Review

Extracellular Vesicles in Airway Homeostasis and Pathophysiology

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Abstract: The epithelial–mesenchymal trophic unit (EMTU) is a morphofunctional entity involved in the maintenance of the homeostasis of airways as well as in the pathogenesis of several diseases, including asthma and chronic obstructive pulmonary disease (COPD). The "muco-microbiotic layer" (MML) is the innermost layer of airways made by microbiota elements (bacteria, viruses, archaea and fungi) and the surrounding mucous matrix. The MML homeostasis is also crucial for maintaining the healthy status of organs and its alteration is at the basis of airway disorders. Nanovesicles produced by EMTU and MML elements are probably the most important tool of communication among the different cell types, including inflammatory ones. How nanovesicles produced by EMTU and MML may affect the airway integrity, leading to the onset of asthma and COPD, as well as their putative use in therapy will be discussed here.

Keywords: asthma; chronic obstructive pulmonary disease; COPD; epithelial–mesenchymal trophic unit; muco-microbiotic layer; nanovesicles; exosomes; outer membrane vesicles; microbiota

1. The Epithelial–Mesenchymal Trophic Unit and the Muco-Microbiotic Layer: Definition, Composition and Functions

Along all the lower airways, except for specific portions, the innermost and proximal layer to the lumen is composed of respiratory mucosa. From a strictly anatomical point of view, in the respiratory mucosa, the outermost layer is made up of a pseudostratified epithelium with mainly goblet and ciliated cells that lie on a basal membrane, below which there is a connective tissue layer with various cells (including fibroblasts, myofibroblasts and immune cells) of mesenchymal origin interspersed in an extracellular matrix (ECM). However, from a morphofunctional point of view, the apical epithelial tissue and the underlying connective tissue within the respiratory mucosa cannot be considered as single, separate entities of their own. Indeed, at the end of the last century, the work of Plopper and Evans focused on the close interconnection between epithelial and mesenchymal cells, providing the basis for the creation of the "epithelial–mesenchymal trophic unit" (EMTU) concept [1]. The role of the interactions between epithelial and mesenchymal elements, although known for some decades at the time of the studies of Evans and Plopper, had been exclusively relegated to airway organogenesis, erroneously assuming that this interaction was limited to intrauterine life [2]. On the contrary, the several cellular and non-cellular components of EMTU in the adult life of individuals are interconnected by

a close communicative relationship that influences many other physiological and pathophysiological aspects, such as cell differentiation, tissue homeostasis, organ remodeling, reparative/regenerative processes, response to external/internal stress stimuli and participation in inflammation/autoimmunity. EMTU processes alterations even contribute to the pathogenesis of some chronic diseases of the airways, e.g., chronic obstructive pulmonary disease (COPD) and asthma [3–5].

Focusing exclusively on the structural components of EMTU, it must be emphasized that, along with its cellular components, the ECM, synthesized mainly by fibroblasts, also plays a very important role [6]. EMTU homeostasis is frequently influenced by the differences in the lymphocyte population as well as by several soluble factors and nanovesicles dispersed in the ECM that determine the outcome of reparative/regenerative processes and the establishment of pathophysiological states [7,8]. Indeed, it has already been demonstrated that variations in the composition of ECM are the basis of asthma and COPD pathogenesis [9–11].

Furthermore, many microbes reside in the mucus constantly produced by goblet cells, constituting the airways' microbiota. Nowadays, we know that this is another fundamental component for EMTU homeostasis. In the gastrointestinal tract, we already proposed the term "muco-microbiotic layer" (MML)—made by microbiota elements (bacteria, viruses, archaea and fungi) interspersed in a mucous matrix—to describe the innermost layer of the intestinal wall. The MML homeostasis is crucial for maintaining the healthy status of these organs and whose alteration is at the basis of gastrointestinal disorders [12]. An MML is also present in airways and, as in the gastrointestinal tract, it takes part in the homeostasis as well as in the pathogenesis of these organs. It is fundamental now to better characterize the constitutive elements of this MML in terms not only of microorganisms that populate it but also of nanovesicles (e.g., exosomes, microvesicles or outer membrane vesicles) that participate in the crosstalk among cells.

