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Transcriptomics of the muco-microbiotic layer of the lower airways: a new frontier for translational research in pulmonology?

Trascrittomica dello strato muco-microbiotico delle vie aeree inferiori: una nuova frontiera per la ricerca traslazionale in pneumologia?

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Summary

The lower airways are traditionally described as comprising three layers: mucosa, fibro-musculo-cartilaginous layer, and adventitia. However, increasing interest in respiratory microbiota has led to the identification of a fourth, innermost component: the muco-microbiotic layer. This dynamic ecosystem consists of airway mucus, resident microbiota, and extracellular nanovesicles (originating from both host cells and microbes). Within this microenvironment, intricate molecular dialogues influence respiratory physiology and the pathogenesis of chronic or infectious diseases.

Transcriptomics, the study of active RNA transcripts, emerges as a key tool to investigate this layer's biological functions. Unlike metagenomics, which captures static genetic potential, transcriptomics reveals real-time gene expression in both microbial and host populations, offering insights into immune modulation, inflammation, and tissue regeneration. Metatranscriptomics enables functional profiling of the airway microbiota in diseases such as COPD, asthma, pulmonary fibrosis, and COVID-19, while extracellular vesicle-associated transcriptomics allows exploration of RNA-mediated cross-talk via exosomes and bacterial vesicles.

This dual-transcriptomic approach facilitates the mapping of intercellular and interspecies signaling networks, potentially revealing early molecular alterations preceding clinical symptoms. It holds promise for non-invasive diagnostics, risk stratification, and personalized therapies – including inhaled probiotics, synthetic RNAs, and engineered vesicles.

However, methodological challenges remain: standardization of sampling, nanovesicle isolation, RNA extraction, and complex data integration. Despite these hurdles, transcriptomics of the muco-microbiotic layer represents a transformative frontier in precision pulmonology, redefining respiratory pathophysiology from a static anatomical model to a dynamic, molecular, and ecological perspective.

Key words: muco-microbiotic layer, respiratory transcriptomics, extracellular vesicles, pulmonary microbiota

Riassunto

Le vie aeree inferiori sono tradizionalmente descritte come composte da tre strati: mucosa, strato fibro-muscolo-cartilagineo e avventizia. Tuttavia, il crescente interesse per il microbiota respiratorio ha portato all'identificazione di una quarta componente più interna: lo strato muco-microbiotico. Questo ecosistema dinamico è costituito dal muco delle vie respiratorie, dal microbiota residente e da nanovescicole extracellulari (di origine sia cellulare che microbica). All'interno di questo microambiente, complessi dialoghi molecolari influenzano la fisiologia respiratoria e la patogenesi delle malattie croniche o infettive.

La trascrittomica, lo studio dei trascritti attivi di RNA, si afferma come strumento chiave per indagare le funzioni biologiche di questo strato. A differenza della metagenomica, che

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rileva il potenziale genetico statico, la trascrittomica rivela l'espressione genica in tempo reale sia delle popolazioni microbiche che di quelle dell'ospite, offrendo nuove prospettive sulla modulazione immunitaria, l'infiammazione e la rigenerazione tissutale. La metatrascrittomica consente la caratterizzazione funzionale del microbiota respiratorio in patologie come BPCO, asma, fibrosi polmonare e COVID-19, mentre la trascrittomica associata alle vescicole extracellulari permette di esplorare i meccanismi di comunicazione mediati dall'RNA tramite esosomi e vescicole batteriche.

Questo approccio duale alla trascrittomica facilita la mappatura delle reti di segnalazione intercellulare e interspecie, con la possibilità di individuare precocemente alterazioni molecolari prima della comparsa dei sintomi clinici. Ciò apre la strada a diagnosi non invasive, stratificazione del rischio e terapie personalizzate, tra cui probiotici inalatori, RNA sintetici e vescicole ingegnerizzate.

Tuttavia, permangono sfide metodologiche: la standardizzazione del campionamento, l'isolamento delle nanovescicole, l'estrazione dell'RNA e l'integrazione di dati complessi. Nonostante tali ostacoli, la trascrittomica dello strato muco-microbiotico rappresenta una frontiera trasformativa nella pneumologia di precisione, ridefinendo la fisiopatologia respiratoria da un modello anatomico statico a una prospettiva dinamica, molecolare ed ecologica.

