small RNAs in EVs. cfDNA fragments represent another important class of nucleic acids detected in EBC. Studies have identified mutations in tumour-associated genes such as TP53 and KRAS [41,42,53] as well as alterations in the epidermal growth factor receptor gene EGFR, in condensate. For example, Zhang *et al.* detected EGFR mutations in EBC from a heavy smoker with squamous cell lung carcinoma [54]. Breath-derived DNA fragments can also reveal microsatellite alterations and genomic instability, early events in carcinogenesis. Carpagnano *et al.* reported microsatellite alterations in EBC from patients with NSCLC [37], and several studies have described epigenetic changes such as promoter methylation of tumour suppressor genes [55–57]. Xiao *et al.* further detected promoter hypermethylation of P16 in EBC, supporting its potential as a non-invasive biomarker for NSCLC diagnosis [55]. Together, these findings position cfDNA in EBC as a complementary genomic readout that can be integrated with ncRNA signatures to characterise tumour biology.

More broadly, cfDNA in EBC is best interpreted as part of a mixed circulating nucleic-acid compartment, rather than as a direct upstream regulator of non-coding RNAs. EBC contains a heterogeneous mixture of cell-free nucleic acids—including DNA, miRNAs, mRNAs, and lncRNAs—released from stressed or dying epithelial, immune, and tumour cells and often stabilised within EVs or protein complexes [6–10]. In cancer and chronic airway diseases, cfDNA measurements primarily capture structural and epigenetic alterations such as mutations, microsatellite instability, and promoter methylation, whereas miRNAs and lncRNAs more directly report on regulatory pathway activity [58,59]. Current evidence therefore supports cfDNA and ncRNAs as co-released readouts of the same airway pathology, rather than demonstrating direct cfDNA-mediated control of ncRNA programmes.

mtDNA has emerged as a biomarker of airway injury and inflammation. Elevated cell-free mtDNA can act as damage-associated molecular patterns (DAMPs) that trigger inflammatory signalling pathways and has been reported in patients with asthma and COPD. Detection of mtDNA in EBC therefore provides a non-invasive means of monitoring lung pathophysiological conditions [40,60,61]. In obstructive lung disease, Carpagnano *et al.* reported increased mtDNA-to-nuclear DNA ratios (mtDNA/nDNA) in EBC, suggesting these ratios as markers of inflammatory and oxidative airway injury [60]. Moreover, mitochondrial genomes are increasingly recognised as sources of small regulatory RNAs capable of influencing both mitochondrial and nuclear gene expression [62]. Because mitochondrial dysfunction and oxidative stress are known to modulate miRNA and lncRNA expression, mtDNA signals in EBC may provide contextual information for interpreting concomitant changes in regulatory ncRNA profiles.

2.3. Pathogen-derived nucleic acids and host–microbe interactions

Besides human cell-free nucleic acids, detection of pathogen-derived genetic material represents an important application of EBC analysis. Multiple viral nucleic acids, including miRNA-like molecules, have been identified in condensate from infected individuals [63]. However, analytical sensitivity is generally lower than that of nasopharyngeal swabs or sputum samples due to dilution and the low abundance of microbial particles in exhaled aerosols. RNA signatures from respiratory pathogens—including influenza A/B, respiratory syncytial virus (RSV), rhinovirus, and SARS-CoV-2—have been characterised in EBC [64]. Detection of SARS-CoV-2 RNA, including targets such as the E gene and ORF1ab, highlights the potential of using condensate as a rapid, non-invasive matrix for infectious-disease diagnostics, respiratory surveillance, and viral transmission studies [6,9,65]. Importantly, many respiratory viruses reprogram host miRNA expression and can produce viral miRNA-like small RNAs (sRNAs) that

influence antiviral immunity, apoptosis, and viral replication [66]. Viral genomes and transcripts can also harbour binding sites for host miRNAs, allowing viral RNAs to act as competitors or sponges within host miRNA–mRNA regulatory networks [67]. In this framework, co-detection of viral RNAs and host regulatory miRNAs in EBC is best interpreted as a snapshot of bidirectional RNA–RNA crosstalk in the infected airway epithelium, rather than as evidence for a single dominant regulatory direction.

EBC also contains genetic material derived from pathogenic bacteria like *Legionella pneumophila*, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Moraxella catarrhalis*, including regulatory sRNAs (approximately 50–500 nucleotides) and DNA, particularly during acute exacerbations of COPD and other infections [16,68]. Although still nascent, fungal RNAs have also been suggested in EBC, particularly in asthma [69]; meta-transcriptomic sequencing and other RNA-based assays are expected to improve detection of such low-abundance microbial RNAs in breath. Recognition of microbial DNA and RNA by innate immune sensors such as TLRs can reshape host miRNA and lncRNA expression in epithelial and immune cells, thereby modulating inflammatory and antimicrobial pathways [70]. In addition, some respiratory microbes release regulatory sRNAs that interact with host transcripts, whilst host miRNAs and lncRNAs can reciprocally regulate microbial gene expression [71]. Because EBC samples airway ALF and recovers both microbial nucleic acids and host regulatory RNAs, ncRNA signatures in condensate are likely to encode integrated, bidirectional host–microbe regulatory interactions within the respiratory tract.