We want to stress here that a crucial element for the interchange of information between EMTU and MML constituents is the trafficking mediated by nanovesicles produced by both parts and called, respectively, exosomes and outer membrane vesicles (OMV). The aim of this paper is to properly highlight the roles that these nanovesicles have in airway homeostasis and disease pathophysiology, i.e., asthma and COPD.

2. Nanovesicles: Exosomes and Outer Membrane Vesicles

The paracrine communication system regulated by extracellular vesicles (EVs) and exosomes plays a key role in the communication of the EMTU, in the maintenance of tissue homeostasis of the airways as well as in many pathogenetic processes affecting the apparatus [13]. The intimate interconnection that interfaces the epithelial layer to the connective layer, synching them in their biological activities, is favored by the vesicular system that traffics nucleic acids (miRNA, siRNA) [14], growth factors, tissue-specific receptors as well as proteases [15].

As with Evans and Plopper's EMTU studies, extracellular vesicles and exosomes have long been erroneously considered as results of "garbage disposal" whose sole purpose was to eliminate waste substances from cells [16,17]. They are actively secreted by all eukaryotic and prokaryotic cells and are part of an articulated cell-to-cell communication system, both in physiological and pathological conditions [18]. In recent decades, several scientific works demonstrated the roles of EVs in a plethora of physiological processes. EVs are involved in cellular homeostasis and signaling. They can act as carriers of an enormous variety of molecules, and they express numerous signal proteins on their surface [19].

EVs are classified according to their size as (1) microvesicles (100–1000 nm in diameter); (2) apoptotic blebs (1000–5000 nm in diameter); and (3) exosomes (diameter 20–150 nm) [20].

On one side, microvesicles and apoptotic blebs originate from the outward budding of the cell membrane, unlike exosomes, which result from the invagination of endosomal membranes [21]. The understanding of the different originative pathways and the sorting

mechanisms spotlighting transport, cargo packing and vesicle exocytosis find practical utility in the isolation studies of EVs for diagnostic and therapeutic purposes.

Therefore, the role of the proteins belonging to the endosomal sorting complex required for transport (ESCRT -0, -I, -II and -III), responsible for the control of the biogenetic and cargo loading processes of the EVs, is crucial [22].

As previously mentioned, the load of the EVs is tissue-specific and related to the function they can perform, i.e., EVs produced by tumor cells have a decisive impact on paracrine signaling mechanisms in support of tumor growth [23–25]; however, under physiological conditions, other EVs perform diametrically opposite roles, such as protection from traumatic tissue events or promoting the tissue healing itself [26].

A determining example might be the EVs produced in the lung microenvironment [26]. In homeostasis conditions, a broad range of cell types, such as fibroblasts, epithelial cells and endothelial cells, also actively secrete EVs: those EVs have been largely characterized, showing that epithelial cells are the main characters on the production of EVs, enriched with secretory and membrane-anchored mucins, which contribute to the mucociliary defense and boosting of innate immune defenses [27].

Not only epithelial cells but also macrophages that are present in BAL fluid play a pivotal role in the inflammatory modulation. It has been demonstrated that alveolar macrophages secrete SOCS-1 and -3 within nanoparticles, which are uptaken by lung epithelial cells. Both SOCS-1 and -3 are negative modulators of cytokine 1 and 3 biogenesis (through the STAT pathway inhibition) [28]. In normal conditions, this can modulate the inflammatory response, but at the same time, this negative modulation of IL-1 and -3 biogenesis seems to be lost in cigarette-smoking subjects, presenting a new model for the control of inflammatory response during inflammation or stress tissue [28].

Thus, with the importance of pulmonary EVs in maintaining homeostasis being confirmed, it is not difficult to think how dysregulations in this sense are closely related to the pathogenesis of various lung diseases [29]. In the following paragraphs, the relationships between microvesicles and chronic diseases of the respiratory system will be analyzed.

