Review

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Review

The New Paradigm: The Role of Proteins and Triggers in the Evolution of Allergic Asthma

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Abstract: Epithelial barrier damage plays a central role in the development and maintenance of allergic inflammation. The rise in airways epithelial barrier permeability alters the tissue homeostasis and allows the allergen and other external agents penetration. Different factors contribute to barrier impairment, such as eosinophilic infiltrate and allergens proteases action: the eosinophilic cationic proteins and the allergens' proteolytic activity have a central role in contributing to epithelial damage. In the airways, the allergens proteases degrade the epithelial junctional proteins allowing the allergen penetration and the uptake by dendritic cells. The increase in allergen-immune system interactions induce the release of the alarmins and the activation of type 2 inflammatory pathways causing, or worsening, the main symptoms at skin, bowel and respiratory levels. We aim to highlight the molecular mechanisms underlying allergenic proteases-induced epithelial barrier damage and the role of immune response in allergic asthma onset, maintenance and progression. We further will explore clinical and radiological potential biomarkers of airways remodeling in allergic asthma patients.

Keywords: allergic asthma; epithelial barrier damage; protease allergen; type 2 inflammation; airway remodeling

1. Introduction

Epithelial barrier damage is a crucial feature of inflammatory allergic diseases. The epithelium plays a double protective action: mechanical, through maintaining skin and mucosal barrier integrity, and immunological, through the action of a rich set of molecules that ensure the immune tolerance.

The epithelial barrier is a structured entity in which cell-cell adhesion complexes ensure integrity and effectiveness [1].

Tight Junctions (TJs) include transmembrane proteins of the claudin family, occludin, tricellulin, junctional adhesion molecules, and cytoplasmic proteins (such as the Zonula Occludens (ZO)-1 ZO-2, ZO-3).

Adherens junctions are composed of cadherin-catenin complexes and they act as key regulators of paracellular permeability [2].

Desmosomes provide mechanical stability and hemidesmosomes contribute to the epithelial layer-basal membrane attachment.

In normal conditions, a functional physical barrier contributes to the regulation of epithelial permeability, cell proliferation and differentiation. The loss of barrier integrity increases the exposure

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to environmental, allergic and toxic substances, breaking the immune tolerance and inducing the activation of different pro-inflammatory pathways.

Epithelial barrier damage characterizes different inflammatory diseases such as asthma, Chronic Rhinosinusitis with Nasal Polyps (CRSwNP), Eosinophilic Esophagitis (EoE) and Atopic Dermatitis (AD); different mechanisms could contribute to the barrier dysfunction.

In allergic diseases, the disruption of the epithelial barrier is associated with TJ defects and with a reduction of the adherence junctions and desmosomes number [3–6].

Zonulin is a regulator of epithelial and endothelial barrier function. It regulates intestinal permeability through disrupting tight junctions. Defective epithelial barrier function is a hallmark of airway inflammation in asthma [3].

Both environmental and genetic factors are involved in barrier damage [7,8].

Several susceptibility genes have been associated with epithelial barrier differentiation and homeostasis [9,10]. Structural airways remodeling signs have been found in genetically predisposed asthma children [11–13].

Moreover, the environmental context with the action of viruses [14], pollutants [15], cigarette smoke [16] and allergens play a central role in epithelial injury. Moreover, industrialization and consumption of highly processed food, can contribute to altering the gut microbiota and the intestinal barrier, increasing the susceptibility to allergic sensitization [17].

Several allergens, or allergens components, such as the House-Dust Mite (HDM) Der p1, have shown the ability to disrupt the TJs through both direct and indirect proteolytic activity [18].

The increase in epithelium permeability leads to Type 2 (T2) cytokines production and eosinophils activation and proliferation in the airways. Both T2 cytokines and eosinophil mediators interfere with TJs, contributing to the lack of barrier response. In asthma, the inflammatory processes start from the release of alarmins; the entity of cytokines' release correlates with the clinical symptoms, the disease severity and the airway remodeling process [19] (Figure 1).

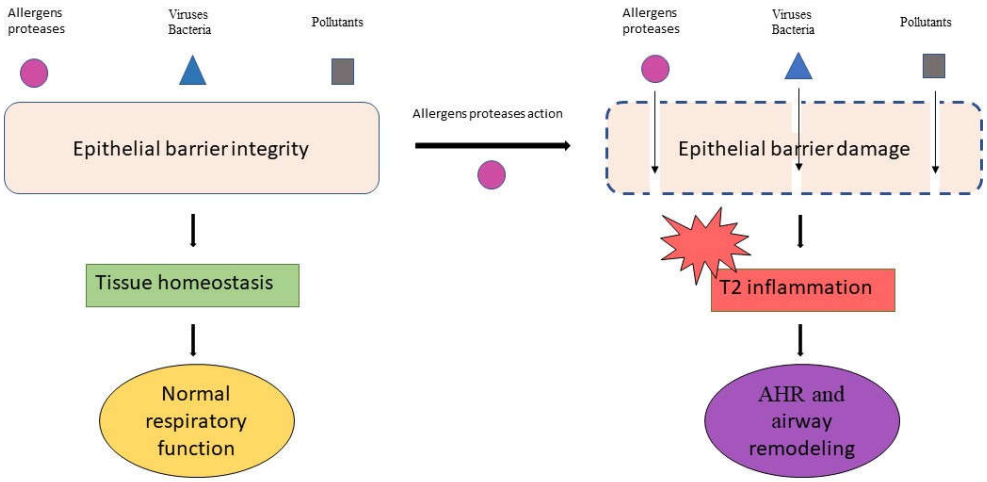

Figure 1. The role of allergens proteases in epithelial barrier damage, chronic inflammation and airway remodeling. AHR: Airway Hyperresponsiveness.

We aim to explore the molecular mechanisms underlying allergenic proteases-induced damage in allergic asthma onset, maintenance and progression. Further, we highlight the role of both epithelial barrier dysfunction and immune response in airways remodeling, exploring potential clinical and radiological biomarkers and the role of different therapeutic options.

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2. Molecular Mechanisms in Allergic Asthma: The Allergens Proteases

Allergens proteases are proteolytic enzymes with a primary role in the pathogenesis of respiratory allergies, facilitating the allergen-host interactions and promoting the development of allergic sensitization.