3. Cell-free nucleic acids in EBC serve as biomarkers for detecting lung cancer and other non-malignant respiratory disease detection

EBC is increasingly being explored as a minimally invasive "liquid biopsy" of the respiratory tract for lung cancer and chronic airway diseases like asthma and COPD. As shown in Table 1, multiple studies now support EBC-derived miRNAs as candidate biomarkers for lung cancer detection, subtyping and risk stratification, including in resource-limited settings.

Several investigations have evaluated specific miRNAs in EBC as potential diagnostic markers for lung cancer. Xie *et al.* showed that serum and EBC miR-186 and IL-1β levels may serve as diagnostic markers for NSCLC [64]. Shi *et al.* identified 24 miRNAs significantly upregulated in EBC from lung cancer patients compared with controls [43]. A pilot study by Rai *et al.* reported 78 upregulated miRNAs in EBC, with candidates such as miR-31-3p subsequently validated as potential biomarkers [26]. Using genome-wide profiling, Pérez-Sánchez *et al.* identified EBC miRNA panels associated with lung cancer diagnosis, staging, and prognosis [33]. Earlier work by Mozzoni *et al.* similarly reported elevated miR-21 and reduced miR-486 levels in EBC, suggesting their potential utility as early biomarkers of NSCLC [72]. Faversani *et al.* further showed that combined EBC–plasma miRNA profiles could distinguish lung adenocarcinoma from pleural mesothelioma and healthy controls [73]. Although reported sensitivities and specificities are encouraging, most studies remain small, single-centre, and pre-validation, underscoring the need for larger prospective cohorts before clinical implementation [74].

Table 1. Representative studies of cell-free nucleic acids in EBC as non-invasive biomarkers for lung cancer.

Authors	Nucleic Acid Type	Sample	Cohort Size	Diagnostic Performance	EV-enriched?	Reference
Mitchell, MI	miRNAs	EBC EVs	69 subjects; independent validation cohort: 6 lung cancer *vs.* 12 controls	Enhanced discriminatory power, no AUC reported	Yes	[12]
Rai, D	miRNAs	EBC	Exploratory: 20 NSCLC *vs.* 20 controls; validation: 10 NSCLC *vs.* 10 controls	AUC 0.865–0.980; sensitivity 70%–90%; specificity 95%–100%	No	[26]
Pérez-Sánchez, C	miRNAs	EBC	21 lung cancer *vs.* 21 healthy controls	LC detection AUC 0.83; subtype AUC 0.98; prognosis/invasion AUC 1.0	No	[33]
Mozzoni, P	miR-21, miR-486	Plasma, EBC	54 NSCLC *vs.* 46 non-cancer controls	Plasma AUC 0.90; sensitivity 87%; specificity 86.5%; EBC AUC 0.68	Yes	[72]
Faversani, A	miRNAs	Plasma, EBC	14 lung AdCa *vs.* 9 controls; MPM cohort: 23 MPM *vs.* 19 controls	Discriminated lung AdCa from controls/MPM, no AUC reported	Yes	[73]
Xie, H	miR-186, IL-1β	Serum, EBC	62 NSCLC *vs.* 60 healthy controls	EBC miR-186 outperformed serum; combined detection AUC 0.863	No	[63]
Kazeminasab, S	cfDNA mutations	EBC	5 lung cancer *vs.* 5 healthy controls	Complete EBC–tumor tissue mutation concordance reported	No	[41]
Ryan, DJ	ctDNA panel	EBC	125 lung cancer patients; no healthy controls	Identified EGFR/KRAS/PIK3CA mutation burden with lower failure rates than NGS	No	[42]
Youssef, O	DNA mutations	EBC	26 lung cancer patients *vs.* 20 healthy controls	NGS detected 39 hotspot mutations; higher mutant allele fractions in patients	No	[53]
Zhang, P	EGFR mutation	EBC	Single case report (1 squamous cell carcinoma patient)	EBC EGFR exon 19 deletion matched tumor biopsy mutation	No	[54]
Xiao, P	P16 methylation	EBC	30 NSCLC *vs.* 30 healthy controls	P16 methylation detected in 40% of NSCLC but not controls	No	[55]

Beyond lung cancer, cell-free nucleic acids in EBC also show promise as biomarkers for other airway diseases, including asthma and COPD, as shown in Table 2. Pinkerton *et al.* first reported differential miRNA expression in EBC from asthma, COPD, and healthy individuals, suggesting that EBC-derived miRNAs may help distinguish these conditions [75]. Pattnaik *et al.* further identified differential expression of inflammatory miRNAs in asthmatic *versus* COPD patients [16], with strong discriminatory power demonstrated by complete differential expression of miR-512-3p and miR-517c secreted by respiratory cells and detectable in EBC. Mendes *et al.* similarly linked miRNA clusters, including miR-155, to clinical parameters such as symptom severity and bronchodilator responsiveness in paediatric asthma [44]. Building on these findings, additional paediatric and longitudinal cohorts have developed and validated multi-miRNA panels in EBC that can endotype childhood asthma and help predict future exacerbation risk. Sinha *et al.* also reported exosome-associated miRNAs in EBC as potential biomarkers for monitoring asthma and other pulmonary conditions [18]. Notably, several miRNA markers correlate with lung function parameters such as FEV_1, indicating that EBC-derived miRNAs may reflect both disease presence and severity. Together, these data support a role for miRNA signatures in both disease discrimination (e.g. asthma *versus* COPD) and activity assessment.