3. Asthma and Nanovesicles in Asthma Pathogenesis

Asthma is a chronic respiratory disease that presents several phenotypes. The global cases of asthma are estimated to be over 300 million by the World Health Organization (WHO) [30]. Asthma is commonly considered a childhood-onset disease; however, adults also can develop asthma later in life. Asthma is caused by both environmental factors such as house dust mites (HDM), particulate matter (PM), cigarette smoke (CS) [31] and genetic factors, among which allele 17q21 is the most studied [32]. Overall, the development of the disease is defined by increased mucus production, thickening of the subepithelial reticular basement membrane (RBM) of the lung mucosa, airway hyperresponsiveness and chronic inflammation [Figure 1]. All these events determine airway remodeling, which leads to the narrowing of the airways [31].

Recently, due to their high therapeutic potential, the emerging role of extracellular vesicles has been investigated in association with asthma pathogenesis [33,34]. Within the respiratory system, several cell types are involved in the release of EVs: for instance, structural cells such as epithelial cells and fibroblasts [35,36], resident immune cells such as dendritic cells (DCs) and alveolar macrophages (AMs) [28,37] as well as recruited immune cells such as eosinophils [38]. The major producer of EVs within the lung are airway epithelial cells [35]. On their membrane, epithelial cell-derived EVs expose several mucins that can neutralize virus and bacteria [27]. Furthermore, it has been shown that EVs produced by epithelial cells can trigger the proliferation of macrophages upon IL-13 release by eosinophils, thereby promoting chronic inflammation [35].

A study by Bartel and colleagues [39] revealed the role in asthma pathogenesis of miRNAs present in epithelial-derived EVs. miR-34a, miR-92b, and miR-210 in EVs can potentially lead to Th2 responses and maturation of DCs in the early development of asthma. Aberrant deposition of extracellular matrix contributes to the RMB thickening

in asthma pathogenesis. On their surface, EVs secreted by fibroblasts expose fibronectin, which can trigger invasion-associated signaling pathways [40]. An in vitro study showed that exosomes produced by the fibroblasts of severe asthmatics contain a low level of TGF2, which inhibits epithelial cells proliferation, contributing to the narrowing of the airways [37]. In order to trigger an allergic response, allergens must be presented to T cells by DCs. In humans, it has been shown how DC-derived exosomes can directly present antigen to T cells [41].