Allergens and pathogens with proteolytic activity can intrinsically overcome the host tolerance, activating various immunological pathways. Proteases enhance antigen-presenting cells in airways, inducing specific Immunoglobulin-E (IgE) production, eosinophils recruitment and inflammatory mediators release in airways, skin or other barrier tissues.

Proteases could be released as enzymatically inactive zymogens, requiring additional adjuvants for the activation, or they could show independent proteolytic activity, such as HDM [20–23].

The HDM major protease Der p 1 is a cysteine protease which has shown self-maturation capacity in acidic ambient [24]; moreover, it has demonstrated to promote the maturation of other HDM proteases, such as requiring enzymatic activation-HDM serine protease Der p 3, Der p 6 and Der p 9 [25].

Beyond activating factors, specific and non-specific protease inhibitors also participate in the regulation of proteolytic activity, ensuring the maintenance of tissue homeostasis [23].

Although HDM Der p 1 has been the first allergen protease to be characterized [26], more recently, different HDM, cockroaches' [27], fungal [28] and plant proteases have been described [29].

Based on the catalysis mechanism, according to the location of the cleavage site and the nature of their active site residues, proteases have been classified into five classes: aspartic, metallo, cysteine, serine and threonine proteases [30].

Although all five classes of proteases are found in the human genome [31], only aspartic, cysteine and serine proteases have been identified as allergens [32].

Most of cysteine proteases share structural homologies with Der p 1, having cysteine-histidine-asparagine as active site residues; while serine protease allergens are structurally similar to trypsin with serine-histidine-aspartic acid as active site residues.

House Dust Mite

Mite allergens proteases include the papain-like cysteine proteases from group 1 (Der p 1, Der f 1); the group 2 (Der p 2, Der f 2), which are lipid-binding proteins causing sensitization in more than 90% of mite-allergic patients [33]; the proteases from groups 3 (Der p 3, Der f 3), group 6 (Der p 6, Der f 6) and group 9 (Der p 9) are serine proteases with trypsin, chymotrypsin and collagenase activity, respectively.

Der p 1 can damage the bronchial epithelial barrier by degrading the endogenous protease inhibitors, such as lung α1-antitrypsin and elafin [34]; moreover, both Der p 1 and Der f 1 can degrade the airway surfactant proteins Sps-A and Sps-D [34,35].

Proteases action favors allergens penetration and, subsequently, allergens-immune cells contact. Der p 1 interacts with multiple molecules involved in the control of IgE synthesis [36,37].

Fungi

Fungal proteases are strong activators of T2 inflammation and play a major role in epithelial damage mechanisms. Fungal proteases, as well as HDM ones, can act as adjuvants of endogenous proteases and degraders of protease inhibitors.

The major fungal proteases are serine proteases, such as those of *Cladosporium cladosporioides* [38], *Penicillium* and *Aspergillus* species [39]; however aspartate proteases have been described in *Alternaria alternata* [40].

Aspergillus fumigatus, *Alternaria alternata*, and *Cladosporium herbarum* proteases induce morphologic changes and cell desquamation in the cultured airway epithelial cells, favoring the release of proinflammatory cytokines [41].

In particular, *A. alternata* proteases induce intense eosinophilic activation: the addition of aspartate protease inhibitors to *A. alternata* extract has shown to attenuate the eosinophils response [40].

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Fungal proteases, as well as Der p1, interact with the kinin system, the coagulation cascade and the fibrinolytic mechanism. The release of fibrinogen cleavage products induced by prothrombinase activity stimulates the innate immune response through the activation of Toll-Like Receptor-4 (TLR4) [42,43]. Moreover, thrombin is involved in different signaling pathways inducing the IgE-independent cytokine production [44].

Cockroaches

The only cockroaches allergen showing proteolytic activity is the *Periplaneta americana* serine protease Per a 10 [45], which provides both self activation and adjuvant effects on inactive proteases. Although *Blattella germanica* extract is rich in proteases that show direct proinflammatory effects on the airway epithelial, none of them have shown a direct proteolytic activity [46]. The aerosolized cockroach extract induced airway eosinophilic inflammation in animal models [47,48].

Foods

Food components, such as melon, kiwi, papaya fruit and others, could induce allergic sensitization and have shown serine and cysteine proteolytic action. The papain, a papaya fruit-derived allergen, belongs to the same family of cysteine proteases of HDM major group 1 allergens. Papain has shown to activate both innate and Th2 immune responses [49], inducing the alarmins release [50,51], the activation of mouse basophils in vitro [52] and lung eosinophilia in mice [53,54].

Pollen

Pollen proteolytic activity has been attributed to both allergenic and non-allergenic cysteine, serine, and metalloprotease [55]. IgE-reactive cysteine proteases are present on the coat of *Cynodon dactylon*, *Sorghum halepense* and *Phleum pratense* pollen [56]. In *Ambrosia artemisiifolia* pollen, the allergenic cysteine protease Amb a 11 has been isolated [29]. *Betula verrucosa* contains proteases potentially homologue to Der p 1 [57].

Taken together, allergens proteases alter epithelial cells and cell junctions, promoting the external agents' penetration and the activation of different inflammatory pathways.

2.1. The Allergens Proteases in Epithelial Barrier Damage and Inflammatory Signals

Airways, skin and gastrointestinal tract are the main tissues involved in allergen proteases-driven epithelial barrier damage [58,59].

In airway epithelium, HDM exerts proteolytic activity through both direct and indirect mechanisms involving the direct occludin and claudin degradation [60,61] and the Protease-Activated Receptors (PARs) activation, respectively [23,60,62]. A primary role has been attributed to Der p 1, although HDM serine peptidases have also shown the ability to damage epithelium [23].

It has been observed that Der p 1 could cause the detachment of bronchial epithelial cells [18,63,64].

The main role of Der p 1 is confirmed by the substantial inhibition of HDM activity if Der p 1-selective inhibition is provided [23].

Similar mechanisms are exploited by the fungal serine proteases Pen c 13 and Asp f 13 and the cockroach Per a 10 [65,66]. Moreover, in human bronchial epithelial cells, the fungal Pen c 13 has shown to downregulate the expression of CD44, which is involved in epithelial repair mechanisms [67].