In addition to regulatory RNAs, mtDNA has emerged as a potential biomarker of airway injury in asthma. Airway epithelial cells are rich in mitochondria and therefore particularly susceptible to mitochondrial stress during inflammation, which can lead to the release of mtDNA into airway secretions, including EBC, where elevated levels indicate oxidative stress [76]. Comparisons of mitochondrial and nuclear DNA have revealed significantly higher mtDNA/nDNA ratios in asthmatic patients than in healthy controls, suggesting this ratio may help endotype severe asthma [60,77]. Current evidence is based on relatively small cohorts, so the robustness of mtDNA/nDNA as a stratification tool still requires confirmation. Because mitochondrial dysfunction and oxidative stress can influence miRNA expression and other ncRNA-regulated pathways, mtDNA signals detected in EBC may provide complementary context for interpreting regulatory RNA signatures associated with airway inflammation [60].

Asthma is also associated with alterations in the airway microbiome. Sequencing bacterial 16S rRNA genes in EBC enables investigation of microbial dysbiosis without invasive procedures such as bronchoscopy [78]. Asthmatic airways often exhibit increased Proteobacteria abundance and reduced microbial diversity, features linked to disease pathogenesis and microbiome-associated asthma phenotypes [79]. These microbial shifts can influence host regulatory RNA networks, as microbial products and innate immune signalling reshape miRNA and lncRNA expression in airway epithelial and immune cells [80]. Consequently, microbial nucleic acids and host ncRNA signatures detected in EBC may together reflect host–microbe regulatory interactions within the asthmatic airway. In this context, microbial DNA/RNA, mtDNA, and regulatory ncRNAs should be viewed as complementary layers of information rather than stand-alone markers.

Table 2. Representative studies of cell-free nucleic acids in EBC as non-invasive biomarker for pulmonary diseases.

Authors	Nucleic Acid Type	Sample	Disease Type	Cohort Size	Diagnostic Performance	EV-enriched?	Reference
Sinha, A	EV miRNAs	EVs in EBC	Pulmonary diseases	10 asthma *vs.* 10 controls; additional 9 TB	EBC miRNA signatures reflected pulmonary disease states	Yes	[18]
Pinkerton, M	miRNAs	EBC	Asthma, COPD, healthy adults	11 asthma, 10 COPD, 12 controls	EBC miRNA signatures differentiated asthma, COPD and controls	No	[75]
Mendes, FC	miRNAs	EBC	Paediatric asthma	71 asthmatic children *vs.* 115 controls	EBC miRNAs and clusters identified asthma traits/endotypes	No	[44]
Mendes, FC	miRNAs	EBC	Asthma	52 asthmatic *vs.* 98 non-asthmatic children	EBC miR-133a-3p differentiated higher dietary acid load	No	[29]
Pattnaik, B	miR-512-3p, miR-517c	EBC	Asthma/COPD	65 asthma, 65 COPD, 65 controls	miR-512-3p/miR-517c differentiated asthma *vs.* COPD with 100% sensitivity/specificity	Yes	[16]
Carpagnano, GE	mtDNA	EBC	Obstructive lung diseases	13 COPD, 14 asthma, 23 ACOS, 12 controls	Exhaled MtDNA/nDNA elevated in COPD and ACOS *vs.* controls	No	[60]
Carpagnano, GE	mtDNA,	EBC	Severe asthma	53 severe asthma, 11 mild–moderate asthma, 12 controls	Increased EBC MtDNA/nDNA identified severe and non-T2 asthma phenotypes	No	[77]
Stachowiak, Z	EV miRNAs	EVs in EBC	Pulmonary exacerbation	18 pediatric CF patients (pulmonary exacerbation) *vs.* 17 stable CF	4-serum-miRNA panel predicted pulmonary exacerbation with AUC = 0.96	Yes	[27]
Pattnaik, B	miRNAs	EBC	Sarcoidosis and mediastinal TB	50 sarcoidosis, 50 mediastinal TB, 50 controls	miR-126/miR-132 differentiated sarcoidosis and mediastinal TB (AUC = 0.618)	No	[25]
Jaya, T	miRNAs	EBC	TB	65 TB and 65 controls; PTB and EPTB subgroups	miR-143 (also miR-139, miR-454) diagnosed TB with 100% sensitivity and specificity	No	[32]
Rai, D	miRNAs	EBC	Pulmonary sarcoidosis, TB	Discovery: 20 PTB, 26 sarcoidosis, 20 controls; validation: 17 PTB, 18 sarcoidosis, 25 controls.	miR-454 and miR-139-5p differentiated PTB from controls with AUCs 0.991 and 0.994	No	[30]

Similar integrative approaches are also being explored in COPD. Although COPD and asthma both involve airway obstruction, their inflammatory mechanisms differ: COPD is typically characterised by neutrophilic inflammation and $CD8^{+}$ T-cell responses, whereas asthma more commonly involves eosinophilic inflammation and $CD4^{+}$ T-helper cell-mediated pathways [81]. These differences are reflected in distinct miRNA expression profiles detected in EBC. For example, Pinkerton *et al.* reported that whilst some miRNAs were shared between asthma and COPD, others were disease specific [75], with similar differences described by Pattnaik *et al.* [16]. Together, these findings suggest that disease-specific EBC-derived miRNA signatures may help distinguish COPD from asthma and healthy smoker populations. However, broader validation across diverse, smoking-stratified cohorts is still needed.