In mice, exosomes, rather than microvesicles (MVs), overexpress allergens leading to the activation of allergen-specific T cells [37]. These findings highlight an important modulatory function of DC-derived exosomes in allergic responses. As epithelial cells, macrophages are a major source of EVs that display multiple functions. Macrophage-derived EVs can promote macrophage differentiation [42] via mRNA-223. During infections, both bacteria and macrophages release MVs, which have a strong proinflammatory effect. At the same time, these MVs can also induce tolerance and promote bacterial shedding [43]. EVs produced by macrophages can also activate toll-like receptors (TLRs) during infection as they contain heat-shock protein 70 (HSP70), which mediates the activation of nuclear factor kB (NFkB) [26].
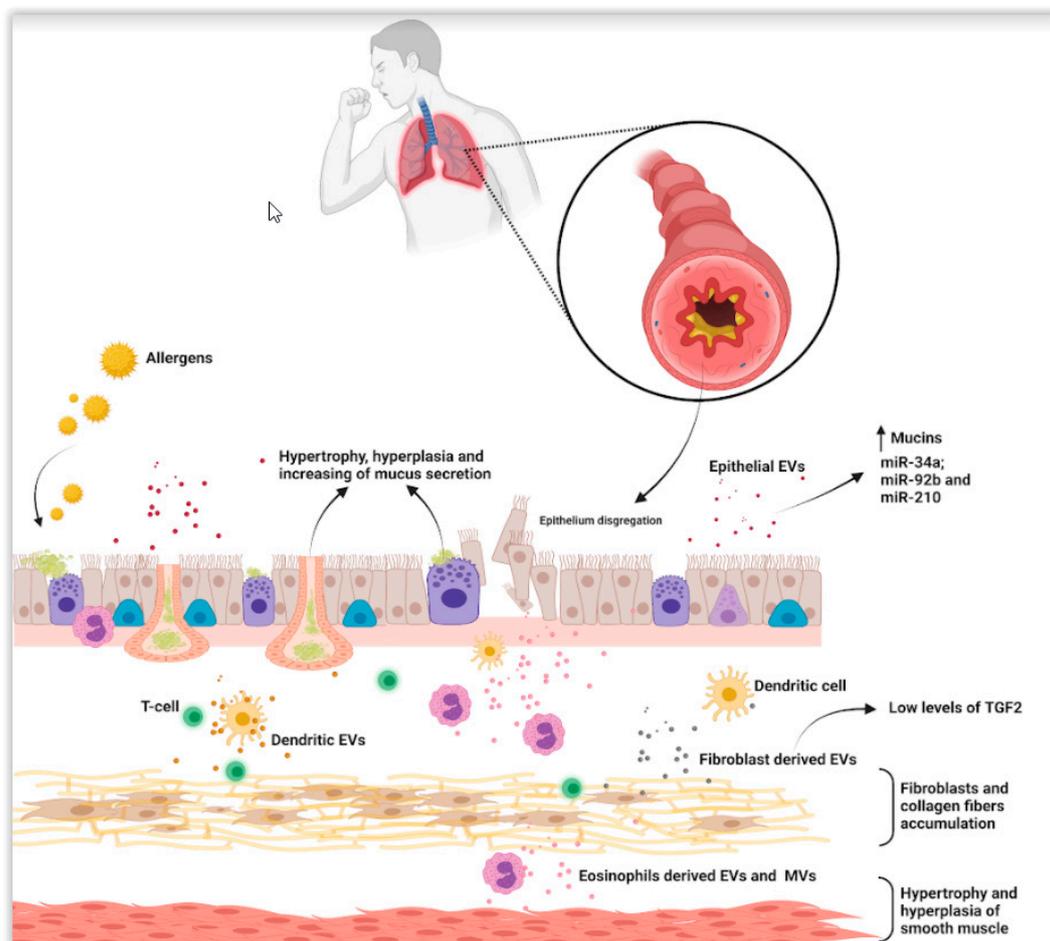

Figure 1. Scheme of allergic asthma pathogenesis. Allergic asthma is triggered by inhaling allergens. (Allergens could have different origins such as dust mites, pet dander, pollen or mold.) During the different phases and chronicization of the pathology, the tissues undergo several modifications. Among these include hyperplasia, hypertrophy of mucous cells and an increase of their secretions, continuity loss of the epithelium and variation in EV content. The extracellular vesicular release appears incremented, modified and responsible for the alterations that occurred.

Eosinophils play a major role in allergic asthma exacerbations [31]. Besides the release of potent Th2 cytokines, eosinophils release both MVs and exosomes [44]. Multiple

effects of eosinophil-derived exosomes have been observed on epithelial cells and smooth muscle cells (SMCs) [45]. In asthmatic patients, these exosomes interfere with epithelial cells, wound healing and SMC proliferation. This novel research on extracellular vesicles, microvesicles and exosomes indicate that future therapies must target these components in the prevention of asthma pathogenesis.

4. Chronic Obstructive Pulmonary Disease and Nanovesicles in Its Pathogenesis

Patients with chronic obstructive pulmonary disease (COPD) face a progressive limitation in airway function. The pathology has remarkable facets and different levels of severity. Several forms of COPD have been studied and described, with a classification that reports the distinct faceting of the pathology [46,47]. The establishment of this chronic disease is due to multiple causes, although it is closely associated with the inhalation of tobacco smoke and other environmental contaminants [48,49] [Figure 2]. Several mechanisms behind COPD pathogenesis have been studied, but we are still a long way from solving the puzzle in its entirety. The number of treatments on a personal basis developed in recent years is constantly increasing, trying to counteract the effects of COPD [50–52]. Despite the progress in therapies, the settled pathology is usually connected with a condition of irreversibility. Therefore, it is even more strategic to understand any biological pathway that participates in the disease onset and its maintenance.