In the gastrointestinal tract, it has been demonstrated that allergen proteases, such as the kiwi fruit actinidin (Act d 1), affect occludin and ZO-1, increasing the intestinal permeability [68].

Damaged epithelia are easily crossed by allergens and other external agents that directly reach Dendritic Cells (DCs), inducing modifications in cell surface receptors and unbalancing the immune response toward a T2 phenotype.

Active proteases induce the proteolytic cleavage of CD40, which results in reducing the Type 1 (T1) inflammatory mediators release, with a weak IL-12 production and, in contrast, increased levels of IL-4 and IL-13 [69].

Der p 1 can not only induce the soluble CD40 directly from DCs surface [69], but also cleave the DC-SIGN (CD209) [70], a receptor involved in T1 cells differentiation [71]. Moreover, Der p 1

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upregulates the expression of CD86 favoring the expression, in DCs, of chemokines involved in T2 response [72].

Der p 1, as well as Per a 10, has been shown to modulate both T and B cells through the direct cleavage of CD25 (the alpha chain of the IL-2 Receptor), and CD23 (the low-affinity Receptor for IgE) [73], resulting in less IL-12 and Interferon (INF) release, increased T2 cytokines levels and IgE synthesis.

In addition, the CD23 cleavage could furtherly increase the IgE synthesis disrupting the negative feedback between the membrane bound form of IgE Receptor and the IgE production [74].

Allergens proteases could activate the mast cells through both IgE-mediated and non- IgE-mediated mechanisms. Non-IgE-mediated mast cells-activation involves the cell surface PARs [75,76].

PAR-1, -3 and -4 are activated by thrombin, while PAR-2 is activated by trypsin, which shares molecular features with allergic proteases [75].

Epithelial cells, mast cells, basophils, eosinophil and other cellular types are all involved in PAR-2 mediated- allergen proteases response, as observed after Der p 1, Der p 3 and Der p 9 exposure [77,78].

In airway epithelial cells, PAR-2 activation induces cellular morphologic changes, cell desquamation and the release of cytokines, growth factors and prostanoids [50].

The inflammatory environment enhances PAR expression [79], this has been demonstrated in asthma patient's bronchial epithelium biopsies, compared with control [80].

Moreover, allergens can upregulate PAR-2 levels on pulmonary and bone marrow derived myeloid Dendritic Cells (mDCs) [81]. An overexpression of PAR-2 and PAR-3 mRNAs has been described in nasal polyp epithelial cells stimulated with *Aspergillus*, *Alternaria*, and *Cladosporium* [82].

PAR-2 favors the alveolar macrophages [83], neutrophil and eosinophil recruitment. In particular, the role of PAR-2 in eosinophils activation has been confirmed by the inhibition of eosinophilic response - stimulated with the cell-free extract of *A. alternata* exposure- in presence of protease ligands and PAR-2 antagonist peptide [40].

Active Der p 1, Der f 1 or papain lead to the superoxide anion production through the direct eosinophil activation [84].

Basophils exposed to proteolytically active Der p 1 or papain secrete Thymic Stromal Lymphopoietin (TSLP) and IL-4 in an-IgE independent way [20,85]; the specific mechanism is unknown, although nociceptive primary sensory neurons, Mas-related G-protein- coupled receptors (Mrgprs), seem to be involved [86].

The early role of type 2 Innate Lymphoid Cells (ILC2) in T2 immune response is well known. Damaged epithelium produces alarmines, such as TSLP, IL-25, IL-33 which activate ILC2 to produce large amounts of IL-4, IL-5 and IL-13, promoting Th2 differentiation. Th2 cells contribute to T2 cytokines release and mediate the allergen-specific IgE production [87].

In allergic patients, Der p 1 and *Aspergillus* have shown to induce the ILC2 recruitment and activation [54,88].

A contribution to tissue injury is provided by the alterations of the protease/anti-protease balance.

As mentioned above, the physiological cellular protective function is ensured by the action of anti-proteases, such as 1-antitrypsin, elafin and Secretory Leukocyte Proteinase Inhibitor (SLPI). The proteases/anti-proteases balance is critical to protect lung tissue being, the loss of their homeostasis, a feature of emphysema and asthma [34].

Beyond the allergen proteases (such as papain, Der p 1, cat skin) degrading effect [34], genetic factors could cause the loss of protease inhibitors expression, contributing to exogenous damage [89,90].

The mechanical and immunological epithelial barrier dysfunction induces molecular, cellular and tissue modifications that are features of allergic asthma.

In this context, the chronic inflammation predisposes to- (and enhances) the airway remodeling and the asthma exacerbations occurrence.

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3. Airway Remodeling and Its Role in Asthma Exacerbations

3.1. Biomarkers of Airway Remodeling

Asthma-related airway remodeling includes structural changes like sub-epithelial fibrosis, thicker Airway Smooth Muscle (ASM), mucous gland hyperplasia, angiogenesis, and damaged epithelial layers, resulting in stiffer airway walls [91]. Such remodeling significantly contributes to persistent symptoms and severity in severe asthma cases [92]. Notably, airway remodeling can begin early, even before asthma diagnosis, as observed in preschool children with confirmed wheezing [93]. The identification of potential biomarkers could aid in detecting early signs of remodeling.

3.1.1. Epithelial Remodeling

Asthma-induced airway epithelium remodeling involves epithelial cell deterioration, loss, decreased ciliated cells, and increased goblet cells [94]. The Epithelial-Mesenchymal Transition (EMT) is crucial in this process, driven by Transforming Growth Factor beta (TGF-β), leading to epithelial cells transforming into mesenchymal cells [95]. Markers include reduced E-cadherin and increased N-cadherin [96]. The IL-33/CD146 axis influences EMT in asthma, with HDM extract boosting IL-33 and CD146. Epithelial cell interactions with the immune system may involve Extracellular Vesicles (EVs), with altered microRNA (miRNAs) content in response to stress or activation, playing a role in asthma development [97]. The communication between lung epithelial cells and the immune system may involve EVs carrying miRNAs . These miRNAs, which change due to cellular stress or activation [98], are crucial in asthma [99–101], showing different levels in asthma patients' bronchoalveolar lavage fluid compared to healthy individuals [102]. Research indicates specific miRNAs in EVs from airway epithelium, like miR-34a, miR-92b, and miR-210, could be key in initiating Th2 responses and asthma development [103].