Accumulating evidence suggests that EBC-derived nucleic acids may also serve as biomarkers in other respiratory diseases, including IPF, sarcoidosis, and tuberculosis (TB) [25,30,32,82]. Emerging work in niche pulmonary conditions, such as cystic fibrosis, likewise implicates EBC-derived extracellular-vesicle miRNAs in exacerbation biology [83]. Although high-resolution CT (HRCT) and surgical lung biopsy remain the diagnostic gold standards for IPF, these approaches carry substantial risks, particularly in patients with advanced disease [84]. Consequently, developing non-invasive approaches to detect disease progression or monitor fibrotic activity has become a major priority in respiratory research. Regulatory miRNAs involved in fibrotic pathways, including miR-21 and members of the let-7 family, have been detected in EBC and may provide insight into fibrotic signalling and disease progression in IPF [85–88]. Consistent with this, growing evidence indicates that multiple classes of non-coding RNAs—including miRNAs, lncRNAs, and circRNAs—contribute to the pathogenesis and progression of interstitial lung diseases [19]. At present, most IPF-related EBC data are hypothesis-generating rather than clinically validated.

EBC analysis has also shown promise in infectious and granulomatous lung diseases. Studies have detected both host miRNA signatures and microbial nucleic acids from Mycobacterium tuberculosis in EBC from patients with active pulmonary TB [25,32]. Rai *et al.* [30] further evaluated EBC miRNAs for distinguishing pulmonary sarcoidosis from TB, reporting significant downregulation of miR-454 ($P < 0.05$) in sarcoidosis compared with TB, although its modest diagnostic performance ($AUC = 0.663$) suggests that multi-marker panels or integration with clinical features may be required for reliable differential diagnosis.

4. Underlying mechanism, detection methods, and limitations

Small RNAs detected in EBC are thought to originate primarily from airway epithelial, immune and tumour cells within the respiratory tract and may be released in EV-encapsulated and EV-free forms [12,19]. Amongst these, miRNAs represent a particularly informative class of post-transcriptional regulators, the expression patterns of which can reflect molecular changes in the airway microenvironment [13–16]. Importantly, similarities between miRNA profiles detected in EBC and those observed in bronchoalveolar lavage samples suggest that EBC can capture representative molecular signatures of the lower airway [12,14,33].

Collection of EBC typically involves condensing devices such as RTube, ECoScreen or Turbo-DECCS, with procedural measures including mouth rinsing and controlled breathing patterns used to minimise oral contamination and improve sampling consistency [21,89,90]. Because nucleic acid yields are generally low (often 75–200 ng DNA/RNA per mL), highly sensitive extraction and detection methods

are required. Common approaches include column-based extraction kits or TRIzol-based protocols, followed by detection using quantitative PCR, probe-based microarrays, or NGS [15,90]. In practice, targeted PCR-based assays often provide greater sensitivity for low-input EBC samples than untargeted metatranscriptomic approaches. Methodological refinements such as universal-tagged RT-PCR and digital PCR have further improved detection of low-abundance nucleic acids, enabling identification of rare genetic or epigenetic alterations in EBC samples [91,92]. In parallel, EV-enrichment strategies followed by EV-focused miRNA profiling have enabled recovery of lung-derived EV cargos from EBC with improved specificity for pulmonary tissue signals [24].

Despite these advances, several technical challenges still limit the broader application of EBC-derived nucleic acids. Key limitations include the inherently low nucleic acid concentration and substantial dilution of ALF by condensed water vapour within the condensate, variability during sample collection (e.g., breathing patterns or device differences). potential upper airway contamination, and the lack of standardised collection and analytical protocols across studies, despite previous calls from the American Thoracic Society (ATS)/the European Respiratory Society (ERS) task forces for harmonisation of EBC methodology [21,93,94]. These factors can reduce analytical sensitivity and reproducibility between cohorts, complicating biomarker validation. Addressing them will require coordinated methodological optimisation and greater standardisation of sampling, extraction, and analytical workflows to support reliable clinical translation [15,94].

Compared with plasma or serum liquid biopsies, EBC sampling is completely non-invasive, repeatable, and enriched for airway-derived signals, which may improve specificity for respiratory diseases whilst avoiding background contributions from extra-pulmonary tissues [5,12,15,18]. However, EBC nucleic acids are substantially more diluted, yields are lower, and pre-analytical variability is higher, creating challenges for sensitivity, standardisation, and multi-centre reproducibility when compared with well-established plasma cfDNA/cfRNA assays [15,21,23,58]. In practice, EBC-based profiling is therefore best considered complementary to blood-based liquid biopsy, particularly for capturing local airway biology that may be less detectable in systemic circulation.

