Lung microbiome and origins of the respiratory diseases

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ABSTRACT

The studies on the composition of the human microbiomes in healthy individuals, its variability in the course of inflammation, infection, antibiotic therapy, diets and different pathological conditions have revealed their intra and inter-kingdom relationships. The lung microbiome comprises of major species members of the phylum Bacteroidetes, Firmicutes, Actinobacteria, Fusobacteria and Proteobacteria, which are distributed in ecological niches along nasal cavity, nasopharynx, oropharynx, trachea and in the lungs. Commensal and pathogenic species are maintained in equilibrium as they have strong relationships. Bacterial overgrowth after dysbiosis and/or imbalanced of CD4$^+$ helper T cells, CD8$^+$ cytotoxic T cells and regulatory T cells (Treg) populations can promote lung inflammatory reactions and distress, and consequently acute and chronic respiratory diseases. This review is aimed to summarize the latest advances in resident lung microbiome and its participation in most common pulmonary infections and pneumonia, community-acquired pneumonia (CAP), ventilator-associated pneumonia (VAP), immunodeficiency associated pneumonia, SARS-CoV-2-associated pneumonia, acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). We briefly describe physiological and immunological mechanisms that selectively create advantages or disadvantages for relative growth of pathogenic bacterial species in the respiratory tract. At the end, we propose some directions and analytical methods that may facilitate the identification of key genera and species of resident and transient microbes involved in the respiratory diseases' initiation and progression.

1. Introduction

A diverse and dynamic microbial communities live in the upper (nasal, mouth, trachea, upper bronchus) and lower (lungs, bronchi, bronchioles, and alveoli) mucosal area of the respiratory tract (Huffnagle et al., 2017; Moffatt and Cookson, 2017; Whiteside et al., 2021; Natalini et al., 2022). A complete understanding of the molecular mechanisms by which host cells and microbial pathogens efficiently survive and multiply in this ecosystem still unknown. Nasal, tracheal and lung tissue microenvironment contain tissue epithelial, vascular endothelial, stromal and immune cells, and the specific extracellular matrix. The trachea and bronchi are covered with the mucus layer made of the heavily glycosylated proteins, the mucins, produced by globlet cells. The cilia carpet of the lung's surface area (alveoli) is covered with a thin layer of lipids and tensoative surfactants, such as SP-A and SP-D proteins (Whitsett and Alenghat, 2015). The bronchi-alveolar fluid regulates the conducting and peripheral airways through the alveoli along ventilation cycles. Some microbes, in special cases, viruses and

bacteriophages, live inside the cells or bacteria, where they directly interfere with host transcription, translation, and DNA repair mechanisms. A variety of proteins, polysaccharides, saturated and unsaturated lipids, and metabolites produced by microbial communities regulate the gene expression programs for cellular development and immunity. The adhesion molecules on the epithelial, ciliated, non-ciliated (clara) secretory cells, and basal cells of the lungs, phagocytes (macrophages and neutrophils), dendritic cells, mast cells, basophils, eosinophils, natural killer (NK) cells and lymphocytes (T and B cells) sense the presence of microbes and trigger the innate immune program. Activation of the Toll-like receptors (TLRs) and its intracellular signaling pathways, in part, via the Jak kinase/STAT3 and/or nuclear factor kappa B (NF-κB) pathways, leads to cytokine production thereby an inflammatory microenvironment. The type 1 pneumocytes, type 2 pneumocytes and immune alveolar macrophages upon interaction with cytokines and growth factors initiate the programs for cellular recognition and differentiation of T and B lineages (Whitsett and Alenghat, 2015). Microbial antigens also gain access directly to peripheral

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lymphoid organs that contain distinctive populations of antigen processing cells (APCs). The first contact of microbial pathogens with immune cells affects the different ways in which immune cells will tolerate or combat the microbial pathogens thereafter (Honda and Littman, 2016; Iwasaki et al., 2017) (see Figs. 1 and 2).

Specific microenvironments present at sites of inflammation cause CD4$^+$ T helper type to differentiate into the types Th1, Th2, Th17, Treg (regulatory T cells) and other specialized subsets (Honda and Littman, 2016; Iwasaki et al., 2017). The differentiation of CD4 T helper 1 (Th1) subset occurs after the recognition of antigen peptide on the surface of major histocompatibility molecule II (MHC II) through T-cell receptor. This triggers the synthesis and release of IL-1β (interleukin), IL-2, IL-12, IFN-γ (interferon) and TNF-α (tumor necrosis factor) and induction of cell-mediated response that is associated with an inflammatory reaction. The differentiation of CD4 T helper 2 (Th2) occurs after expression of interleukin-4 (IL-4), IL-5, IL-10 and IL-13. IL-4 induces B cell proliferation and differentiation and also proliferation of mast cells (IL-3, IL-4), and the differentiation and proliferation of eosinophils (IL-5). Th2 immune response is observed during asthma and many other allergic inflammatory diseases. The transition from one phenotype into another plays a critical role in the orchestration of the immune response in many pathological settings. Thus, some respiratory diseases are characterized and classified according with changes in Th1/Th2 balance, leukocytes ratio, genetic background, exposure to respiratory viruses, bacteria co-infection and inhaled noxious environmental pollution (Barnig et al., 2018; Lamarche et al., 2018; Mendez et al., 2019). Microbes do not exist in isolation; biofilm communities respond to intrinsic quorum-sensing mechanisms, ecological and environmental factors. This connected-network between microbes-microbes and microbes-host cells is partially understood (Mukherjee and Bassler, 2019). As front defense mediators, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by various chemical reactions lead to damage of cell membranes, tissue destruction, and cell death of host cells and microbial pathogens (Sangiuliano et al., 2014). Therefore, one common approach is to classify respiratory diseases based on the type of cell or tissue affected. For example, diseases that primarily affect the airways, such as asthma and chronic obstructive pulmonary disease (COPD) are often classified as obstructive lung diseases. In contrast, diseases that primarily affect the lung parenchyma, such as pulmonary fibrosis are often classified as restrictive lung diseases.

It is now widely acknowledged that a dysbiosis, or alteration in the composition of certain microbial genera or species in the microbiome population, can result in infections, inflammatory reactions, and the development of acute or chronic diseases (Dickson et al., 2015; Belizário and Napolitano, 2015). Currently, the investigations are being focused on defining the microbial signatures, cells, genes, molecules and specific innate immune signaling pathways that is triggered in epithelial and immune cells, and how they contribute collectively to stability, maintenance, and loss of homeostasis of this complex ecosystem. Nonetheless, the exact role of microbiome, whether causal or bystander factor, in the pathogenesis of lung diseases still being elucidated. Here we will overview some recent studies on basic, translational and clinical microbiomics that have provided further understanding of the pathogenesis of respiratory tract diseases and perspective for possible therapeutic interventions to treat them.