3.1.2. Reticular Basement Membrane Thickening

Research links Reticular Basement membrane (RBM) thickening in asthma to gene expressions influencing airway growth and fibrosis, affecting various physiological processes [104]. Identifying specific fibrocytes in Bronchoalveolar Lavage Fluid (BALF) marked by CD34/CD45RO/α-SMA/procollagen I, indicative of basement membrane thickening, suggests a role in mild asthma's airway remodeling, with future non-invasive detection possibilities [105]. A study on severe asthma identifies galectin-3 as a biomarker in omalizumab-treated patients, distinguishing responders by their protein profiles related to smooth muscle and extracellular matrix [106].

3.1.3. Subepithelial Fibrosis

TGFβ plays a crucial role in asthma by transforming airway fibroblasts into myofibroblasts, leading to subepithelial fibrosis [107]. The severity of fibrosis correlates with TGF-β1 mRNA levels in bronchial biopsies [108], and elevated αvβ8 integrins in asthma indicate their potential as biomarkers [109]. Periostin, associated with IL-4 and IL-13, impacts fibrosis and inflammation, marking the efficacy of Th2 antagonists [110]. Follistatin-like 1 (FSTL1)-induced autophagy may promote epithelial-mesenchymal transition, suggesting new asthma treatments [111].

3.1.4. Airway Smooth Muscle

Many ASM cell mitogens are involved in asthma, such as Platelet-Derived Growth Factor (PDGF), TGF-β, Epidermal Growth Factor (EGF), Heparin-Binding EGF, and Vascular Endothelial Growth Factor (VEGF) [112].

ASM, histologically assessed by endobronchial biopsies, has been recognized as a valuable biomarker in phenotyping airways disease, especially in the context of personalized medicine [113].

TGF-β stands out as a potential biomarker for this particular mechanism, as it becomes activated when ASM cells and the airways contract. TGF-β is known as a cytokine that promotes remodeling processes [114]. Additionally, pharmacological means to inhibit Transient Receptor Potential

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Vanilloid-1 (TRPV1), a factor that can influence the tone of ASM and effectively mitigate airway remodeling in living organisms, are promising [115].

Recent research highlights the absence of a complete molecular marker system for ASM cells (ASMCs), yet remains hopeful for future developments. It has been discovered that Myosin Heavy chain 11 (MYH11) serves as a marker for mature SMCs, and Transgelin (TAGLN) indicates early SMC differentiation. This suggests the possibility of using various molecular markers or their combinations to identify the properties and origins of increased ASMCs in asthma-related airway remodeling, depending on the stage of differentiation and research requirements [116].

DNA methylation changes in severe asthma, particularly in ASMCs, illuminate disease mechanisms. Asthma shows reduced methylation in the Phosphodiesterase 4D (PDE4D) promoter area, impacting ASMCs proliferation [117]. These patterns relate to asthma's severity and correlate with gene and miRNA changes, affecting ASMCs function. This suggests the potential use of demethylating agents in severe asthma treatment [118]. Integrins, crucial in ASM contraction and remodeling, mediate ASM and extracellular matrix interactions. Fibronectin-binding $\alpha 5\beta 1$, $\alpha 2\beta 1$, and $\alpha 9\beta 1$ integrins could be therapeutic targets [112].

3.1.5. Mucus

In asthma, the hypersecretion of mucins MUC5AC and MUC5B by goblet cells contributes to airway remodeling. While MUC5B has essential homeostatic roles, targeting MUC5AC secretion could be a potential therapeutic strategy [119,120].

3.1.6. Vasculature

Many studies have observed changes in the bronchial vascular network in asthma, including increased blood vessel number, size, density, vascular leakage, and plasma engorgement. This neovascularization, a key element of airway remodeling, has uncertain effects on bronchial walls and lung function. Contributing factors include extracellular matrix alterations and growth factor dysregulation [121]. VEGF, a key stimulator of endothelial cell growth and vascular permeability, is elevated in asthma, and specific integrins like $\alpha v\beta 3$ and $\alpha v\beta 5$ play vital roles in blood vessel development [112].

The markers/biomarkers of airway remodeling are summarized in Tables 1 and 2.

Table 1. Markers of airway remodeling.

Marker	Description	Reference
High-resolution CT (HRCT)	HRCT is crucial for identifying static and dynamic airway changes in asthma, revealing details as small as 1 mm in diameter.	Brown RH, Mitzner W, 1985; Silva CI, Colby TV, 2004
Bronchial Wall Thickness (% WT)	% WT, the bronchial-to-arterial diameter ratio (BA ratio), and the level of airway collapsibility (AC) are acknowledged as efficient measurements for assessing airway remodeling in CT scans.	Chae EJ, Kim TB, Cho YS, et al., 2010
Wall Area Percentage (WA%)	WA% is a crucial marker for assessing airway remodeling in severe asthma, with a negative correlation between WA% and FEV1 observed, indicating the relationship between airway wall thickness and lung function impairment.	Hartley RA, Barker BL, Newby, 2016
Quantitative CT (qCT) scans	QCT scans serve as effective biomarkers for airway remodeling, enhancing the precise analysis of severe asthma. Biomarkers such as wall thickness percentage (WT%), wall area percentage (WA%), and	Walker C, Gupta S, Hartley et al., 2012; Aysola RS, Hoffman EA, et al., 2008; Trivedi A, Hall C, Hoffman EA, 2017; Adams JE, 2009

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	air trapping are higher in asthma patients and particularly elevated in severe cases.	
Bronchial Wall Thickness (BWT) and Emphysema	BWT and emphysema are more prevalent in patients with severe asthma, indicating their role as radiological markers for lung function changes in asthma.	Gupta S, Siddiqui, 2009; Harmanci E, Kebapci M, 2002; Gupta S, Hartley R, Khan UT, et al., 2014; Wang D, Luo J, Du W, Zhang LL, 2016

Table 2. Biomarkers of airway remodeling.