Research on microbial sRNAs in EBC remains nascent and underexplored. Most studies focus on microbial genomic DNA or RNA detected in EBC rather than regulatory small RNAs such as bacterial sRNAs (approximately 50–500 nt) or viral miRNAs [13,15,95]. Distinguishing microbial regulatory RNAs from host-derived transcripts in low-biomass samples remains technically challenging and will likely require advanced sequencing approaches, including small-RNA NGS applied to EV-enriched EBC fractions [2,15,96]. Future studies would benefit from integrating improved sequencing strategies with advances in EV isolation and ultrasensitive detection technologies to facilitate identification of microbial regulatory RNAs as biomarkers of respiratory infections. In addition, many studies to date involve small cohorts and geographically limited populations, leading to variable findings across studies. Clarifying microbial sRNA signatures in EBC will also be important for interpreting host ncRNA changes that arise from innate immune recognition of microbial DNA and RNA in the airway lining fluid [12,96]. Large-scale, multicentre investigations will therefore be essential to validate EBC-derived nucleic acid biomarkers and establish their clinical utility across diverse patient populations. Integrating EBC nucleic acid analysis with complementary biomarker modalities—such as VOC profiling or imaging diagnostics—may further enhance diagnostic performance and support precision medicine approaches in respiratory disease.

5. Potential of EBC-containing miRNAs as indicator for other organ-associated diseases

Human and microbial nucleic acids detected in EBC are generally considered to originate primarily from the local pulmonary microenvironment (e.g., airways and alveoli), as aerosolised droplets of ALF capture EVs and cell-free nucleic acids released by epithelial, immune, and tumour cells during tidal breathing. The workflow of EBC collection and nucleic acid analysis is summarised in Figure 3. During tidal breathing, exhaled air passes through a cooled condensation device, allowing respiratory microdroplets and water vapour to condense into liquid EBC. Following collection, nucleic acids can be isolated, converted to cDNA when appropriate, and analysed using PCR-based or sequencing-based approaches for biomarker detection. Accordingly, EBC has been treated as a lung-focused "breath biopsy" and used mainly for respiratory biomarker discovery, rather than as a window on systemic disease. However, direct evidence that organ-enriched regulatory RNAs from distant tissues are consistently detectable in human EBC remains sparse, and much of the current understanding derives from preclinical models and indirect observations. Organs undergoing injury, ischaemia, or malignancy—including the liver, kidney, heart, and tumours—release EVs and extracellular nucleic acids into circulation that can accumulate within the pulmonary microvasculature, raising the possibility that systemic nucleic acids may occasionally enter airway secretions and exhaled droplets. Owing to its extensive capillary network and large vascular surface area, the lung also serves as a major interface for circulating EVs, where vesicles from distant tissues may transiently accumulate before interacting with pulmonary immune or epithelial cells. Supporting this concept, prenatal diagnostic studies detected Y-chromosome DNA in EBC from pregnant women carrying male foetuses using digital droplet PCR, though this signal represents circulating foetal DNA rather than RNA [97]. Nevertheless, overall evidence remains sparse.

Although liver-enriched miRNAs such as miR-122 have not yet been directly demonstrated in human EBC, the liver–lung axis provides a well-characterised mechanistic model of inter-organ RNA communication. miR-122 is abundantly released by hepatocytes during acute or chronic liver injury. During conditions like ischaemia–reperfusion injury or hepatitis, hepatocytes release large quantities of miR-122 into the circulation in both EV-associated and protein-bound forms. Circulating miR-122 can subsequently accumulate in the lung and be taken up by alveolar macrophages [98], and liver-to-lung miRNA transfer has also been demonstrated in models of ischaemia–reperfusion-induced liver injury [99]. Matrix metalloproteinase-2 (MMP2) present on hepatic EV surfaces has been proposed to facilitate traversal of extracellular matrices and enhance uptake by recipient alveolar macrophages, providing a potential mechanism for efficient hepatic EV delivery to the lung [100]. Within these immune cells, hepatocyte-derived miR-122 activates TLR7 signalling pathways, triggering cytokine production and inflammatory responses [101]. Together, these findings demonstrate that liver-derived miR-122 can reach and function within the lung via systemic circulation. On this basis, it is plausible that liver-derived miRNAs, including miR-122, could be intermittently detectable in EBC—particularly under inflammatory conditions that increase alveolar-capillary permeability—and might therefore serve as indirect indicators of hepatic injury [98,102,103]. However, the presence of liver-enriched miRNAs in human EBC has not yet been demonstrated and remains an important evidence gap. More broadly, these observations raise the possibility that lung tissue can acquire "foreign" miRNAs from distant organs or metastatic

tumours, which may subsequently modulate inflammatory signalling pathways within the pulmonary microenvironment [104,105].