***Parole chiave**: strato muco-microbiotico, trascrittomica respiratoria, vescicole extracellulari, microbiota polmonare*

Introduction

The wall of the lower airways is traditionally described as being composed of three layers or tunics: 1) the mucosa, 2) the "submucosa" (although we do not favor this term, as it is anatomically more accurate to refer to a submucosa only in the hollow organs of the alimentary canal; we prefer to refer to this layer as the "intermediate layer", or even better, the "fibro-musculocartilaginous layer", to encompass all three structural components that constitute it), and 3) the adventitia.

In recent years, however, growing interest in the role of the microbiota in pulmonary pathophysiology has led to the definition of a new layer, which in living subjects represents the innermost tunic, facing the lumen: the muco-microbiotic layer of the lower airways. This three-dimensional ecosystem, first described by us in the alimentary canal [1,2] (to which the lower airways are embryologically connected), is composed of three main elements: 1) mucus secreted by airway epithelial goblet cells; 2) the resident microbiota; and 3) extracellular nanovesicles, derived both from microorganisms (e.g., outer membrane vesicles, OMVs) and from the host's epithelial cells (such as exosomes) (Fig. 1) [3].

Within this highly dynamic and regulated microenvironment, complex biomolecular dialogues take place, influencing both respiratory physiology and the development of chronic inflammatory or infectious conditions. In this context, transcriptomics - the study of the complete set of RNA transcripts present in a given cellular or microbial population at a specific time - may emerge as a key technology to analyze the active functions of the muco-microbiotic layer of the lower airways, including both the respiratory microbiota and the nanovesicles hosted within this layer. This aspect is particularly important considering that nanovesicles can also carry RNA capable of modulating gene expression in target cells.

Unlike metagenomics, which provides a snapshot of the overall genetic potential, transcriptomics allows the identification of genes that are actively expressed, both by microorganisms and host cells. This opens up new perspectives for understanding the molecular mechanisms underlying complex respiratory diseases and offers innovative tools for early diagnosis, patient stratification, and the identification of novel therapeutic targets.

The airway microbiota and its transcriptional functions: how does it work?

The microbiota of the lower airways, once thought to be scarce or even absent, is now recognized as a stable and functionally active component of the respiratory environment. Under physiological conditions, resident microbial communities contribute to the maintenance of immune homeostasis, the maturation of respiratory mucosal cells, and protection against opportunistic pathogens [4].

Studying the active functions of the microbiota, however, requires tools capable of going beyond mere taxonomic identification. In this context, metatranscriptomics - the application of transcriptomics to the entire microbial ecosystem of the muco-microbiotic layer - may allow real-time analysis of gene expression, highlighting how the microbiota responds to environmental, inflammatory, or therapeutic stimuli. The resulting transcriptional profiles enable the identification of genes involved in metabolism, toxin production, immune modulation, and inter-microbial communication.

Numerous studies have begun applying transcriptomics to patients with COPD, severe asthma, idiopathic pulmonary fibrosis, and recurrent respiratory infections (including SARS-CoV-2 infection), showing that the dysbiosis associated with these conditions is not only quantitative (reduced diversity) but also functional: the expression of key microbial genes changes, such as those regulating the production of anti-inflammatory metabolites or immunostimulatory molecules [5].

Moreover, transcriptomics can be applied in experimental models in vitro or ex vivo, such as respiratory cell

lines or tissue biopsies cultured with samples of the respiratory muco-microbiotic layer, to study the "molecular dialogue" between host and microbiota. These approaches will allow the reconstruction of microbial signaling networks involved in modulating the mucosal environment, with important implications for understanding the progression of respiratory diseases and for developing targeted therapeutic interventions.

Nanovesicles and cross-kingdom communication in the muco-microbiotic layer: what is the state of the art?