The onset of COPD was recently found to be closely associated with the biological airway senescence process [53]. An increase in the release of exosomes was found precisely during senescence. A variation in the molecules contained within these extracellular vesicles was observed [54]. The habits of a patient suffering from COPD can also greatly influence pathology development by directly affecting the different EVs. For example, cigarette smoke leads to a massive release of exosomes by mononucleated cells and, as a consequence, IL-8 production by the respiratory epithelium increases. The result is the construction of a microenvironment ideal for a widespread inflammatory state [55]. In particular, Fujita's group reported an increased expression of miR-210 within exosomes released by bronchial epithelial cells in smokers [56]. The direct consequence of this miR-210 overexpression is a variation in the number of myofibroblasts within the airways. This variation is due to the suppression of the ATG7 pathway biologically implicated in their autophagy phenomena. The efficiency of exosomes in transmitting long-distance messages is, in this case, a double-edged sword. CS acts on epithelial cells by upregulating CCN1 expression in exosomes. These exosomes are now able to spread inflammatory states to other distant portions of the airways as well [57]. However, CCN1, as a result of chronic exposure to cigarette smoke, is released directly into the bronchial fluids (in a truncated isoform). Extracellular matrix degradation and increased cell death are a direct consequence of this abnormal release of CCN1 outside exosomes [58]. The variation in COPD status from stable to exacerbated is also associated with a variation in exosome release. The variation in COPD status is also associated with a variation in exosome release [59]. Exosomes with CD31, CD62E and CD144 were notably reduced in stable patients than in patients with COPD exacerbation [60]. A further fascinating correlation concerns the forced expiratory volume in 1 s (FEV) and the number of exosomes present in sputum [61]. In recent years, the number of miRNAs with an assigned biomarker role for COPD is constantly increasing. miR-203, miR-4455, miR4785, miR-218-5p, miR-29c and miR-126 were analyzed by different research groups, and for each of them, a variation in patients with COPD was found. This discovery makes the miRNAs potential biomarker candidates for a more precise diagnosis and progression of COPD [62–65].

The interaction between the immune system and pathogens plays a key role in COPD as in many other complex multi-factor pathologies. Recently, the focus has also been on the EVs released by pathogenic bacteria and not only on bacteria per se. The set of extracellular vesicles released by pathogens during infection is, today, one of the mechanisms used to reveal different parameters (type of pathogen, state of infection, etc.) [66,67]. In COPD subjects, analysis of EVs showed a different lung microbiome distinct from "standard"