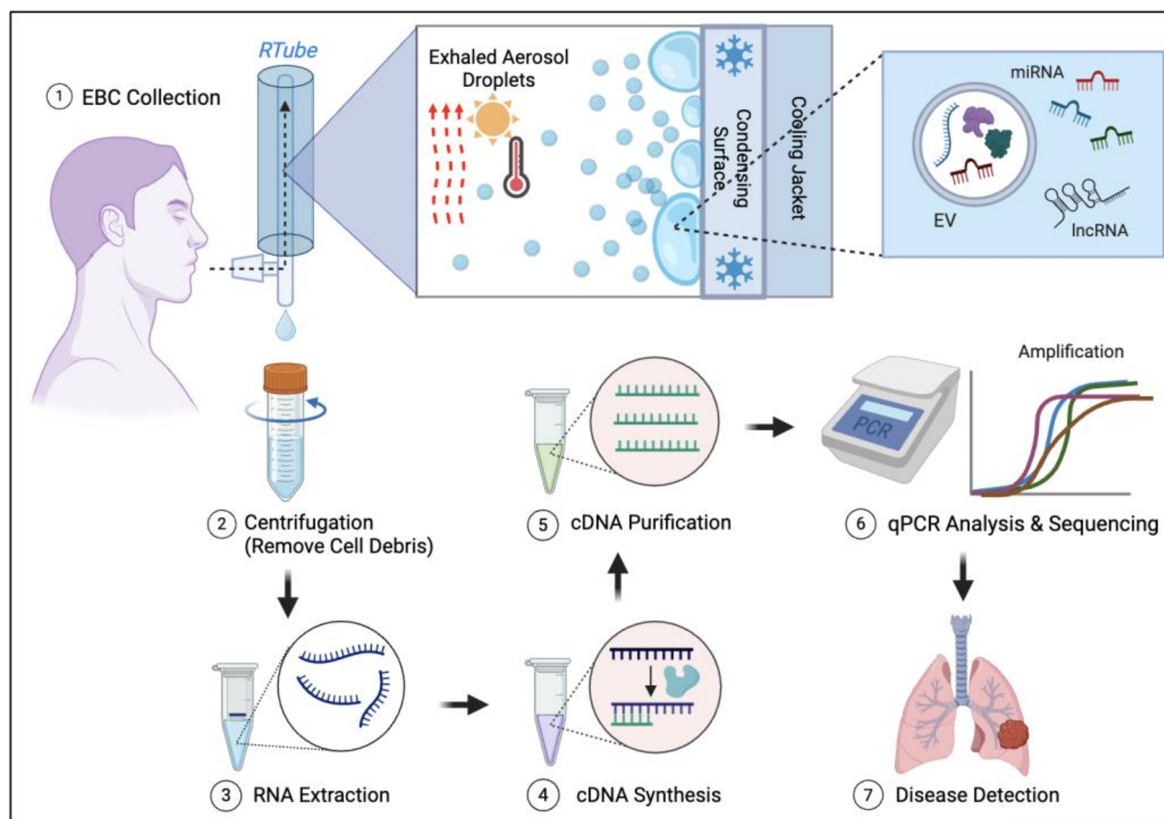

Figure 3. Working model of EBC collection process. Exhaled air is directed through a mouthpiece into a cooled condensation device (e.g., RTube), where reduced temperature causes water vapor and respiratory microdroplets to accumulate on chamber surface as liquid condensate, which gets collected. This exhaled breath condensate contains soluble and vesicle-associated biomolecules, including cell-free nucleic acids (RNA) that can be recovered by extraction, cDNA synthesis and purification, and subsequently analysed with PCR-based amplification and related molecular assays.

Beyond passive transfer, pulmonary cells may act as intermediate relays of systemic RNA signalling. Alveolar macrophages and epithelial cells can internalise circulating EVs and subsequently resecrete miRNA cargo through newly formed vesicles [106,107]. How these vesicles or their RNA cargos traverse the alveolar-capillary barrier into airway lining fluid is not yet fully resolved. Potential routes include transcytosis across endothelial and epithelial cells, fusion or uptake by resident immune cells like alveolar macrophages with subsequent re-secretion of miRNA-containing EVs into the airway, and transfer via injured or permeable alveolar–capillary interfaces in inflammatory states; however, these mechanisms have not been directly demonstrated in human EBC. Conceptually, this positions the lung not only as a passive filter for circulating EVs but also as an active "signal-processing hub" that can transform incoming extra-pulmonary RNA cues into secondary, lung-derived miRNA signatures measurable in EBC.

Another important example of systemic communication is the gut–lung axis, a well-established bidirectional pathway through which intestinal microbiota influence lung health and immune responses [108–111]. Although no direct evidence for substantial translocation of intact gut-derived microbial RNA (e.g., mRNA or rRNA transcripts) to the lung exists, EVs released from intestinal microbes may transport bacterial proteins, lipids, and nucleic acids to distant organs [112]. Bacterial

components, including lipopolysaccharide and outer membrane vesicles, are known to translocate during conditions involving intestinal barrier disruption—such as inflammatory bowel disease, sepsis, or experimental colitis—allowing gut-derived microbial products to enter systemic circulation and influence pulmonary inflammation. In pre-clinical models, including induced colitis in mice, bacterial translocation from the intestine to the lung has been observed, with colonies derived from gut-tagged bacteria detected in lung tissue, although these findings are largely based on DNA- or culture-based detection methods [113]. It is therefore plausible that a fraction of these microbial nucleic acids contributes to the heterogeneous pool of extracellular DNA and RNA present in ALF and, by extension, in EBC. Mechanistically, this suggests that nucleic acids detected in EBC could, in rare and highly inflammatory states, encode composite information reflecting both pulmonary and gastrointestinal barrier integrity; however, such cross-origin signatures have not yet been directly quantified in EBC. However, evidence for microbial nucleic acids reaching the lung largely comes from animal models and DNA- or culture-based assays, and any contribution of gut-derived RNAs to human EBC is therefore speculative at this stage.