2. The lung microbiome: sources and functions

Specific sampling approaches and whole genome metagenomic DNA sequencing methods have been used to determine the structure, taxonomic composition, gene content and dynamics of functional and resident respiratory microbiome (Dickson et al., 2014; Man et al., 2017; Huffnagle et al., 2017; Moffatt and Cookson, 2017; Whiteside et al., 2021; Natalini et al., 2022). The studies have revealed that the mucosal surface of 50–75 cubic meters of respiratory tract is colonized by complex ecosystems comprised of thousands of bacteria, archaea, viruses, bacteriophages and fungi species.

There are different methods to assess respiratory tract microbiome, as follows: oral swabs, pharingeal secretions, sputum, endotracheal aspirate (ETA), bronchoalveolar lavage fluid (BAL), microlavage (mBAL), bronchoscopy aspiration, bronchial biopsies and exhaled breath condensates. Sputum is deemed a poor surrogate, mixing lower and upper tracheobronchial secretions with upper digestive ones. Nasal swabs are routinely used for SARS-CoV-2 diagnosis, and oral swabs are favored by some, yet neither one can be assumed to trustfully reflect bronchoalveolar bacterial population. Tracheal swabs are practical only in intubated patients, and lung biopsy is too aggressive for routine employment. A resident microbiota in nasal and lung tissues is shaped
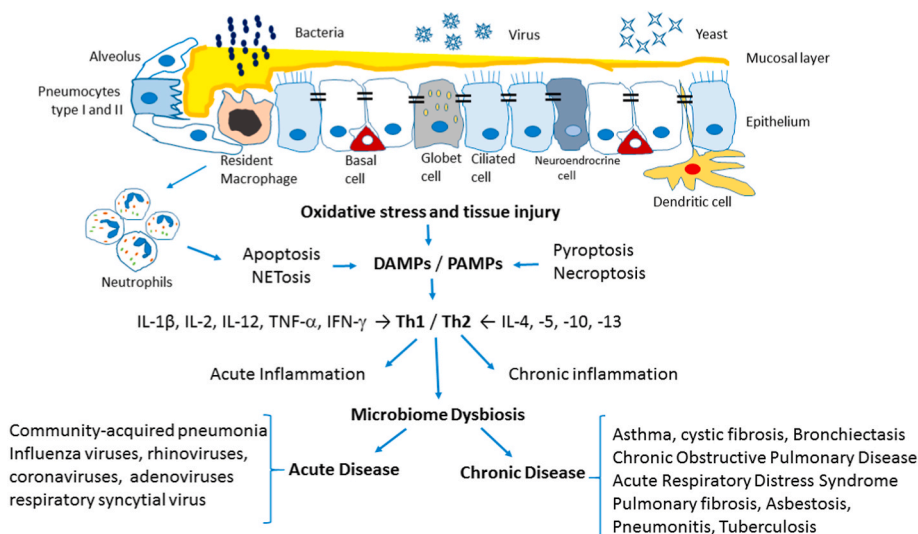

Fig. 1. Cellular and molecular signaling pathways of the acute and chronic lung diseases. Airway epithelial cells, parenchymal cells, goblet cells and immune cells such as neutrophils, macrophages and dendritic cells recognize pathogenic microbes, via pattern recognition receptors (PRRs) such as TLRs and NLRs, and assembly the inflammasomes. The activation of these intracellular complexes results in the release of antimicrobial peptides, secretory immunoglobulin A, cytokines and chemokines. The induction of program cell death by apoptosis, pyroptosis, necroptosis and NETosis contribute to further liberation of DAMPs and PAMPs and inflammatory reaction. Efferocytic resolution of dead epithelial cells, dendritic cells, neutrophils and pathogens contribute to the regulation of inflammation. CD4$^+$ T cells under the stimulation of a set of cytokines undergo proliferation and differentiation into Th1 or Th2 subsets, and Th17 or Treg cell subsets, all of which are important in the initiation and maintenance of systemic immune responses. Sustained inflammation, tissue injury by cytotoxic immune cells, and overgrowth of microbial pathogens (bacterial, virus or yeast species) cause the shift or dysbiosis in the composition (genera and species) of lung microbiome. All these processes contribute to the onset, progression, or exacerbation of various inflammatory and immune reactions and development of acute and chronic respiratory diseases. Abbreviations: TLR, Toll-like receptor; NLR, NOD-like receptor; PAMPs, pathogen-associated molecular pattern; DAMPs, damage-associated molecular pattern; Treg, regulatory T cells.
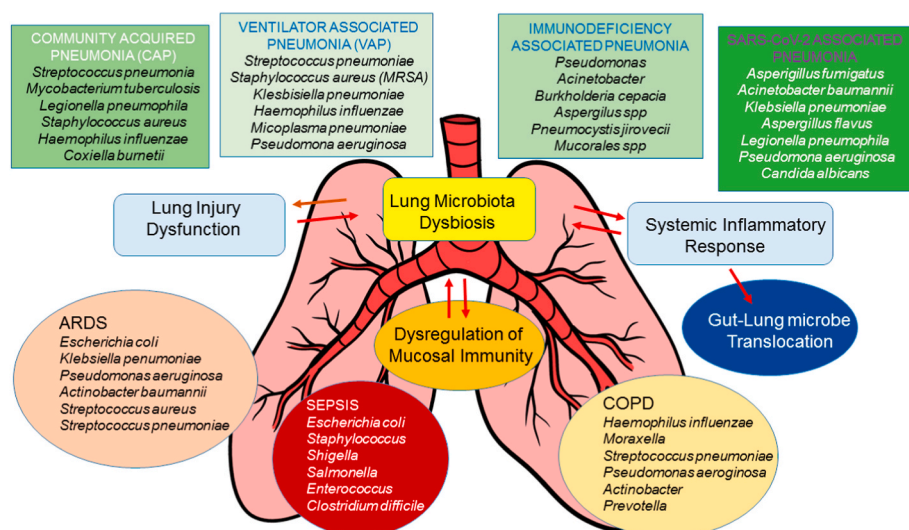
Fig. 2. Microbial species involved in the physiopathology of pneumonias and respiratory diseases ARDS and COPD. Numerous bacterium species from environmental and host microbiota can migrate and infect lung tissue causing pneumonia. Deficiencies in specific lung functions and mucosal immune system lead to increased susceptibility to specific pathogens. Alterations in the pulmonary microbiome contribute to lung injury by promoting increased pulmonary vascular permeability, low oxygen gradients, and a flow of inflammatory molecules for bacterial growth. Ecological analysis revealed that gut dysbiosis is likely the source of bacterial species in the lung that leads to VAP and ARDS. Microbiota translocation from gut to lungs is enhanced during critical illness. Furthermore, lung pathogenic microorganisms are dominated by gut-associated commensal bacteria after sepsis onset. Both pathogenic bacteria and fungi can signal fatal outcomes. Abbreviations: ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; VAP, ventilator associated pneumonia.

continuously by differential selective pressure mediated by innate and adaptive immune defenses, alveolar ventilation, oxygen tension, temperature, pH and nutrient availability. These physiological and environmental gradients determine the niche-specific selective growth conditions that ultimately shape the microbial communities along the respiratory tract. To a certain extent this issue plagues respiratory microbiome research to this day, interfering with comparison of results among different investigators, each group employing specific techniques and thus often reaching diverging conclusions.