One of the most fascinating and least explored components of the muco-microbiotic layer of the lower airways is represented by extracellular nanovesicles [6,7]. These structures include, on one hand, OMVs produced by Gram-negative bacteria, as well as Gram-positive extracellular vesicles (GpEVs) produced by Gram-positive bacteria; on the other hand, they include exosomes derived from host epithelial cells. All of these vesicles play a crucial role in molecular communication among the different compartments of the ecosystem.

These nanovesicles can contain proteins, lipids, and - most relevant to this work - messenger RNAs (mRNAs) and small non-coding RNAs (e.g., microRNAs, bacterial small RNAs), which act as intercellular and interspecies signaling molecules [6,7]. Analyzing their transcriptomic content allows for the study of molecular messages exchanged between the microbiota and the respiratory mucosa, opening up new avenues for understanding local regulatory mechanisms of inflammation, immune response, and epithelial regeneration.

Transcriptomics applied to nanovesicles – recently referred to as "extracellular vesicle-associated transcriptomics" [8] – enables the analysis of both free and encapsulated RNAs in respiratory fluids (e.g., BAL fluid, sputum) as well as in experimental models. This approach allows researchers to identify actively exported bacterial transcripts (e.g., virulence genes, immunomodulatory factors) as well as epithelial transcripts released in response to microbial or inflammatory stimuli.

It is therefore possible to map an integrated molecular network in which transcriptomic information is not confined within cells but is disseminated through mucus and nanovesicles, contributing to the maintenance or disruption of local homeostasis. Under pathological conditions, this communication circuit may become dysregulated, and transcriptomics offers valuable tools to detect functional alterations at an early stage, even in the absence of macroscopic structural changes.