In several disease contexts, including COPD exacerbations, acute lung injury, and critical illness, intestinal dysbiosis and barrier disruption have been associated with altered pulmonary immune responses and increased susceptibility to lung inflammation [114,115]. Under such conditions, gut-derived microbial products that reach the lung or systemic circulation are thought to be carried predominantly as bacterial fragments or EV-associated cargo, rather than as abundant intact microbial transcripts, as translocation typically arises from bacterial debris or vesicles rather than viable organisms [116–118]. Accordingly, breath-derived nucleic acids might, in rare and highly inflammatory states, carry indirect signatures of systemic or gastrointestinal perturbations, although this remains to be demonstrated experimentally.

Based on these findings, we proposed a working model for EBC-containing miRNAs as potential indicator for other organ-associated diseases (Figure 4). According to this model, circulating EVs and extracellular RNAs released from distant tissues may accumulate within the pulmonary microenvironment, undergo uptake and processing by lung immune and epithelial cells, and subsequently contribute—directly or indirectly—to the extracellular nucleic acid signatures measurable in EBC. Taken together, these inter-organ axes highlight three key research priorities: (i) targeted assays for organ-enriched miRNAs (e.g., liver, cardiac, and renal signatures) in EBC from patients with well-phenotyped extra-pulmonary diseases; (ii) mechanistic models tracing labelled EV-associated RNAs from distant organs into ALF and EBC; and (iii) integrative analyses comparing EBC, plasma, and tissue miRNA profiles to disentangle lung-intrinsic from systemic contributions. However, establishing whether and under what conditions extra-pulmonary RNAs make a measurable contribution to EBC will be essential before EBC can be reliably advanced as a biomarker source for non-respiratory diseases.

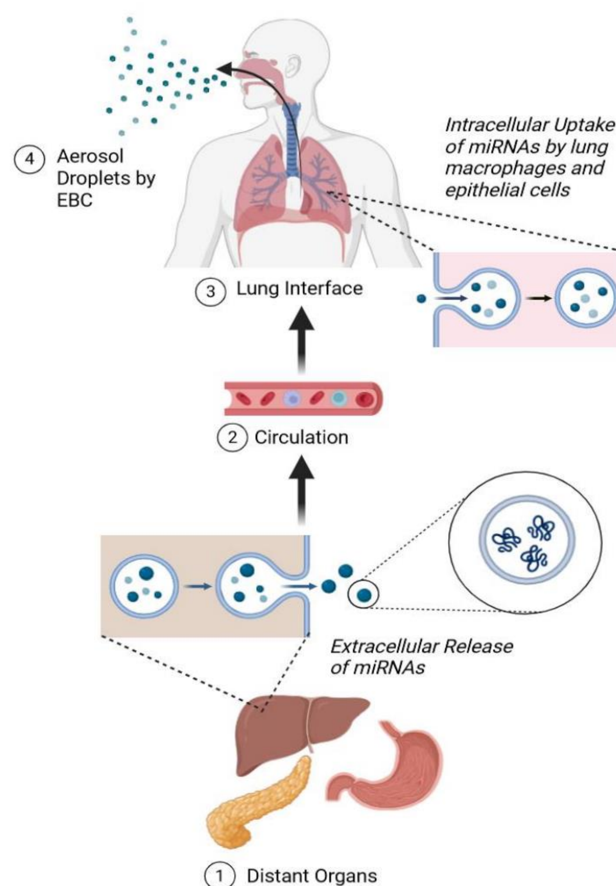

Figure 4. Proposed systemic EV/RNA trafficking model contributing to nucleic acids detected in EBC. Extracellular miRNAs released from distant organs particularly through EVs during injury, inflammation, or malignancy may enter the systemic circulation and accumulate within the pulmonary microvasculature. Circulating EVs can subsequently interact with alveolar macrophages and epithelial cells, potentially leading to secondary lung-derived EV or RNA release into the airway lining fluid. These nucleic acids may ultimately contribute to the heterogeneous pool of extracellular RNAs detectable in EBC. Solid arrows indicate processes with experimental support in preclinical or related human studies; dashed arrows indicate proposed or incompletely resolved mechanisms.

6. Conclusions and future perspectives

The study of EBC has progressed from a technical innovation to an informative molecular lens on respiratory biology and disease. This condensed ALF contains extracellular nucleic acids that report on tumour biology, inflammation, infection, and tissue injury within the airway microenvironment. Amongst these, regulatory non-coding RNAs are particularly informative because of their stability, disease-specific dysregulation, and central roles in post-transcriptional gene regulation.