Lung microbiome is predominantly comprised of the phylum Bacteroidetes, Firmicutes and Proteobacteria, followed by lesser proportions of Actinobacteria (Man et al., 2017; Carney et al., 2020; Whiteside et al., 2021; Natalini et al., 2022). The lung microbiome often overlaps with the oral microbiome, however, the genus Prevotella occurs at high levels in the oral cavity, and it is less abundant in healthy lungs. It is less well documented and thus somewhat more controversial than the gastrointestinal, urogenital or skin microbiome. The main reason is probably the difficulty for noninvasively collecting samples of the lower respiratory tract (LRT) and relation to the upper respiratory tract (URT), which includes the anterior nares, nasal passages, paranasal sinuses, the nasopharynx and oropharynx. UTR comprises bronchoalveola region, which is associated with the most relevant clinical conditions namely lung diseases. Sequence-based studies typically report taxa or genera in terms the nine hypervariable regions (V1–V9) of the 16S eRNA that differ across taxa. Metagenomics is a powerful tool for the rapid and accurate diagnosis of pathogenic organisms allowing differences between taxonomic composition. BAL and ETA samples give much more precise resolution than sequence-based of standard cultures. The nasal cavity contains 10^3 units of bacterial density, being the most common species *Staphylococcus* spp., *Propionibacterium* spp., *Corynebacterium* spp., *Moraxella* spp. And *Streptococcus* spp (Dickson et al., 2014; Dickson et al., 2015, Man et al., 2017). The high microbial density niche is observed in the nasopharynx and oropharynx compartments, which contains approximately 10^6 bacteria per cm^3 of air. *Moraxella* spp., *Staphylococcus* spp., *Corynebacterium* spp., *Dolosigranulum* spp., *Haemophilus* spp., *Streptococcus* spp., *Rothia* spp., *Veillonella* spp., *Prevotella* spp. And Leptotrichia spp., are the most representative species. The number of bacteria in internal lung tissue is estimated in 10^2 bacteria, with high abundance of *Prevotella* spp., *Veillonella* spp., *Streptococcus* spp. And *Tropheryma whipplei*. The absolute number is not given as colony forming units (CFU) because not all species are cultivable, thus their genome is still poorly characterized.

The lung microbiome has links to diets, gender, genetic polymorphisms and environmental conditions, as it has been demonstrated in the studies of the gastrointestinal and genitourinary tract and skin microbiomes. The transfer of bacteria from gut microbiome to lungs via reflux, lymphatic and systemic circulation may contribute to inflammatory airway diseases (Singhania et al., 2020, Ma et al., 2022). Furthermore, inhaled and exhaled breath contain thousand metabolites, volatile organic compounds (VOCs) and the inorganic and endogenous gaseous transmitters, as examples: nitric oxide (NO), carbon dioxide (CO_2), carbon monoxide (CO), hydrogen cyanide (HCN), and hydrogen sulfide (H_2S) originated from external environment and respiratory and gastrointestinal tract microbiomes (Belizário and Napolitano, 2015; Belizário et al., 2021). The continuous uptake and release of VOCs and bioactive small molecules is very important for controlled expression of genes that regulate the major biological processes of cells, tissues and organs of the human body.

Given the dearth of lifelong studies of the respiratory microbiome in large populations, its absolute composition and natural history is still incompletely known even in healthy people (Man et al., 2017). Most current studies thus focus on qualitative changes in the community diversity namely alpha and beta diversity, as potential markers of dysbiosis and susceptibility to pathogen invasion (Whiteside et al., 2021; Natalini et al., 2022). Alpha diversity (Shannon index) refers to species level abundances or richness in a given sample, while beta diversity quantifies dissimilarities between different samples or microbial communities. Many specialized clinical centers postulate that the main source of respiratory germs is the oropharynx, at least in adults (Pettigrew et al., 2021; Fromentin et al., 2021). In children the nasopharynx could contribute as well as, by means of microaspiration or mucosal translocation. The preferred term for some is transcolonization or the migration of extra respiratory microbes to the subglottic airways (Soussan et al., 2019). The most relevant tracheobronchial respiratory phyla in all circumstances seem to be Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria, while *Prevotella*, *Streptococcus*, and *Veillonella* are the most common genera (Flanagan et al., 2007; Huffnagle et al., 2017; Moffatt and Cookson, 2017; Kumpitsch et al., 2019). Their shifts depend on the spatial context and the underlying disease. This general pattern should not be surprising, given that in the gastrointestinal tract a roughly similar profile is encountered, only enriched by further phyla such as Actinobacteria and Fusobacteria. One respiratory feature that is not mimicked to the same degree by the gut microbiome is the obvious increase in bacterial density whenever a pathogen is associated with major infections.

Multiple tools and metadata analyses have been used to integrate and analyze the human microbiome datasets and systemic distribution of key signatures of microbial species, genes, functions, metabolic

pathways and metabolites (Lloyd-Price et al., 2017; Gupta et al., 2020). Studies now suggest that the gut-lung axis connecting the intestine and lung alveoli exists and may exert relevant biological functions as gut-brain axis (Ma et al., 2022). The total respiratory microbiome is incomparably smaller than the gastrointestinal one, both regarding total population and occupied area (Natalini et al., 2022; He et al., 2022a,b). Systemic distribution of growth hormones, cytokines, chemokines, antimicrobial proteins, lipid mediators, microbial metabolites, endotoxins, amino acids, VOCs and hormones with significant pro- or anti-inflammatory effects can exacerbate or attenuate either the lung or gut diseases at different clinical setting (Rooks and Garrett, 2016; Khan et al., 2021). In this context, bacterial taxa involved in the production of important mediators such as arginine, proline, glutamate, tyrosine, and short-chain fatty acids (SCFAs) could be important allies to prevent the gut and airway respiratory diseases (Rooks and Garrett, 2016). In any case, many more studies need to be done to decipher the physiological cooperation and competition within the lung and gut microbiomes, and when deciding whether or not to initiate antibiotic therapy for the resolution of an infectious disease.