Biomarker	Description	Reference
Sub-epithelial fibrosis	Characterized by thicker airway smooth muscle, mucous gland hyperplasia, angiogenesis, and damaged epithelial layers, contributing to stiffer airway walls.	Kozlik P, 2020; Aysola RS, chest 2008
Epithelial remodeling	Involves deterioration of epithelial cells, loss of ciliated cells, and an increase in goblet cells. The epithelial-mesenchymal transition (EMT) driven by TGF-β is a key process, with markers like reduced E-cadherin and increased N-cadherin.	Bahmer T, Sand JMB, 2020; Yang ZC, Qu ZH, Yi MJ, 2019; Rout-Pitt N, Farrow N, 2018
Reticular Basement Membrane (RBM) thickening	Linked to gene expressions affecting airway growth and fibrosis. Identifying specific fibrocytes in BALF as markers suggests a role in airway remodeling.	Bazan-Socha S, Buregwa-Czuma, 2021; Nihlberg K, Larsen K, e coll., 2006
Subepithelial Fibrosis	TGFβ's role in transforming airway fibroblasts into myofibroblasts leading to subepithelial fibrosis. The severity of fibrosis correlates with TGFB1 mRNA levels, and periostin's association with IL-4 and IL-13 impacts fibrosis and inflammation.	Sidhu SS, Yuan S, Innes AL, 2010; Vignola AM, Chanez P, 1997; Izuhara K, Arima K, Ohta, 2014
Airway Smooth Muscle (ASM)	ASM cell mitogens, such as PDGF, TGFβ, EGF, are involved in asthma. Histology assessed through endobronchial biopsies serves as a valuable biomarker.	Joseph C, Tatler AL, 2022; Sha J, Rorke S, Langton D, et al., 2019
Mucus	Hypersecretion of mucins MUC5AC and MUC5B by goblet cells contributes to airway remodeling, with targeting MUC5AC secretion as a potential therapeutic strategy.	Bahmer T, Sand JMB, Weckmann, 2016; Lai HY, Rogers DF, 2010

3.2. Airway Remodeling: Radiological Pathways and Key Points

High-resolution Computed Tomography (HRCT) is crucial in identifying radiological markers in asthma, revealing both static and dynamic airway changes as small as 1 mm in diameter [122,123]. In patients with stable asthma who undergo computer CT scans, three primary measurements are acknowledged as efficient in assessing airway remodeling: the percentage of bronchial Wall Thickness (WT%), the Bronchial-to-Arterial (BA) diameter ratio (BA ratio), and the level of Airway Collapsibility (AC) during both inhalation and exhalation. This evaluation of airway remodeling relies on the post-bronchodilator [124].

In a significant study, about 80% of severe asthma patients showed chest CT abnormalities, highlighting CT's value in assessing this condition [125].

Hartley et al. discovered a negative correlation between Wall Area percentage (WA%) and Forced Expiratory Volume in 1 second (FEV1) in non-smoking asthma patients. This indicates that

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WA% is a crucial marker for assessing airway remodeling in severe asthma, highlighting the relationship between airway WT and lung function impairment [126].

Quantitative CT (QCT) scans are effective biomarkers for airway remodeling, significantly enhancing the precise analysis and understanding of severe asthma [92,127–129]. QCT biomarkers like WT%, WA%, and air trapping (measured as low attenuation area) are higher in asthma patients compared to controls [130] and are particularly elevated in severe cases [131]. These QCT measures correlate closely with asthma severity, and histological findings, making them effective for both studying and monitoring asthma [132].

3.2.1. Radiological Indicators for Assessing Severity, Early Identification, and Involvement of Small Airways

Bronchial WT (BWT) and emphysema are more common in patients with severe asthma compared to mild asthma [125,133–135]. However, other studies have not found a link with the severity of asthma [133,136,137]. WT% is a meaningful radiological marker in assessing lung function changes in asthma. In the Severe Asthma Research Program (SARP) study, which focused on never smokers or ex-smokers with less than a 10-pack year history, it was found that the WT% was notably higher in asthmatics who experienced a significant decline in lung function over a three-year period compared to those whose lung function remained normal or improved [138]. Similarly, in HRCT imaging, patients with lower bronchodilator-responsive FEV1 had twice the WT compared to those with normal FEV1 (about 90% predicted), underscoring a significant relationship between increased BWT and diminished lung function [139].

Emphysema-linked changes notably impact lung function in asthma patients, regardless of smoking habits [140,141] indicates the permanent nature of persistent airway obstruction in severe asthma patients, particularly in those with a significant reduction in baseline bronchodilator-responsive FEV1. In the study by Kim YH et al., emphysema scores were four times higher in the Tr5 group compared to the Tr4 group, a trend also observed among non-smokers [139]. Research has shown that 15% to 39% of people with asthma, including non-smokers, experience these changes [142]. CT-measured air trapping in asthma patients is linked to the severity of their asthma and an increased likelihood of experiencing severe exacerbations [143]. Patients undergoing three months of inhaled corticosteroid therapy showed reduced air trapping in CT scans [144]. Additionally, a study by Haldar et al on 26 patients with severe, eosinophilic, refractory asthma revealed that one-year treatment with mepolizumab, an anti-IL5 monoclonal antibody, significantly lowered average wall area compared to a placebo [145].

Delta Lumen, defined as the percent change in airway lumen area between Functional Reserve Capacity (FRC) and Total Lung Capacity (TLC), is a new metric in a study of 152 asthma patients. It negatively correlates with wall thickness WT% and low attenuation area, especially in severe cases like refractory asthma requiring systemic corticosteroids or hospitalization due to exacerbation. This suggests that a reduced Delta Lumen, as measured by QCT, could be a useful biomarker for identifying severe, unstable asthma [146].

The more pronounced thickening of airway walls observed in HRCT images can act as an early indicator of airway remodeling in asthma cases, even when lung function tests like FEV1 are normal [147].

In persistent asthma, the tBTW is linked to increased resistance and reactance in peripheral airways, a higher frequency of severe exacerbations, and the presence of nasal polyposis [148]. QCT showed a strong correlation between bronchial lumen area and inner diameter with lung function tests in a study of 83 long-term asthma patients. Notably, these measures were reduced from the seventh to the ninth bronchial generations, indicating airway remodeling predominantly in medium and small airways [149].