Across respiratory diseases, EBC-derived nucleic acids capture pathological processes occurring at the airway–alveolar interface. In lung cancer, dysregulated miRNA networks detected in EBC show promise for early detection, molecular stratification, and prognostic assessment, particularly when combined with cfDNA mutation or methylation profiling. In chronic airway diseases such as asthma and COPD, altered small RNA profiles reflect inflammatory signalling and immune activation, whilst elevated mtDNA levels indicate mitochondrial injury and oxidative stress. In interstitial, infectious, and

granulomatous lung diseases, the concurrent detection of pathogen-derived nucleic acids and host immune-regulatory miRNAs further supports the concept of a non-invasive "breath biopsy" integrating regulatory RNA dynamics with genomic and microbial signals from ALF.

Beyond their utility as individual biomarkers, EBC nucleic acids should be viewed as components of broader regulatory and pathophysiological networks. cfDNA frequently carries tumour-associated mutations or epigenetic modifications, reflecting genomic instability and cellular turnover, whereas mtDNA reports on oxidative stress and mitochondrial damage—processes that can reshape ncRNA programmes. Microbial nucleic acids recovered from EBC reflect airway colonisation or infection and engage innate immune receptors that in turn alter miRNA and lncRNA expression in epithelial and immune cells. Together, these complementary molecular layers can provide a more integrated view of disease biology than any single marker class alone. Conceptually, this positions EBC not only as a local "breath biopsy" of the airway–alveolar interface but also as a potential reporter of systemic extracellular RNA communication, albeit one where clinical evidence is still emerging and often indirect.

A key conceptual advance is the recognition that EBC may reflect not only local pulmonary pathology but also systemic extracellular RNA trafficking. Experimental models show that miRNAs released from injured tissues, including the liver, can circulate within EVs, accumulate in the pulmonary microvasculature, and be internalised by alveolar macrophages, where they modulate inflammatory signalling. These observations support a model in which the lung acts as a "signal-processing hub" for circulating EV-associated RNAs, with EBC potentially capturing both direct systemic RNA transfer and secondary lung-intrinsic responses. However, direct evidence that extra-pulmonary regulatory RNAs contribute measurably to human EBC profiles remains limited, and determining when such signals are detectable is important. In vitro studies exposing airway epithelial cells and alveolar macrophages to organ-derived EVs could determine whether circulating regulatory RNAs are internalised and repackaged for release into airway secretions or instead trigger secondary ncRNA responses within lung tissue. Complementary *in vivo* models of multi-organ injury and malignancy will be needed to trace regulatory RNAs from their tissue of origin through the circulation to the lung and into EBC, including across physiological axes like the gut–lung interface.

Going forward, solving these three core questions will be important to shape the field of EBC cell-free nucleic acids as non-invasive biomarker for disease diagnosis: (1) What degree of methodological standardisation in EBC collection, EV enrichment, and nucleic acid detection is necessary to enable harmonised and clinically deployable assays, and how can these workflows be integrated with existing plasma-based liquid biopsy pipelines? (2) Which combinations of EBC-derived nucleic acids—including miRNAs, lncRNAs, cfDNA, mtDNA, and microbial RNA/DNA—together with complementary modalities such as volatile organic compound (VOC) profiling, yield robust and reproducible disease signatures across large multicentre cohorts? (3) Under what physiological and pathological conditions do extra-pulmonary EV-associated RNAs make a measurable contribution to human EBC profiles, and how can these signals be distinguished from lung-intrinsic responses? Addressing this will require mechanistic *in vitro* and *in vivo* models capable of tracing labelled EV-associated RNAs from distant organs through the circulation to the lung and into EBC. Continued advances in EV isolation, ultrasensitive sequencing, and digital PCR technologies, alongside integrative profiling strategies combining EBC, plasma, and tissue analyses, will likely be central to establish EBC as a clinically informative platform linking local airway biology with broader systemic disease processes.

Supplementary data

Supplementary Table S1 summarises studies reporting extracellular nucleic acids detected in human exhaled breath condensate, including nucleic acid class, disease context, analytical methods, and key findings.

Declaration of generative AI and AI-assisted technologies

The authors did not use generative AI or AI-assisted technologies in the writing of this manuscript.

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Authors' contribution

Conceptualisation: J.L.Z. and G.B.H.; methodology: J.L.Z.; literature review and formal analysis: J.L.Z.; writing—original draft preparation: J.L.Z.; writing—review and editing: J.L.Z. and G.B.H.; visualisation: J.L.Z.; supervision: G.B.H.; funding acquisition: G.B.H. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare or other disclosures.

Abbreviations

ALF, airway lining fluid; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; cfDNA, cell-free DNA; COPD, chronic obstructive pulmonary disease; circRNAs, circular RNAs; cfNAs, cell-free nucleic acids; DAMP, damage-associated molecular pattern; EBC, exhaled breath condensate; EVs, extracellular vesicles; FVC, forced vital capacity; H_2O_2, hydrogen peroxide; IPF, idiopathic pulmonary fibrosis; IL-6, interleukin-6; lncRNA, long non-coding RNAs; MMP, matrix metalloproteinase; mRNA, messenger RNA; miRNA, microRNAs; NGS, next-generation sequencing; nt, nucleotides; NF-κB, nuclear factor kappa B; NSCLC, non-small cell lung cancer; qPCR, quantitative polymerase chain reaction; SP-A/D, surfactant protein A/D; sRNAs, small RNAs; TLR, toll-like receptor; TNF-α, tumour necrosis factor-alpha; VOCs, volatile organic compounds.

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