Knowledge gained from genomic and clinical studies have enabled us to further interrogate the disease pathogenesis in the complex milieu of the microbiota among healthy people and patients with specific lung diseases. There has been intense research on pneumonia, cystic fibrosis, asthma, acute respiratory distress syndrome (ARDS), community-acquired pneumonia (CAP) and chronic obstructive pulmonary disease (COPD), also called chronic bronchitis and emphysema (Dickson et al., 2014; Shukla et al., 2017; Iwasaki et al., 2017; Barnig et al., 2018; Lamarche et al., 2018, Mendez et al., 2019). Nosocomial infection of the lung by *Staphylococcus aureus* or *Streptococcus pneumonia*, which share common clinical features, is a major cause of community-acquired pneumonia, and morbidity world-wide. Nonetheless, non-infectious CAP develops as a consequence of neoplastic lesions, pulmonary edema, pulmonary embolism, drug-induced pneumonitis, among other causes. The symptoms of viral and bacterial pneumonia overlap. Thus, it has been difficult to distinct the clinical symptoms of viral infection caused by either influenza A and B, parainfluenza 1, 2 and 3, respiratory syncytial virus, hantaviruses, metapneumoviruses, rhinoviruses, adenovirus, or novel acute respiratory syndrome (SARS-CoV) coronaviruses (Molyneaux et al., 2013; Man et al., 2017; Linden et al., 2019; Clementi et al., 2021). Therefore, innovative methods are needed for discovering and validation of microbial community members (commensal or pathogenic), which contribute positive or negatively to lung diseases. It is also necessary to validate complementary cellular and plasma biomarkers that could precisely define the heterogeneity of molecular pathways associated with phenotypes and endotypes of the respiratory diseases.

3. Acute pulmonary infections and pneumonia

Pneumonia is a heterogeneous cluster of illnesses that encompasses community acquired pneumonia (CAP), ventilator associated pneumonia (VAP) and immunodeficiency associated pneumonia (Quinton et al., 2018; Long et al., 2022). CAP is caused by various bacteria strains including *Streptococcus pneumonia*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Coxiella burnetii* and other species (Nizio et al., 2016; Ahmed et al., 2018; van Vliet et al., 2017; Kuruvilla et al., 2019). In addition, over 1200 viruses can infect the respiratory tract. Classical studies of microbiology neglected the roles of resident communities and dynamic interplay played by pathogenic and resident microbes, immune system and the host environment. CAP occurs along with more or less specific modalities linked to immune compromised subjects (post trauma, post transplantation) as well as to ARDS or tuberculosis (Pettigrew et al., 2021; Rello et al., 2021). Of course, viruses, notably SARS-CoV-2, and fungi are recognized etiological agents, and non infectious inflammatory lung conditions should not be forgotten in the diagnosis. As alluded to

respiratory microbiome information about these multiple contexts is far from abundant, however, this is a dynamic field, with new contributions occurring all the time.

While recommended as primary treatment, unspecific antibiotic therapy acts as an accelerator to a large number of resistance mechanisms to practically all bacteria strains associated with pneumonia (Galán et al., 2013). In addition, early childhood antibiotic exposure to antibiotics (as example, macrolides) can disrupt the healthy lung bacterial and viral microbiome formation, and increase the risk for asthma and allergies as well as neurological diseases (Lim et al., 2015; Shukla et al., 2017). The airborne exchange of antibiotic resistance genes between the environment bacteria and clinical human pathogens and vise-versa appears to be an important avenue to the pathogenesis and cause of morbidity and mortality worldwide (Gwenzi et al., 2022). Thus, the therapeutic use of probiotics such as *Lactobacillus rhamnosus, Bacillus subtilis, Enterococcus faecalis, Faecalibacterium prausnitzii and Akkermansia muciniphila*, has attracted attention as an alternative treatment of respiratory diseases.

The cellular and molecular pathways to lung infections includes neutrophil recruitment by the chemokines CXCL2, CXCL5 and CCL2, expression of growth factor such as GM-CSF (granulocyte-monocyte-colony stimulating factor) and cytokines IL-1β, IL-6, TNF-α, and the extravasation of plasma constituents from the vascular space into the alveolar space. These events recruit and activate more neutrophils, macrophages, eosinophils, basophils, dendritic cells, as well as NKs at sites of infection and injury. Upon viral or bacterial infection, infected cells can undergo different cell death pathways, which includes apoptosis, necroptosis, pyroptosis and NETosis (Place et al., 2021). Apoptosis is a cell death program regulated by caspase-dependent pathways with low inflammatory response, in which the intracellular TNF receptor complex plays a central regulatory role. Necroptosis and pyroptosis are two highly inflammatory cell death programs mediated by caspase-8/receptor-interacting protein kinase (RIPK1/3)/Mixed lineage kinase domain-like protein (MLKL) and the caspase-1/5/7 and the gasdermin (GSDM) pathways, respectively (Place et al., 2021). However, at an early stage of infection, many bacteria (as examples *S. Aureus, L. pneumophila, M. tuberculosis, Yersnia*, etc) and viruses (rhinovirus, adenovirus, herpesvirus, and bocavirus) can either accelerate or inhibit a host cell survival and cell death pathways, via the expression effector and inhibitor proteins to enhance their replication and survival (Kitur et al., 2015; Jorgensen et al., 2017; Feng et al., 2022). Dead microbial, immune, endothelial and epithelial cells release a variety of endogenous molecules such as lipopolysaccharides, lipid A, peptidoglycans, ATP (adenosine triphosphate), HMGB1 (high-mobility group box 1), HSP (heat shock proteins), S100 proteins, antibiotic peptides, cytokines, chemokines and uric acid, collectively named pathogen-associated molecular pattern (PAMPs) and damage-associated molecular pattern (DAMPs) (Sangiuliano et al., 2014). These inflammatory mediators bind to specific TLRs, retinoic acid-inducible gene-I-like (RLRs) and NLS (NOD-like receptors) and activate the intracellular inflammasomes, as example: NLRP3 (NACHT-domain-leucine-rich-repeat-and-PYD-containing protein-3), leading the production of cytokines interleukins IL-1β and IL-18 (Broz and Dixit, 2016). Along the process known as NETosis (classical or suicidal), the release of so-called neutrophil extracellular traps (NETs) contributes to the development of complications of the lungs infectious diseases (Ebrahimi et al., 2018, Sauler et al., 2019). Aberrant NET production and cycles of neutrophilic mucosal inflammation have been reported in patients with asthma, cystic fibrosis and ARDS (Uddin et al., 2019). More studies need to be done to understand how lung NETosis contribute to CAP, ARDS, COPD, as well as bronchial asthma.

