Review

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Serum biomarkers of remodeling in severe asthma with fixed airway obstruction and the potential role of KL-6

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Abstract: Over 3% of asthmatic patients are affected by a particularly severe form of the disease ("severe asthma", SA) which is often refractory to standard treatment. Airway remodeling (AR), which can be considered a critical characteristic of approximately half of all patients with SA and currently thought to be the main mechanism triggering fixed airway obstruction (FAO), seems to be a key factor affecting a patient's outcome. Despite the collective efforts of internationally renowned experts, to date only a few biomarkers indicative of AR and no recognizable biomarkers of lung parenchymal remodeling have been identified. This work examines the pathogenesis of airway and lung parenchymal remodeling and the serum biomarkers that may be able to identify the severe asthmatic patients who may develop FAO. The study also aims to examine if Krebs von den Lungen-6 (KL-6) could be considered a diagnostic biomarker of lung structural damage in SA.

Keywords: airway remodeling; asthma; biomarkers.

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Introduction

It is well established that over 3% of asthmatic patients are affected by a particularly serious pathological form ("severe asthma", SA) that does not respond to standard treatment [1]. The European Respiratory Society (ERS) and the American Thoracic Society (ATS) have recently defined SA a form which requires high-dose inhaled corticosteroids (ICS) together with a second controller with or without systemic corticosteroids to achieve asthma control or which essentially continues to be uncontrolled presenting frequent asthma exacerbations (AEs) despite specific therapy [2]. SA has been found to be a heterogeneous disease affecting patients of both genders and different ages at onset often presenting other symptoms and disturbances, such as allergies, and biochemical features including sputum or blood eosinophils [3].

Depending on the patient's airway inflammation, SA has been distinguished into: type 2-high (T2-high) characterized by eosinophilic infiltrate and T_H2-dependent cytokine overexpression (IL-4, IL-5, and IL-13) and type 2-low (T2-low), characterized by neutrophilic and pauci-granulocytic inflammation promoted by IL-6, IL-8, IL-17, IL-22, and epithelial cell-derived cytokines; the latter is rarely reported in clinical practice [4, 5].

Affecting both categories of airways (small and large), airway remodeling (AR) is frequently defined as any alteration, with respect to a normal state, in composition, distribution, thickness, mass or volume and/or number of the structural components of the airway wall [6]. AR, a cardinal feature of SA, may explain the onset of fixed airway obstruction (FAO), and may be a key factor affecting an asthma patient's outcome. Disease-related changes in the mechanical properties of the lung parenchyma may also contribute to causing FAO in these patients.

State-of-the-art, reliable, reproducible, cost-effective biomarkers could optimize how asthma patients are diagnosed and managed as well as help to predict patients' functional and clinical outcomes. But despite the collective

efforts of internationally renowned experts, to date only a few biomarkers of AR and none of lung parenchymal remodeling have been identified.

This work is concerned with the pathogenesis of airway and lung parenchymal remodeling and the serum biomarkers that may be able to pinpoint the patients with severe asthma who may develop FAO. It also intends to examine if Krebs von den Lungen-6 (KL-6), a sialylated glycoprotein, can be considered a serological biomarker of lung damage in SA.