3.2.2. Radiological Pathway in Patient Phenotyping

Various attempts have been made to phenotype asthmatic patients through radiological pathways.

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The WA% is significantly higher in asthma patients than those with Eosinophilic Bronchitis (EB), with a difference of 72 (3.1)% versus 54 (2.1)%. This suggests that in asthma, increased WA% might play a more critical role in airway hyperresponsiveness than factors like air trapping or centrilobular prominence, which are typically considered. In contrast, the WA% in EB patients is not as prominently different [150].

In a cluster-based study of asthma patients, clusters with higher bronchial wall area in the right upper lobe's bronchus, as assessed by CT, were associated with elevated sputum neutrophil levels [151]. In 2014, Gupta et al., through cluster analysis, identified three novel asthma phenotypes with unique clinical and radiological characteristics. Cluster 1 showed an increase in the lumen volume and a decrease in the percentage wall volume of the right upper lobe apical segmental bronchus. In contrast, Cluster 3 had the smallest lumen volume but the highest percentage wall volume in the same bronchus. Cluster 2, however, displayed an absence of proximal airway remodeling. These findings suggest distinct structural changes in the airways of different asthma phenotypes [152].

In a HRCT study of 109 untreated asthma patients, key findings included airway remodeling, bronchiectasis, and mucus plugs, more pronounced than in healthy individuals. A notable inverse relationship existed between airway WT and mid-expiratory flow [153].

In a study of 61 asthmatic patients, four QCT-based clusters were identified, differing in asthma severity and lung function decline over five years. Cluster C1 consisted of non-severe asthmatics with increased wall thickness; C2 had a mix of severe and non-severe cases with limited bronchodilator response; C3 and C4 included severe asthmatics, with C3 focusing on severe allergic asthma without small airway disease, and C4 on ex-smokers with significant small airway disease and emphysema [154]. Kim S. et al. categorized asthma airway remodeling into three types: Large Airway Involvement (LA), Small Airway Involvement (SA), and Normal/Near-Normal (NN). In their study of 91 severe asthma patients, 81.3% showed bronchial thickening and bronchiectasis, 6.6% had small airway remodeling associated with airflow obstruction and smoking, while 26% displayed no significant remodeling and required fewer oral corticosteroids [155].

4. Relevant Therapeutic Options in Airway Remodeling

4.1. The Role of Standard Therapy in Airway Remodeling (LAMA)

Prior randomized trials have shown that Inhaled Corticosteroids (ICS) can lead to a reduction in subepithelial fibrosis [156,157]. The use of Inhaled Corticosteroids/ Long-Acting Beta2 Agonists (ICS/LABAs) is known to reduce airway inflammatory and remodeling pathways. For instance, in post-ICS-LABA treatment, there's a noted downregulation in the expression of various elements like nuclear receptor transcription coactivator, N-acetyltransferase, protein tyrosine kinase, nuclear receptor, and RNA polymerase II-activating transcription factor [158].

Muscarinic M1-3 receptors, present in the lungs, are crucial for the bronchodilatory effect of Long-Acting Muscarinic Antagonists (LAMAs), primarily through M3 inhibition. M3 receptors also influence mucus secretion, making LAMAs effective in reducing it. Muscarinic receptors are found in various lung cells, including epithelial cells, fibroblasts, smooth muscle cells, and inflammatory cells. This indicates that non-neuronal cells can also produce and release Acetylcholine (ACh), contributing to different biological responses in an autocrine or paracrine manner [159].

In animal and in vitro studies, LAMAs have shown significant anti-inflammatory and anti-proliferative effects. They are capable of inhibiting airway remodeling triggered by allergens [160].

ACh plays a role in airway inflammation and remodeling, also influencing the growth of ASM. Studies have shown the benefits of using muscarinic ACh Receptor (mAChR) antagonists, especially long-acting types [161] (LAMAs), in targeting these effects by blocking ACh's activation of mAChRs.

In earlier stages of the condition, the challenge in prescribing LAMAs lies in the high variability of patient responses and the lack of detailed patient phenotyping. Enhancing the characterization of parasympathetic tone activity could lead to more effective LAMA prescriptions [162].

Adding LAMAs to ICS/LABA therapy enhances lung function, decreases exacerbations, and slightly improves asthma control in moderate to severe asthma patients not fully controlled by

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ICS/LABA alone. LAMAs are effective across various asthma phenotypes and endotypes. Three LAMA molecules- Tiotropium (TIO), Glycopyrronium (GLY), and umeclidinium- have been studied as add-ons, each with slightly different action onsets and half-lives. GLY, in particular, acts slightly faster than TIO and umeclidinium may have similar properties [163].

The impact of anticholinergic drugs on airway remodeling remains unclear. Further research is needed to understand the anti-inflammatory effects of anti-muscarinic drugs on human airway inflammation and remodeling processes.

4.2. The Role of Biological Drugs in Airway Remodeling

The specific mechanisms of how environmental factors trigger inflammatory responses leading to airway remodeling in asthma are not completely clear. Alarmins, cytokines from epithelial cells, start these immune processes, contributing to remodeling. Biological therapies can improve airflow by addressing inflammation and may reverse fixed remodeling caused by structural changes. Differentiating the immediate and long-term effects of biologics is vital for evaluating their impact on severe asthma's airway remodeling [164].

Omalizumab, a humanized IgG1-κ monoclonal antibody, targets the Fc fragment of IgE [165]. It has been shown to reduce the thickness of the basement membrane and decrease fibronectin deposits in the airways of asthma patients [166].

Mepolizumab treatment in asthma patients not only reduced the number of eosinophils in the bronchial passages but also decreased TGF-β1 positive eosinophils, the thickness and tenascin immunoreactivity of the airways, and the levels of TGF-β1 in bronchoalveolar lavage fluid [167].

In biopsies from severe eosinophilic asthma patients, benralizumab significantly reduced eosinophils in the bronchial lamina propria and airway smooth muscle mass, without affecting myofibroblast numbers. This reduction was linked to the depletion of TGF-β1 positive eosinophils [168]. Additionally, a single dose of benralizumab notably improved ventilation in patients with uncontrolled asthma and significant mucus plugging [169].