TLRs and NF-κB pathways play important roles in maintaining tissue homeostasis by regulating the inflammatory mediators' synthesis and tissue repair responses after injury, a process named resolution of inflammation (Barnig et al., 2018; Panigrahy et al., 2021). Macrophages display at least two activation or polarization states. M1 state, in which

they are pro-inflammatory and cytotoxic against microbes. M2 state, in which they play a pivotal role in wound healing and tissue remodeling. On one hand, M1 airway macrophages and dendritic efficiently phagocyte (efferocytosis) apoptotic epithelial cells, neutrophils, cellular debris, and microbes. In the other hand, M1 air macrophages via TLR4 and NFκB-dependent signaling induce cyclooxygenases (COX-1/2) expression that mediates the synthesis of prostanoids, including prostacyclin, thromboxanes, prostaglandins and leukotrienes and other early pro-inflammatory mediators derived of arachidonic acid metabolism. At resolution phase, M2 airway macrophages switch the production bioactive lipid mediators by activating the lipoxygenase (LOX)-catalyzed conversion of polyunsaturated fatty acids (omega-6 arachidonic acid, omega-3 docosahexaenoic acid and eicosapentaenoic acid) into specialized pro-resolving mediators (SPM): lipoxins, resolvins, protectins and maresins (Barnig et al., 2018; Panigrahy et al., 2021). These lipids mediators target many inflammatory cell types and inhibit their motility, granulation and production of cytokines while stimulate the synthesis of amphiregulin, which binds to the epithelial growth factor receptor (EGFR) to promotes proliferation of epithelial cells to regenerate damaged pulmonary tissue. Thus, the balance of anti-inflammatory and pro-inflammatory cytokines and lipids play a key role in modulating the immune response and tissue repair.

A diverse array of metabolic and biochemical indicators and biomarkers of airway dynamics, blood coagulation and inflammation have been used to monitor and predict the disease activities and stratification of patients at risk. Among of more reliable biomarkers, CRP (C-reactive protein), PCT (procalcitonin), IL-6, nCD64 (Neutrophil CD64 Receptor) and D-dimer (a product of fibrin degradation) are most frequently used. Other biomarkers that require further validation are as follows: NLRP3, NT-proBNP (N-terminal pro-B-type natriuretic peptide), and also various cytokines, chemokines, and acute-phase proteins (Karakioulaki and Stolz, 2019). Many other metabolic and biochemical mediators, including cytokines, chemokines, growth factors and plasma proteins are engaged in these multistep processes. Currently, research efforts are focused on delineating the molecular mechanisms that underlie the transition from acute to chronic lung inflammation, as well as elucidating how immune-related genetic variations influence susceptibility to pneumonia, how they could contribute to clinical outcomes.

4. Ventilator associated pneumonia (VAP)

Ventilator associated pneumonia is a modality that is best served by microbiome investigations (Zakharkina et al., 2017; Kitsios et al., 2018; Fenn et al., 2022). This is because it is common and serious among hospitalized patients, but also due to the fact that tracheobronchial secretions can be easily collected in this population, thus enabling microbial longitudinal studies. Indeed, the tracheal tube acts as a lifesaving device, providing easy and reliable connection to mechanical respirators, at the same time as it allows aspiration and analysis of accumulated secretions. Nevertheless, it dangerously disrupts lung physiology and ecology. Inspired air is not heated, humidified and partially filtered at the nose and upper airways, being promptly injected downstream. The tube itself behaves as a foreign body and irritant interfering with the efficiency of cough, and precipitating mucus accumulation, as well inflammation of delicate bronchoalveolar pathways. Moreover, most perivascular luid cuffs fail to prevent microaspiration of oropharyngeal and gastric secretions, which remarkably increase in sedated and curarized subjects. As a consequence, widespread contamination is almost inevitable, even though the mere presence of pathogens in swabs and collected samples does not demonstrate the occurrence of pneumonia, as will be discussed further on.

General microbiomic patterns in VAP have alpha and beta diversity almost always decrease in mechanically ventilated subjects, whose suffering could be attributed to substantial loss of potentially protective commensals. The colonization by reduced groups of higher pathogenic potential (dysbiosis), not uncommonly including Gram-negative Proteobacteria, presumably of gastrointestinal origin (Pettigrew et al., 2021; Rello et al., 2021). Thus, the standard of low bacterial density and high diversity of species is progressively disrupted. Of course, this is not just a result of tracheal intubation and mechanical ventilation. The use of antibiotics, and shifts in local ecology represented among others by fluctuations in mucus viscosity, tissue pH, and O_2 tension are believed to influence immune response, microbiome abundance and composition as well as susceptibility to pneumonia (Dickson et al., 2014).

Some advocate certain paradigm shifts. For instance, instead of a disease caused by a single bacterial pathogen acquired through microaspiration, the occurrence of VAP would underscore major lung dysbiosis, enhanced by immune aberrations and variable degrees of pathogen invasion (Rello et al., 2021). For others the emphasis should be on dysbiosis in the upper digestive tract, nominally oropharyngeal communities. This would pave the way for pathogen invasion and subsequent respiratory illness (Pettigrew et al., 2021). Still the new paradigms would not encompass abandoning traditional tracheal aspirates for diagnosis, even though microbial culture can be misleading (Fernández-Barat et al., 2020). Their role would be to complement the work up with metagenomic studies of the lung ecology, along with the upper gastrointestinal tract (Fernández-Barat et al., 2020). It is worth mentioning that all those changes (dysbiosis, DNA sequences consistent with multiple pathogens, and weakened immune response) may be detected in mechanically ventilated patients without evidence of VAP (Pettigrew et al., 2021). Interestingly some series failed to detect a damaging impact of systemic antibiotics on the respiratory microbiome pattern, only mechanical ventilation robustly interfering on its diversity (Zakharkina et al., 2017). In contrast, other investigators (Kitsios et al., 2018) do incriminate broad spectrum empiric antibiotics in lung ecology deterioration.

By the same token many authorities posit a role for the intestinal microbiome within the context of all bacterial pneumonias (Singhania et al., 2020; Ma et al., 2022). Yet aspiration or translocation of intestinal bacteria does not strictly depend on the presence or absence of intestinal dysbiosis, as aspiration is a mechanical phenomenon, which apparently affects all mechanically ventilated subjects, and even the non-intubated during sleep or sedation (Wheatley et al., 2022). Translocation in turn is typically linked to mucosa barrier dysfunction, a problem associated with dysbiosis as well as many other circumstances such as intestinal diseases, shock, burns, malnutrition and organ failures.