The pathogenesis and biomarkers of airway remodeling

AR in asthmatic patients may involve epithelial fragility, goblet cell hyperplasia, enlarged submucosal mucus glands, angiogenesis, elevated matrix deposition in the airway wall, increased airway smooth muscle mass, wall thickening and elastin fiber abnormalities [7]. According to recently published data eosinophils may contribute to the pathogenesis of allergic airway inflammation in AR. Although some have suggested that persistent inflammatory infiltrate characterized by eosinophilic granulocytes is critical to the definition of AR in asthmatics [8], it is still unclear if different types of infiltrates define specific remodeling patterns and in what way they could be linked to divergent clinical or inflammatory phenotypes. Further unanswered questions are if and how neutrophils contribute to AR [9] and if an activation status could be more important than the actual number involved [10]. Although how environmental factors trigger inflammatory responses, in turn triggering AR, is still incompletely understood, there are three recognized integrated and dynamic processes underlying AR in allergic asthma: (1) the role of epithelial cells in initiation; (2) the role of immune cells in amplification; and (3) the function of mesenchymal stem cells [11]. The airway epithelium, a critical part of the innate immune system, functions as the primary interface between the environment and the lung. Considerable epithelial denudation has been found in severe as well as in newly diagnosed patients; this would suggest that mucosal inflammation is an early development. Epithelial cells respond to allergens, respiratory viruses, bacteria, cigarette smoking, and airborne pollutants by secreting soluble factors that recruit and activate immune cells [12]. Indeed, activation of epithelial pattern recognition receptors (PRR), which are expressed transmembrane or intracellulary and possibly capable of detecting molecules derived from pathogens (pathogen associated molecular patterns, PAMPs) and/or ligands derived from damaged host cells (damage associated molecular patterns, DAMPs), triggers the release of alarmin cytokines, in particular thymic stromal lymphopoietin (TSLP), interleukin-33 (IL-33), and interleukin-25 (IL-25) [7]. These alarmins are crucial inflammatory mediators that initiate immunological events through the recruitment/activation of dendritic cells (DCs), type 2 innate lymphoid cells (ILC2s), mast cells, eosinophils, and other immune cells. TSLP, which is constitutively expressed by human bronchial epithelial cells, is overexpressed in the airways of SA patients. Of interest, a bronchial allergen challenge in an asthmatic patient leads to higher TSLP+ cell expression in the epithelium and submucosa [13]. TSLP promotes allergic responses and AR by inducing the differentiation of naïve CD4+ T cells into Th2 cells and the survival of ILC2s as well as by activating epithelial-mesenchymal transition (EMT) in airway epithelial cells [14, 15]. IL-33 activates ILC2s and induces both type 2 cytokine (i.e., IL-4, IL-5 and IL-13) production in human mast cells and fibronectin release from airway fibroblasts. In turn, IL-4 and IL-13 enhance subepithelial fibrosis, mucous hyperplasia, and collagen deposition [16]. IL-33 also triggers the release of angiogenic and lymphangiogenic factors [17], leading to vascular congestion which is mirrored by increased vessel area possibly contributing to airway wall thickening [18]. Finally, IL-25, also called IL-17E, activates ILC2s, induces EMT of alveolar epithelial cells (AECs) and local tissue remodeling, and up-regulates cytokine expression in lung fibroblasts [19]. Alveolar and interstitial macrophages, which represent the most abundant immune cell population in the healthy lung, may also contribute to AR by producing Transforming Growth Factor beta (TGF-β), a multifunctional cytokine that induces EMT and releases matrix metalloproteinases (MMPs). MMPs are a family of zinc-dependent endopeptidases that degrade the extracellular matrix (ECM) causing structural changes in the airway structure. Collagen fibers, fibronectin, and tenascin are the primary components of the ECM in the asthmatic lung [20]. Airway smooth muscle (ASM) hypertrophy/hyperplasia, a hallmark of AR, is an important driver of SA pathophysiology, independently of the underlying inflammatory context. It has been estimated that increases in ASM mass range between 50 and 200% in non-fatal asthma and between 200 and 400% in fatal asthma [21]. Increases in the ASM mass contribute to bronchial obstruction, lung function decline, and heightened susceptibility to external triggers [7]. Another distinct hallmark of AR is that the layer immediately below the true basement membrane is thicker. This process, which is called subepithelial fibrosis, is chiefly mediated by submucosal resident fibroblasts that

proliferate and differentiate into myofibroblasts. Together with airway resident fibroblasts, the number, activation, and differentiation of circulating bone marrow-derived fibrocytes appear to be correlated with asthma severity [22]. Of importance, the subepithelial layer appears to thicken with disease severity, but not necessarily with its duration [23]. Despite the overlapping links between airway inflammation and remodeling, some investigators have suggested that AR may present as an initial event, i.e., even before any inflammatory process has been triggered and asthma has become symptomatic [24].