In a mouse model of asthma, the use of dupilumab, which blocks both IL-4 and IL-13, was effective in preventing eosinophils from infiltrating lung tissues, while it did not impact the levels of circulating eosinophils [170]. In a different mouse model, blocking the IL-4Rα receptor improved lung function. This effect was achieved by influencing various factors involved in inflammation and the remodeling process in the lungs [171].

TSLP, overexpressed in asthmatics' airway epithelium, activates lung fibroblasts, promoting airway remodeling [172]. Tezepelumab, a human IgG2-λ monoclonal antibody, targets TSLP. Studies show TSLP' role in fibrotic lung disease and its blockade reduces inflammation, TGF-β1 levels, and airway remodeling in animal models [173,174]. The CASCADE study revealed Tezepelumab significantly reduces airway submucosal eosinophils in moderate-to-severe asthma patients compared to placebo [175]. Lebrikizumab, a humanized monoclonal antibody, targets and inhibits soluble IL-13, blocking its downstream signaling. In exploratory analyses, treatment with lebrikizumab has been linked to a decrease in subepithelial fibrosis, a characteristic of airway remodeling [176].

The EMT in airway remodeling is influenced by the IL-33/CD146 axis. IL-33, derived from HDM extract-treated alveolar epithelial cells, stimulates CD146 expression. This process promotes EMT in the context of chronic allergic inflammation caused by HDM exposure. These findings highlight the potential of targeting the IL-33/CD146 pathway as a therapeutic approach in airway remodeling [97].

5. Discussion

The loss of epithelial barrier function and the airways remodeling are both features of allergic asthma.

The first one, generally occurs in the early stages of the disease, not rarely preceding the allergic sensitization. The second one has been longer considered as resulting from a long lasting disease, however, evidence has shown that airway remodeling may occur in asthma patients even prior to diagnosis [93].

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The barrier damage and the airways remodeling appear to be linked, defining a more complex clinical phenotype: epithelial permeability is higher in severe asthma compared with mild asthma [177], as well as in CRSwNP compared with CRS without nasal polyps [178].

Allergen proteases, such as Der p 1 or Asp f 13, directly induced Airway Hyperresponsiveness (AHR) in animal models [179,180] and provoked morphological and molecular modifications in human ASM cells. These effects have been described not only in allergic patients, but also in subjects without prior allergic sensitization [87].

The complete inactivation of *Aspergillus* protease activity totally prevented the T2 airway inflammation in a murine model of asthma [21]. Moreover, selective inhibition of Der p 1 not only reduced the levels of blood allergen-specific IgE, but also suppressed AHR in rats, avoiding the chronic inflammation and the predisposition to airway remodeling [181].

The redox ambient in bronchial lumen regulates the response to allergens proteases. Der p 1 activity is enhanced by the bronchial epithelium-secreted glutathione-*S*-transferase-pi and by the presence of the antioxidant glutathione, both of which are highly present in the human epithelial lung fluid [182]. In damaged epithelia of asthmatic patients this effect is favored by anti-protease and mucociliary clearance impairment [183]. Moreover, in allergens proteases-induced inflammation, the production of mitochondrial Reactive Oxygen Species (ROS) is increased, feeding the inflammatory vicious circle. Downregulation of Indoleamine 2,3-dioxygenase (IDO) is observed in bronchial epithelial cells after *Aspergillus,* Der p 1 and HDM extracts exposure [184,185].

Cystatin SN (CST1) inhibits the cysteine protease activity and its expression is enhanced in asthma epithelium. In a recent study, sputum and serum CST1 protein levels were negatively correlated with lung function in asthma patients; CST1 protein levels were significantly lower in the serum of HDM-specific IgE-positive asthmatics than in sIgE-negative asthmatics. Moreover, the HDM-induced epithelial barrier function disruption was suppressed by recombinant human CST1 protein in vitro and in vivo, reducing asthma symptoms. CST1 has been considered as a potential biomarker for monitoring asthma control [186].

The complex of different chemical and biological agents, which humans are daily exposed to, is known as the "exposome" [187] and includes microorganisms, pollution, hygiene-derived products, HDM, natural toxins, and food additives. The exposure to these factors alters the cell functions and favors the allergic response activation [188,189].

Proteases deriving from microorganisms, chemicals, environmental pollution, cigarette smoke and other noxious agents may damage the epithelial barrier, similarly to allergen proteases, contributing to the inflammatory process, the AHR and the airway remodeling [190].

Considering infective agents, the exposure to viruses during infancy and childhood predisposes to asthma development [191].

Staphylococcus aureus produces a wide range of proteins including toxins, serine-protease-like proteins, and protein A and its role in severe asthma and CRSwNP is well known. Staphylococcal enterotoxin B-IgE sensitization has been considered as a possible independent risk factor for asthma development and, in severe asthma patients, it has been associated with the presence of CRSwNP as comorbidity [192]. Moreover, *Staphylococcus aureus*, regardless of enterotoxins production, may damage the airway epithelial cells, inducing the release of IL-25, IL-33 and TSLP, which can activate the ILC2 and the T2 response [193].

Recent evidence has demonstrated that, in the skin of a preclinical mouse model, eosinophil-recruiting chemokines (and eosinophil infiltration) are induced after *Staphylococcus aureus* epicutaneous exposure, the IL-36α-IL-36R pathway is involved [194].

The exposure to tobacco smoke has been strongly associated with asthma prevalence in children [195] and exacerbates asthma and rhinitis symptoms in adults, decreasing muco-ciliary clearance [196]. The cigarette smoke can directly damage the TJ of the pulmonary epithelium [197], promoting the T2 response through epigenetic modifications, such as decreasing gene methylation of IL-4, IL-13 or increasing FOXP3 methylation after HDM challenge [198,199].

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The airborne microplastics inhalation caused pulmonary inflammatory cell infiltration, bronchoalveolar macrophage aggregation and increased the TNF-α levels in both healthy and asthmatic mice [200].