5. Immunodeficiency associated pneumonia

Immunodeficiency can be separated into three categories; those caused by neutrophil dysfunction; and those caused by humoral or cellular immunodeficiency (Soler-Palacín et al., 2018). Their primary origin is genetic, however, immunodeficiency can be acquired, as example, acquired immunodeficiency syndrome (AIDS) after infection with the human immunodeficiency virus (HIV). The diagnosis of severe respiratory infections in immunodeficient patients is very difficult and early antibiotic therapy is recommended but this decreases the chances of identifying the causative microorganism species (Azoulay et al., 2020). *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Haemophilus* spp., *Stenotrophomonas* spp., and methicillin-resistant *Staphylococcus aureus* are the most frequently causative pathogens in this population. Invasive fungal infection, as example Aspergillus, Mucorales, and *Pneumocystis jirovecii*, is almost always observed HIV-infected patients and those with systemic autoimmune diseases (Dunbar et al., 2020).

Immunodeficiency disorders also occur in patients receiving long-term (>3 months) high doses of steroids or other immunosuppressant drugs, after solid-organ transplant or chemotherapy for treating hematological neoplasia. Immunocompromised patients such as those affected post-hematopoietic stem cell transplantation (HSCT) pneumonia are a case in point. *Pseudomonas*, *Acinetobacter*, *Burkholderia*, and *Mycobacterium* tend to be frequently identified, and *Enterococcus* has been significantly associated with mortality in one series (He et al.,

2022a,b). Nominally for *Pseudomonas pneumonia* gut translocation is emphasized rather than usual aspiration of oropharyngeal contents. In this sense, interventions that reduce intestinal colonization by this Gram-negative have been advocated for prophylaxis. In contrast, previous prescription of carbamapenems such as meropenem for urinary infection triggered antibiotic resistance in one series (Wheatley et al., 2022). The herpes simplex viruses 1 and 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), and cytomegalovirus (CMV) are most frequent responsible for systemic viral infection in immunocompromised patients (Dunbar et al., 2020). New molecular biology tools such as real-time PCR (RT-PCR), transcriptomics, and next generation sequencing (NGS) are most reliable methods to identify rapidly co-infection of bacteria, fungi, and viruses in fluids and respiratory specimens from patients with severe pneumonia.

6. SARS-CoV-2-associated pneumonia

The major clinical and pathologic manifestation of severe COVID-19 mediated by SARS-CoV-2 infection of the lung is pneumonia. With the severity of disease, more than half the patients develop community-acquired pneumonia, ventilated-associated pneumonia and ARDS, as measured by standardized intensive care unit (ICU) predictive tools (Budinger et al., 2021). In patients with SARS-CoV-2 pneumonia, it is clinically indicated the analysis of BAL fluid with quantitative bacterial cultures, and bacterial and fungal multiplex PCR analyses as well as antigen detection to guide antimicrobial therapy. The bacterial species living in the upper respiratory tract, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*, among others, are commonly associated with bacterial co-infections or secondary bacterial infections in COVID-19 patients. The most common fungal infection reported are candidiasis, aspergillosis and *Pneumocystis jirovecii*.

Except for transplanted patients and other high risk immune deficient groups, viral pneumonia tends to be underdiagnosed and even overlooked in the general population. Not in the context of the SARS-CoV-2 pandemic, during which all of the world became appalled by the significant morbidity and mortality of advanced forms of the respiratory illness and its sequelae. Is the regional microbiome involved in the pathology? Kullberg et al. addressed a cohort of Covid-19 and ARDS patients. Elevated lung bacterial and fungal burden signaled difficulty for extubation and increased mortality (Kullberg et al., 2022). Overexpression of tumor necrosis factor-α and other cytokines followed microbial burden and similarly predicted mortality. In contrast subjects with less deranged respiratory microbiomes were more easily extubated and survived (Kullberg et al., 2022). Elevated levels of neutrophils in the blood of the patients infected with SARS-CoV-2 indicate severity of the disease and poor prognosis (Zhu et al., 2022). The increased level of NETosis as detected by the release of biomarkers: circulating cell-free DNA, myeloperoxidase and neutrophil elastase-DNA complexes and citrullinated histone H3, have been associated coagulopathy and immunothrombosis found in blood samples from COVID-19 patients (Zhu et al., 2022). These findings did not entirely coincide with those of Sulaiman et al. pointing towards an unfavorable outcome in mechanically ventilated COVID-19 cases fundamentally precipitated by the primary disease, namely heavy viral burden and weak antibody response against SARS-CoV-2. Still a change in the bronchoalveolar bacterial microbiome, more specifically abundance of the oral commensal *Mycoplasma salivarium*, was endowed with an ominous prognosis. Thus, these authors are in favor of a microbiome survey as well (Sulaiman et al., 2021).

Bubonic plague epidemics devastated Medieval Europe (the famous black death) and are still endemic in certain parts of the world. The pneumonic variety of plague has not been historically registered and is usually classified as an emergent respiratory pathogen (Zimbler et al., 2015). Severe forms are classically associated with a marked cytokine release syndrome which seems to precipitate mortality (Pechous et al.,

2013; Zimbler et al., 2015). The clinical course is fulminant, with nearly 100% fatality rate, and the same pattern of cytokine storm, severe tissue destruction and organ failures is recorded (Pechous et al., 2013). Certain viral pneumonias such as those induced by SARS-CoV-2 have been connected to cytokine storm, which seem to be intrinsic to the poor evolution of severe cases (Fajgenbaum and June 2020). Does the same phenomenon occur in conventional pneumonia and is it a marker of ominous prognosis? In tuberculosis pneumonia, increased expression of cytokines is announced in comparison to community acquired pneumonia and lung cancer, and correlated with the pattern of respiratory dysbiosis in this population. However prognostic implications are not anticipated (Xia et al., 2022; Zhang et al., 2022). This type of pneumonia is also comparatively rare in Western countries.