AR, which is driven by persistent eosinophilic inflammation and one of asthma's main features, is the pathological change that may explain the development of FAO. The ATS and ERS technical statement asserts that FAO is a condition characterized by a post-bronchodilator forced expiratory volume in the first second (FEV_1)/forced vital capacity (FVC) ratio of less than 0.7 or below the Lower Limit of Normal (LLN) [25]. FAO is present in approximately half of all SA patients and appears most frequently in those with long disease duration, inadequate symptom control, and insufficient response to treatment [26, 27]. Some computed tomography (CT) studies have demonstrated that airway wall thickness is increased in patients with FAO with respect to those without, and several investigators have described the adverse effects of AR on airflow obstruction and lung function in asthma patients [28, 29]. In the light of these considerations, effective biomarkers capable of early detection of AR would be able to help physicians to diagnose and to treat asthmatics thus preventing or at least mitigating the severe damage linked to FAO. While Shimizu et al. uncovered no links between eosinophilic or neutrophilic markers and airway/parenchymal remodeling parameters [30], some investigators have hypothesized that other clinically utilizable biomarkers may be able to identify changes in airway structure.

There is growing evidence that galectin-3 (Gal-3), one of the components of the macrophage scavenger receptor family, may be a diagnostic and prognostic biomarker associated with a wide range of diseases and disorders, including cancer, cardiovascular disease, autoimmunity, wound healing, allergies, and chronic inflammation in general. Elevated Gal-3 levels have been also reported in eosinophilic asthma [31]. Moreover, Gal-3 concentrations have been found to decrease by between 5- and 50-fold in the bronchial specimens of patients with severe allergic asthma receiving omalizumab, an anti-IgE monoclonal antibody (mAb). Since the reduction was correlated with an improvement in eosinophilic inflammation and remodeling and in particular with a decrease in reticular basement membrane thickness, Riccio et al. [32] hypothesized that Gal-3 positivity at baseline and improved pulmonary function test results following omalizumab treatment might be correlated. Recently Riccio et al. reported finding a significant difference in baseline plasma Gal-3 levels in responders and non-responders to omalizumab; the functional responders were defined by an increment of 0.1 L in FEV_1 at the 12 months timepoint with respect to the baseline value. Those investigators concluded that baseline Gal-3 plasma levels higher than 11 ng/mL can be considered a predictive marker identifying functional responders and super responders to omalizumab [33].

Some have hypothesized that chitinases, which are hydrolases characterized by their affinity to cleave chitin, play a role in remodeling and regulating the ECM. The chitinase-3-like protein 1 YKL-40 is a secreted glycoprotein that is normally expressed by macrophages, neutrophils, and airway epithelium. YKL-40 levels, which can be detected in both the serum and in the airways, are higher in patients with asthma and chronic obstructive pulmonary disease (COPD) with respect to healthy controls. They are also associated with subepithelial basement membrane thickness in adults as well as in children [34]. Although the biological role that YKL-40 plays in asthma patients continues to elude investigators, many reports have demonstrated that it is associated with lung function and parameters of AR [35]. In this regard, YKL-40, which studies have showed is induced by IL-6/sIL-6R but not by IL-4/IL-13, stimulates IL-8 production in bronchial epithelial cells, presenting a possible pathway leading to bronchial smooth muscle proliferation and AR [36]. When Kimura et al. evaluated the association of circulating YKL-40 levels with morphologic changes in the airways and lung parenchyma and with the longitudinal progression of airflow limitation in SA patients, they reported that the glycoprotein levels were significantly associated with CT indices of proximal AR, i.e., the proximal wall area percentage and airway fractal dimension ($r=0.25$, $p=0.01$; $r=-0.22$, $p=0.04$, respectively); those investigators thus concluded that YKL-40 is strictly associated with the pathogenesis of AR in SA patients [37].

Stimulated by IL-4 and IL-13, periostin, which is an ECM protein secreted by bronchial epithelial cells and fibroblasts, plays an important role in amplifying and prolonging the chronic inflammation linked to allergies [38]. A T2-related biomarker, serum periostin has been associated with subepithelial fibrosis and FAO in several clinical studies [39, 40]. In this regard, an inverse relationship was found between the periostin level and the degree of airflow obstruction, as measured by FEV_1% predicted [41]. Moreover, a randomized controlled trial (RCT) focusing on the effect of inhaled corticosteroids (ICS) on serum periostin levels showed that they had a significant effect on both

serum periostin and sputum eosinophil count and that the reduction was associated with improved lung function and AR [42]. Other studies have instead underlined the discordance between serum periostin and sputum eosinophilia [43]. To date, the use of periostin has been limited by the lack of standardized measuring techniques and validated predicted values.