microbiomes usually present in healthy lung tissue [67]. Outer membrane vesicles (OMVs) released by Gram-negative bacteria, for example, contain several molecules such as LPS, invasion proteins, adhesion proteins, and immunomodulatory factors [68]. Augustyniak et al. showed the potent proinflammatory effects of *Moraxella catarrhalis* OMVs in COPD. Briefly, they demonstrated how OMVs promote an inflammatory state by activating neutrophil degranulation and modifying the activation of the hBD-2 promoter in epithelial cells. An initial in vitro treatment was successfully carried out to counteract the interactions of OMVs with neutrophils and epithelial cells. This treatment is a clear example of how increasing knowledge in this specific area could, in the future, bring significant benefits to those with COPD [69]. An additional level of complexity is given by respiratory viruses that have evolved to use EVs as means of transport to spread inside the organism [70,71]. Even viruses, like bacteria, are a source of COPD exacerbation and it is crucial to monitor any viral infections to limit the course of the disease. Recent work from Roffel and her team demonstrated the role of miR-223 on the regulation of several gene expressions, providing a further example of the therapeutic potential associated with the study of different EVs [72].
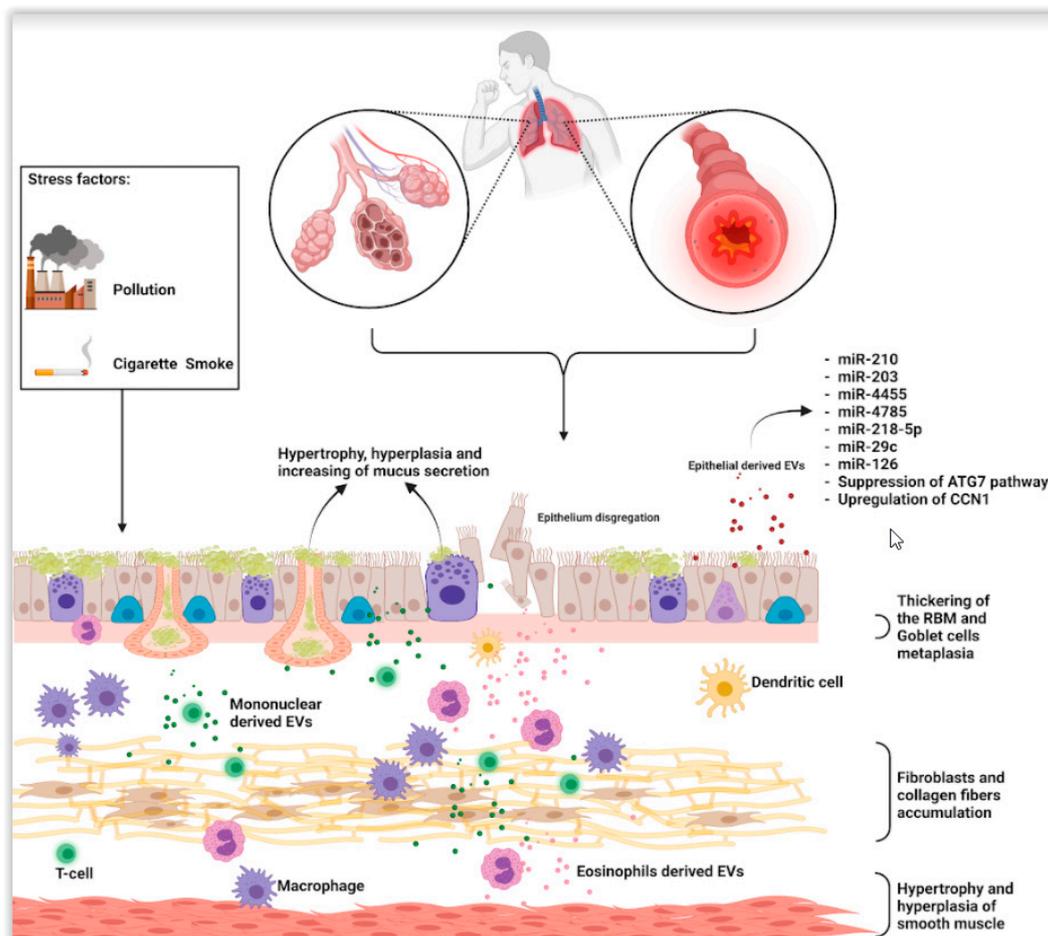

Figure 2. Scheme of COPD pathogenesis. Chronic external stimuli generate massive deregulation of lower airways. Chronic external stimuli generate massive deregulation or lower airways. EVs mediate these tissue changes directly and indirectly. Their production is also increased by the perennially present phlogistic status established with the progress of the pathology. The immune cell component contributes greatly to the release of EVs that affect all cell populations of the respiratory mucosa.