SARS-CoV-2 infection triggers the expression various cytokines including IL-1, IL-6, and TNF-α, and consequently the NF-κB, JAK/STAT3 kinases and mTOR-related pathways. Thus, some of biological immunotherapies, mainly monoclonal antibodies, have been evaluated aiming at regulating the excessive immune response seen in SARS-CoV-2 patients with severe infection. The two classes of IL-6 inhibitors, the monoclonal antibodies against the IL-6 receptor, including sarilumab, tocilizumab, clazakizumab, olokizumab and the anti-IL-6 monoclonal antibody siltuximab, were investigated in various clinical trials and no clear beneficial were found (Baracaldo-Santamaría et al., 2022). In addition, it was reported that the treatment with these antibodies caused acute pneumonitis, idiopathic pulmonary fibrosis, and exacerbation of rheumatoid arthritis-associated interstitial lung disease in a fraction of patients. Baricitinib, an inhibitor JAK (Janus kinase), known to reduce IFN signaling in alveolar macrophages, was approved as an alternative treatment option to manage patients with respiratory insufficiency and hyperinflammation (Lin et al., 2022). We are still learning whether or not the current Covid-19 vaccines prevent SARS-CoV-2 infection and help patients from rapid recovery of respiratory illness.

7. Acute respiratory distress syndrome (ARDS)

ARDS is a heterogeneous syndrome with variable clinical and pathological features. Patients may present with different phenotypes or endotypes based on clinical and physiological characteristics or response to treatment (Huang et al., 2017; Matthay et al., 2019; Reilly et al., 2019). According to the 2012 Berlin definition, ARDS is a syndrome caused by the acute onset of hypoxia (poor oxygenation) and bilateral pulmonary opacities not fully explained by a cardiac cause (Fergenson et al., 2014). ARDS is defined by T PaO_2/FiO_2 ratio that is determined by the patient's oxygen in arterial blood (PaO_2) to the fraction of the oxygen in the inspired air (FiO_2). The clinical disorders associated with ARDS including smoke inhalation, acute exacerbations of interstitial lung disease, sepsis, and primary graft dysfunction following lung transplantation, among other causes. Based on underlying pathological mechanism, ARDS syndrome is commonly described in phenotypes and subphenotypes Matthay et al., 2020. Hypoxic phenotype is characterized by severe hypoxemia and respiratory failure, often requiring mechanical ventilation with high levels of positive end-expiratory pressure (PEEP) to maintain adequate oxygenation. Patients with this phenotype often have diffuse alveolar damage and increased lung injury. Hyperinflammatory phenotype is characterized by a robust inflammatory response, with elevated levels of pro-inflammatory cytokines and chemokines in the plasma and bronchoalveolar lavage fluid. Patients with this phenotype may have a more severe illness periods and a higher mortality rate. Reactive phenotype is characterized by much more mild forms of lung injury, with a lower degree of hypoxia and less diffuse lung involvement. Patients with this phenotype may have a better prognosis and will respond well to lower levels of PEEP. Unresolved phenotype is characterized by persistent hypoxia and lung injury despite aggressive treatment, including mechanical ventilation with high levels of PEEP, prone positioning, and neuromuscular blockade. Patients with this phenotype may have a

higher mortality rate and may require extracorporeal membrane oxygenation (ECMO) or lung transplantation (Matthay et al., 2020). In addition to clinical physiological and radiographic imaging information, each biologic phenotype is evaluated based on BALF and plasma protein biomarkers, differential gene expression, and the presence of either bacterial, viral or fungal infection, as well the presence of co-morbid conditions (Matthay et al., 2020). The evolution of ARDS phenotypes and endotypes is differentiated using distinct clinical, radiologic, plasma and biological bio-markers. Potential useful and relevant biologically ARDS biomarker are angiopoietin-2 (Ang-2), von Willebrand factor (VWF), surfactant protein D (SP-D), IL-6, interferon-γ, plasminogen activator inhibitor-1, the receptor for advanced glycation end-products (RAGE), and many other putative candidates.

Multiple biologic pathways and gene signatures have been implicated in ARDS (Lynn et al., 2019). The pathological process develops from an acute-onset of tissue hypoxia that is followed by lung infiltration, diffuse alveolar damage, chaotic inflammation, activated coagulation, pulmonary vascular microthrombosis and lung fibrosis. In ARDS, the increased permeability to liquid and protein across the lung endothelium leads to edema in the lung interstitium (Lynn et al., 2019; Matthay et al., 2019). The edematous fluid then moves to the alveoli causing injury to normally tight barrier and alveolar epithelium of the lung. The epithelium can be injured directly, for example, by bacterial products, viruses, organic acid, oxygen toxicity (hyperoxia), hypoxia and mechanical forces, or by inflammatory cells or their products, as seen in sepsis (Lynn et al., 2019; Matthay et al., 2019). The activation of the complement system, recruitment and activation of immune cells such as neutrophils and macrophages, and the release of cytokines and chemokines such as IL-6, IL-8, and TNF-α are some of the molecular events that contribute to the systemic inflammatory response in ARDS. As an inflammatory syndrome generally requiring intubation and mechanical ventilation, it may or may not be followed by pneumonia. ARDS patients in ICU, with severe trauma or nosocomial infection (mainly sepsis), have very poor outcomes.

PaO_2/FiO_2 ratio is commonly used to classify physiologically patients into mild, moderate, or severe phenotypes and to determine the supportive management of mechanically ventilated patients (Ferguson et al., 2012; Matthay et al., 2020). The major treatment strategy in ICU is focused on increasing oxygen delivery. One comparatively common feature in severe forms of the ARDS syndrome is oxygen toxicity, potentially further injuring lung parenchyma. Experimental findings in both germ-free and conventional mice suggest that deleterious shifts in the alveolar-capillary interface, induced by high fractions of inspired oxygen, could be mediated by the lung microbiome (Ashley et al., 2020). Germe-free mice were protected by hyperoxia-induced growth on oxygen-tolerant respiratory microbial species, including *Staphylococcus aureus*. Qualitative and quantitative alterations in bacterial communities could be induced as early as 24 h after hyperoxia exposure and these communities could contribute to lung injury (Ashley et al., 2020). Indexes and biomarkers of such phenomena are mostly lacking in clinical populations, and relevant investigations have been emphasized, aiming to enhance the definition of lung injury phenotypes and clinical outcomes (Matthay et al., 2020).

Imbalance of the microbiome is prominent in ARDS, especially concerning robust enrichment by gut-associated bacteria of the Enterobacteriaceae family such as Gram-negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, as well as Gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, many of them found in the respiratory niches (Panzer et al., 2018; Dickson et al., 2020). Respiratory viruses such as influenza virus, coronavirus (including SARS-CoV-2), respiratory syncytial virus, adenovirus, and herpes simplex virus, as well as *Mycoplasma* spp., *Chlamydia pneumoniae* and *Bordetella pertussis* can also cause ARDS. It is important to mention that the use of all invasive sampling procedures for respiratory specimen collection, including bronchoalveolar lavage, have failed to give exact identification of pathogens in some patients. The percentage of patients with ARDS with no identified organisms can be higher than 50%. The NGS is a promise tool to precisely diagnose lung infection in ARDS patients.