Air pollutants exalt the action of aeroallergens, damaging the pollen cell wall and facilitating the release of allergenic proteins into the environment [201,202]. The direct allergenic proteins - air pollutants contact promotes chemical protein modification before inhalation and deposition in the respiratory tract. Especially high ozone (O3) and nitrogen dioxide (NO2) levels have been shown to efficiently nitrate and cross-link the proteins [203,204]. The concentrations of smog and industrial contamination-associated O3, NO2 and particles in suspension are geographically associated with a higher rate of infant asthma [205]; also maternal exposure to NO2 leads to enhanced sensitivity to allergens and increased AHR [206].

Increased T2 response and accumulation of ILC2 cells was observed in a diesel exhaust-enhanced allergic mice model [207]; O3 and NO2 promote the release of cytokines and chemokines, such as IL-33, IL-25 and TSLP, in both normal and asthmatic patients' bronchial epithelial cells [208,209].

Recently it has been observed that TLR4 is enhanced in Phl p 5, but not in Bet v 1, after the ROS and nitrogen species exposure; subsequently, chemical modification and increased protein-receptor interactions occur. These events might contribute to the growing prevalence of respiratory allergies in industrialized countries [210].

The allergens proteases cause the airways remodeling both directly and indirectly, through chronic inflammation-induced modifications.

HDM proteses directly induce CX3CL1 chemokine activating the T2 response [211] and the proliferation of ASMCs [212].

HDM proteases, signaling through EGF receptor and TGF-β1, have shown to promote epithelial-to-mesenchymal transition in human bronchial epithelium cells, contributing to airway remodeling in asthma [213].

The chronic inflammation induces a chronic repair reaction leading to the continuous release of growth factors, the uncontrolled proliferation of fibroblasts, ASMCs, goblet cells, and the deposition of extracellular matrix molecules [214,215] which are all features of the airway remodeling process.

Due to the different emerging asthma phenotypes and to the increasing number of factors included in the exposoma concept, which contribute to inflammatory damage and enhance asthma incidence and severity, the identification of clinical and radiological biomarkers is a concrete need in asthma. In a previous study, we have explored the role of serum free light chains -κ and -λ in asthma patients, showing their value as qualitative and quantitative (severity indicator) potential biomarkers, respectively [216].

Focusing on airway remodeling, the examination of the physio-pathological and radiological features in allergic asthma should be considered for patients' clusterization.

6. Conclusions and Future Directions

The role of allergen proteases in the pathogenesis of allergic asthma has been early identified and it is currently well known. Molecular mechanisms underlying the allergen proteases-induced damage, starting from the epithelial barrier loss, have been better defined over the years and new allergen proteases have been identified.

In our review, the clinical features and the radiological patterns of airways remodeling have been explored, in order to emphasize the importance of biomarker identification in a disease with multiple endotypes and phenotypes.

The aim of this review was to draw a continuous thread between molecular mechanisms of allergen protease exposure, epithelium damage, chronic inflammation and airway remodeling in allergic asthma patients.

Although, as previously remarked, these “steps” are not necessary subsequently, in many cases they may take part in an evolutive process. The lack of available biomarkers, in particular for

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monitoring airway inflammation and remodeling, doesn't allow an optimization for the therapeutic management and follow-up of allergic asthma patients.

Considering T2 inflammation, beyond the blockage of IL-5, IL-4, IL-13 and IgE, the epithelial damage-derived cytokines are a new available therapeutic target.

In a future perspective, a better endo- pheno-typization of asthmatic patients will ensure the selection of the appropriate therapeutic option, between the increasing number of available drugs. Currently, different available molecules have shown positive effects on airway remodeling. However, the identification of new potential therapeutic targets in the molecular pathways involved in the airway remodeling process should be achieved, considering the increase of allergic, environmental, and chemical stimuli in the industrialized exposome.

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Abbreviations

Tight Junctions	TJs
Zonula Occludens	ZO
Chronic Rhinosinusitis with Nasal Polyps	CRSwNP
Eosinophilic Esophagitis	EoE
Atopic Dermatitis	AD
House-Dust Mite	HDM
Type 2	T2
Immunoglobulin-E	IgE
Toll-Like Receptor-4	TLR4
Protease-Activated Receptors	PARs
Dendritic Cells	DCs
Type 1	T1
Interferon	INF
myeloid Dendritic Cells	mDCs
Thymic Stromal Lymphopoietin	TSLP
type 2 Innate Lymphoid Cells	ILC2
Secretory Leukocyte Proteinase Inhibitor	SLPI
Airway Smooth Muscle	ASM
Epithelial-Mesenchymal Transition	EMT
Transforming Growth Factor-β	TGF-β
Extracellular Vesicles	EVs
microRNA	miRNAs
Reticular Basement membrane	RBM
Bronchoalveolar Lavage Fluid	BALF
Follistatin-like 1	FSTL1
Platelet-Derived Growth Factor	PDGF
Epidermal Growth Factor	EGF
Heparin-Binding	EGF
Vascular Endothelial Growth Factor	VEGF
Transient Receptor Potential Vanilloid-1	TRPV1
ASM cells (ASMCs); Myosin Heavy chain 11	MYH11
Transgelin	TAGLN

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Phosphodiesterase 4D	PDE4D
High-resolution Computer Tomography	HRCT
Wall Thickness percentage	WT%
Bronchial-to-Arterial	BA
Airway Collapsibility	AC
Wall Area percentage	WA%
Forced Expiratory Volume in 1 second	FEV1
Quantitative CT	QCT
Bronchial WT	BWT
Severe Asthma Research Program	SARP
Functional Reserve Capacity	FRC
Total Lung Capacity	TLC
Eosinophilic Bronchitis	EB
Large Airway Involvement	LA
Small Airway Involvement	SA
Normal/Near-Normal	NN
Inhaled Corticosteroids	ICS
Inhaled Corticosteroids/ Long-Acting Beta2 Agonists	ICS/LABAs
Long-Acting Muscarinic Antagonists	LAMAs
Acetylcholine	Ach
muscarinic ACh Receptor	mAChR
Tiotropium	TIO
Glycopyrronium	GLY
Airway Hyperresponsiveness	AHR
Reactive Oxygen Species	ROS
Indoleamine 2,3-dioxygenase	IDO
Cystatin SN	CST1
Toll-like Receptor	4TLR4
Ozone	O3
Nitrogen dioxide	NO2

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