5. Microbiome Extracellular Vesicles and Chronic Respiratory Diseases

Human cells are not the only ones to produce microvesicles that affect the status of the respiratory system. Above the respiratory mucosa is a mucous layer in which all the microorganisms that make up the airway microbiota are settled [73]. The main

communication pathway between the cells of our body and the microbiota is via EV [18]. Communication via EV guarantees an interaction between host and microbiome without direct contact and in a bidirectional manner. This interaction is physiologically present in a state of health, and its alteration can trigger pathogenetic processes [74]. Commensal bacterial species that constitute our microbiome are not the only EV users, but also the pathogenic infectious species could exploit this communication pathway [75]. Among the most significant aspects affected by EVs is that of immunomodulation [76,77]. One of the most studied interactions is between *Pseudomonas aeruginosa* and airway epithelial cells. *P. aeruginosa* releases outer membrane vesicles (OMVs) in the mucus layer; OMVs fuse with cellular membranes on the epithelial cells apical side and deliver a 23-nucleotide tRNA (sRNA-52320). sRNA-2320 reduces IL-8 secretion and the migration of neutrophils into the lungs and suppresses the immune response to bacterial infection by targeting several genes in the LPS-stimulated MAPK signaling pathway [76,78,79]. In recent years, specific interactions between microbiome EVs and chronic diseases have been shown. In patients with asthma, *Sphingomonas, Akkermansia, Methylophaga, Acidocella*, and *Marinobacter* were significantly more abundant. It is interesting to note how this notion was obtained indirectly by analyzing the EVs released by these bacterial species. Thus, microvesicles can be used for diagnostic purposes (in integration with other specific examinations) [80]. Urine-released microbial extracellular vesicles can be potential and novel biomarkers for chronic respiratory diseases. The analysis of EVs is, in fact, also possible through an investigation of the urine of patients [81]. By this analytic method, the different microbiome composition of asthmatic patients has been detected. The diversity also appears to correlate with IgE levels and eosinophil % [82]. These findings suggest that they may play important roles in allergic-based airway diseases. The analysis and monitoring of COPD also benefit EVs studies. Altered miRNA profiles in COPD have been discovered analyzing EVs. Sundar et al. used different EV isolation and purification methods to characterize the plasma-derived EV miRNAs from nonsmokers, smokers, and patients with COPD [83]. They analyzed plasma-derived EVs from smokers, nonsmokers and patients with COPD, discovering how EVs vary in their dimensions, distribution, concentration and phenotypic characteristics. They concluded that plasma-derived EV miRNAs are novel circulating pulmonary disease biomarkers. miR-21 to miR-181a levels have been monitored by Xie et al. In particular, heavy smokers without diagnosed COPD were taken into consideration. The levels of the two miRNAs have a dichotomous pattern: the levels of miR-21 were significantly higher in the COPD patients and asymptomatic heavy smokers than in the healthy controls (HC), while miR-18a levels were significantly lower in the COPD patients and asymptomatic heavy smokers than in the HC [84]. The ratio of these miRNA levels could be used as a potential biomarker of early COPD pathogenesis.

6. Conclusions

Asthma and COPD are, in themselves, very complex and multi-factorial diseases. These pathologies are characterized by airway inflammation, airflow reduction, and airway remodeling. For years, the scientific community has been looking for a solution to this complex puzzle without success. Probably, crucial pieces were missing before they could get the entire picture: the EVs. This carrier is used to transport different molecules and cellular material, not only by our tissues but even by any pathogens eventually present. This review tried to highlight EVs' importance as a biomarker and a potential therapeutic target in two complex chronic airways pathologies: asthma and COPD. Therefore, an accurate understanding of EVs' roles in these pathologies could lead to a more precise diagnosis and more effective treatments for patients. The contribution made by the microbiome should not be underestimated. The bacterial populations usually present, the opportunistic pathogens, and the possible infections are all able to condition the microenvironment of the airways through the EVs. The possibility of having additional biomarkers available could be essential to make an early diagnosis or analyze the state of progression of the pathologies.

Finally, in the future, the analysis of EV pathways may provide new instruments to contrast the development and progression of chronic respiratory diseases.

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