Despite the dearth of well-designed randomized trials, useful retrospective observations are progressively emerging. In a series addressing sepsis-induced ARDS, patients were divided according to infection location (Zhang et al., 2022). There were 111 patients with intrapulmonary (Group I ARDSp) and 45 with extrapulmonary infections (Group II ARDSexp), along with 28 controls (Group III). In Group I ARDSp was associated with reduced pulmonary microbiome diversity, contrasting with Group II ARDSexp in which diversity increased, mainly on account of conditionally pathogenic bacteria and intestinal microbes. Death was associated with elevation of *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, enteric microbes, or conditional pathogens in Group I ARDSp, however just with Bilophila in Group II ARDSexp. Evidence in favor of a protective impact for Hydrobacter was detected in Group I ARDSp (Zhang et al., 2022). Investigations on the relationship between the lung microbiome and the evolution of ARDS phenotypes is still in its early stages.

8. Chronic obstructive pulmonary disease (COPD)

COPD is a chronic lung disease that is caused by long-term exposure to irritants that damage the lungs and airways, leading to inflammation and obstruction of airflow (Christenson et al., 2022). The primary cause of COPD is tobacco smoke, which is responsible for approximately 85–90% of cases. Other risk factors for COPD include exposure to air pollution, occupational dust and chemicals, and genetic factors. COPD is a progressive disease that worsens over time, leading to symptoms such as shortness of breath, coughing, wheezing, and chest tightness, and can significantly reduce the quality of life, being one of the leading causes of death worldwide. Research has shown that changes in the lung microbiome can contribute to the development and progression of COPD (Wang et al., 2016). Studies have found that individuals with COPD have a different lung microbiome compared to healthy individuals. In particular, there is an increase in the abundance of potentially harmful bacteria, such as *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Haemophilus influenzae*, in the lungs of individuals with COPD. There are many factors and changes that can lead to chronic inflammation and damage to the airways, exacerbating COPD symptoms. The gut microbiome is the main enhancer of innate host immunity against infections via regulation of and differentiation of Th17 T cells that secrete cytokines (IL-17 and Il-22) that promote neutrophil accumulation, changes in barrier function, and inflammation. A complex interplay of viral-bacterial coinfection, deficient host response to bacteria, and the gut and lung microbial changes cause gradual exacerbation and severity of COPD (Ritchie and Wedzicha, 2020; Chiu et al., 2022). Studies suggested that exacerbations result from dysbiosis caused by changes in preexisting bacterial composition, with an increase in Proteobacteria and a decrease in Actinobacteria, Clostridia, and Bacteroidia (Wang et al., 2016; Chiu et al., 2022). Both steroids and antibiotics treatment can promote an enrichment of multiple taxa, including members of Proteobacteria and reduction of Bacteroidetes and Firmicutes (Wang et al., 2016; Wheatley et al., 2022). The network analyses revealed that several sputum biomarkers, including sputum interleukin (IL)-8/chemokine (C-X-C motif) ligand (CXCL)8, matrix metalloproteinase (MMP)-7 and MMP-were negatively correlated with the increase of *Haemophilus*, *Moraxella* and *Streptococcus* species during COPD exacerbation (Wang et al., 2016). A recent study demonstrated that a lipopolysaccharide compound derived from a gut commensal bacterium *Parabacteroides goldsteinii*, by acting as an antagonist of TLR4 signaling pathway, is anti-inflammatory and significantly ameliorates COPD (Lai et al., 2022). Clinical interventions aimed at modulating the lung microbiome, such as antibiotic treatment or probiotic supplementation, may have potential as a therapeutic strategy for COPD. However, more

research is needed to fully understand the complex interactions between the gut and lung microbiomes and COPD. Future development of effective treatments depend on this understanding.

9. Conclusions and research targets

Elucidating the functional virome, mycobiome and microbiomes poses several methodological challenges with significant promises for delineating novel microbiome-oriented strategies for preventing and treating respiratory diseases. Many viruses and bacterial pathogens, especially those capable of invading and replicating intracellularly, have undertaken evolutionary changes to colonize the microbiomes in the exterior and interior of the human body. They interact with the host's cells through an enduring mutualistic partnership with the immune system and conserved metabolic signaling. How site specific-microbiome profiling and characterization of those resident microbiomes can help predict and monitor lung diseases? Much more standardized approaches, analytic methods, and reliable and reproducible datasets are needed for identification of key genera and species differences in longitudinal microbiome studies. We propose some directions for future research, as it follows.

- Which is the most reliable method(s) to assess respiratory tract microbiome: oral swabs, pharyngeal secretions, sputum, endotracheal aspirate (ETA), bronchoalveolar lavage fluid (BAL), microlavage (mBAL), bronchoscopy aspiration, plasma samples?
- How does one define the normal respiratory tract microbiome, with quantitative and qualitative cut-off points for dysbiosis? Is the approach defended by some investigators acceptable, in the sense that dysbiosis coincides with low alpha and beta diversity and less abundance of protective oral-origin commensal bacteria? Which limits should be considered?
- Specifically for pneumonia (community acquired, nosocomial and other variants), pathogen cut-off points are essential, as standard microbiome sequencing might elicit many potential agents not necessarily involved in lung disease. Does over 50% relative abundance or a massive pathogen burden as anticipated by some (4, 20) implicate an etiological role?
- Is a sharper distinction between the respiratory microbiomes possible, in such diseases as acute respiratory distress syndrome, community acquired pneumonia, ventilator associated pneumonia, and pneumonia in immunodeficient populations?
- What are the implications of identification of Candida spp (and other yeasts) in tracheal and alveolar fluids? Should a deleterious association with bacterial pneumonia be suspected? Is an antifungal intervention warranted?
- What are the impact of antibiotics or steroids on bacterial and fungal composition and richness of microbiome? What are airway microbiomes that predispose to a disease and then to recurrent exacerbations?
- Novel efficacious mucosal vaccines to respiratory viruses, including respiratory syncytial virus, coronaviruses, paramyxoviruses, rhinoviruses and respiratory enteroviruses might be the best solution to prevent both the upper and lower respiratory tract diseases.

Authors' contribution

José E Belizário: Conceptualization, design, literature paper selection, Funding acquisition, data acquisition, Writing – original draft, Writing – review & editing, drafting and editing of the manuscript. Joel Faintuch: Conceptualization, design, literature paper selection, Funding acquisition, data acquisition, Writing – original draft, Writing – review & editing, drafting and editing of the manuscript. Miguel G. Malpartida: Reviewing and comments and final approval of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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