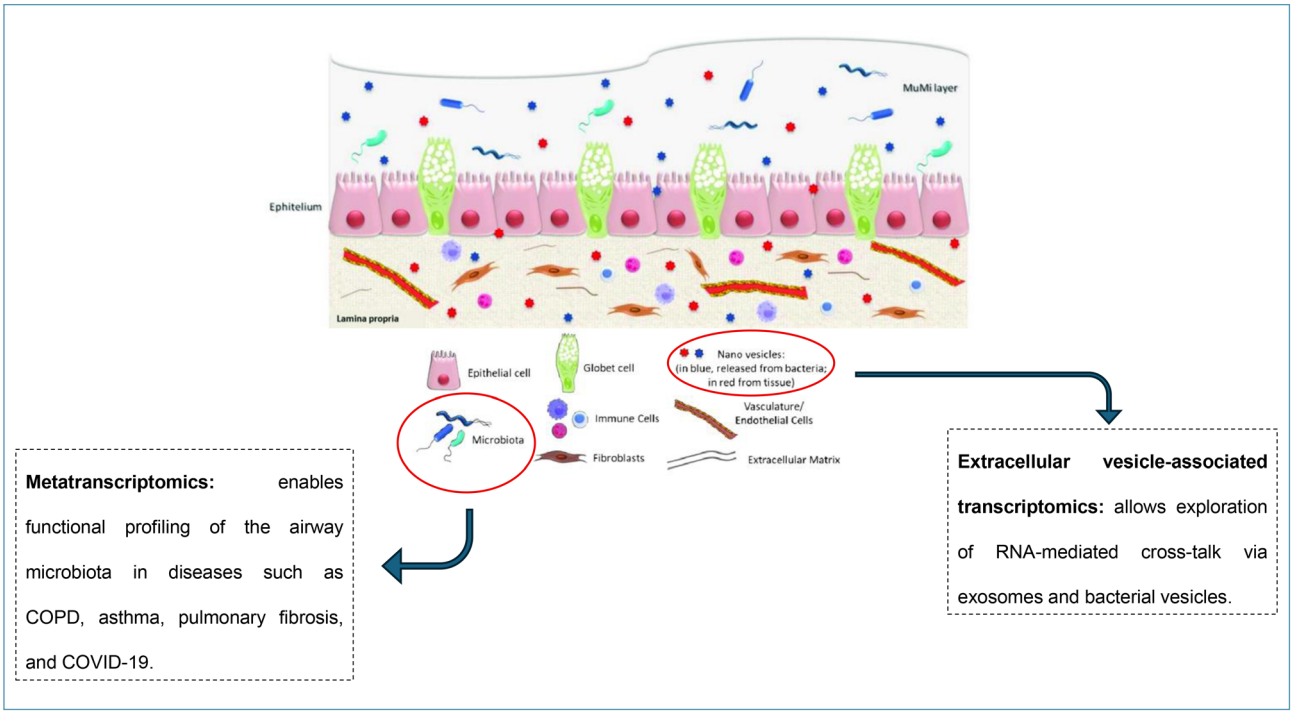

Figure 1. This figure summarizes the main components of the mucomicrobiotic layer (mucus, microbiota and nanovesicles) as well as its relationship with the layer of the airway mucosa (i.e., epithelium and lamina propria). We focus in particular on the microbiota and the nanovesicles since the proposed techniques (*Metatranscriptomics* and *Extracellular vesicle-associated transcriptomics*) refer specifically to them. Further details are available in the text of the manuscript. The figure has been modified and adapted from Fucarino et al. [2].

Clinical potential: what are the prospects for its use in the future?

The integration of transcriptomics in the study of the muco-microbiotic layer of the lower airways can open innovative scenarios in diagnostics, prognosis, and therapeutics. This approach allows going beyond the analysis of microbiota composition to access a functional and dynamic dimension, potentially more closely correlated with health or disease states.

In diagnostics, transcriptomic profiles of the muco-microbiotic layer can provide early and specific molecular fingerprints of complex respiratory diseases such as COPD, severe asthma, idiopathic pulmonary fibrosis, and infectious bronchopneumopathies. These signatures may derive from microbial gene expression (e.g., genes involved in pro-inflammatory or immune evasion mechanisms), altered epithelial transcripts, or RNAs carried by extracellular nanovesicles.

From a prognostic perspective, transcriptomics of the muco-microbiotic layer could contribute to patient stratification by highlighting subgroups with specific functional patterns associated with higher risks of exacerbations, disease progression, or treatment resistance. In this sense, the combination of metatranscriptomics and extracellular vesicle-associated transcriptomics represents a potential tool for personalized medicine.

Therapeutically, transcriptomic analysis of the muco-microbiotic layer could guide the development of interventions aimed at selectively modulating microbial or immune functions. Examples include the use of respiratory prebiotics and probiotics, such as inhalable formulations, synthetic small RNAs, or recombinant nanovesicles capable of restoring a balanced molecular dialogue between host and microbiota.

However, it remains essential to address several methodological challenges: standardizing protocols for collection and preservation of muco-microbiotic layer samples (mucus? BAL? sputum?), effective separation and characterization of nanovesicles, optimizing RNA yield, and the bioinformatic analysis of highly complex data. Moreover, integrating transcriptomic data with other omics dimensions (metabolomics, lipidomics, proteomics) will be crucial to obtain a comprehensive systemic view of the muco-microbiotic layer.

In summary, transcriptomics applied to the muco-microbiotic layer may represent a promising technological frontier for precision pulmonology, still underexplored but already rich in potential implications.

Conclusions

We hope that the study of the muco-microbiotic layer of the lower airways will emerge as a new ecological and functional perspective in understanding respiratory diseases. In this context, transcriptomics is proposed as a key technology for the integrated analysis of the active functions of all ecosystem components: from the respiratory microbiota to the extracellular nanovesicles that convey molecular signals.

This dynamic vision allows overcoming a purely descriptive or taxonomic approach, offering tools to identify early functional alterations often invisible with conventional methodologies. Clinical applications would be manifold: from early diagnosis to risk stratification, up to the identification of molecular targets for targeted therapeutic interventions.

For these potentials to translate into clinical practice, it is necessary to promote translational studies on well-characterized cohorts, strengthen interdisciplinary collaborations between clinicians and basic researchers, and develop shared protocols for the collection and analysis of biological samples.

In conclusion, the transcriptomic approach to the muco-microbiotic layer will not only represent a technological advancement but also a paradigm shift: from a static and compartmentalized view of respiratory mucus to an ecological, molecular, and dynamic conception that can substantially contribute to advancing morpho-functional and pathophysiological knowledge of the airways.

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