Vitamin D is commonly known to suppress AR by blocking the activity of profibrogenic mediators such as TGF-β1 or Th1 inflammatory cytokines. An experimental Sprague-Dawley rat model study showed that treating the rats with 100 ng/mL vitamin D considerably reduced the thickness of the ASM, the deposition of collagen, the alpha-smooth muscle actin (α-SMA) mass and the inflammation of the airway [44]. Another recent experimental study showed that calcitriol pre-treatment heightened the inhibitory effect of beclomethasone 17-propionate and montelukast sodium on the expression of ACTA2, *a* marker of myofibroblasts (p=0.0072), and vimentin, an EMT marker (p=0.0002) in the HFL1 cell line [45]. Despite its critical role, Vitamin D is not generally used as a biomarker of anti-AR activity given the wide variability of serum 25(OH)D3 values associated to patients' gender, age, and concomitant chronic diseases and the season [46].

The principal features of AR biomarkers are outlined in Table 1.

The pathogenesis and structural characteristics of lung parenchymal remodeling

The pulmonary alveoli are lined in healthy subjects with types I and II alveolar epithelial cells (AEC1 and AEC2, respectively); the latter are roughly cuboidal cells mostly concentrated at the alveolar septal junctions. While flat-shaped AEC1 facilitate the transport of oxygen in the blood stream, AEC2 are a highly specialized subpopulation of the respiratory epithelium; they make up about 15% of the distal lung cells and occupy approximately 5% of the alveolar surface [47]. Several major functions such as the synthesis and secretion of surfactant, a protein-lipid complex and surface-active material, ion transport, and alveolar repair in response to injury have been attributed to AEC2 [48, 49]. Ultrastructural criteria used to identify AEC2 include the presence of lamellar bodies, apical microvilli and specific junctional proteins. AEC2 contribute to maintaining the integrity of alveolar epithelium by proliferation (and AEC1 differentiation) in response to injury, and they tightly regulate transepithelial water transport via a number of mechanisms. Consequently, when AEC2 are injured, normal alveolar epithelial fluid transport and alveolar edema fluid clearance are affected [50].

Lung parenchymal remodeling causing important structural and functional alterations in the elastic properties of the lung has been recently reported in severe asthmatic patients [30], and ever more data is being published demonstrating that the loss of lung elasticity and/or alveolar destruction, leading to mild alveolar dilation and reduced radial traction and mechanical support of the airways, may have an important effect on the development of FAO and the longitudinal decline in FEV_1 [51]. Indeed, parenchymal tissue changes seem to be critical for lung elastic recoil, which helps to maintain the airway driving pressure and subsequently airway caliber during exhalation. In particular, the integrity of the elastic fibers is critical for maintaining the interplay between the airway and the parenchyma. According to the "waterfall" model proposed by Pride et al., once the driving pressure reaches a certain point, the upstream and downstream segments are separated by a narrowing of the channel. Beyond this specific point along the airway (i.e., in the more central airways near to the mouth), no changes in the downstream resistance or further increases in the driving pressure produce any additional increase in the airflow. In this condition, the maximal airflow depends exclusively on elastic recoil, which in turn can be reduced by lung parenchymal remodeling leading to loss of elasticity [51] (Figure 1). When Gelb et al. [52] analyzed the data collected during a study focusing on a group of older non-smoking asthmatic adults with persistent maximal expiratory airflow limitation, they found that reduced elastic recoil was responsible for as much as 35–55% of the decrease in maximal flows at high lung volumes. Some investigators have reported that the loss of lung elastic recoil in asthmatic patients is associated with aging, longer disease duration and progressive expiratory airflow limitation [21].

Table 1: Characteristics of serum biomarkers for airway remodeling in severe asthma. ICS, Inhaled corticosteroids.

Biomarker	Advantages	Limits
Galectin-3	Predictive of response to biologic therapies [32, 33]	Lack of specificity [31]
YKL-40	Predictive of poor prognosis [37]	No clear relationship with other adult-onset asthma biomarkers [37]
Periostin	Predictive of response to treatment with ICS [42]	Lack of validated predicted values [38]
Vitamin D	Commonly tested in clinical practice	Affected by multiple confounders; low specificity [46]

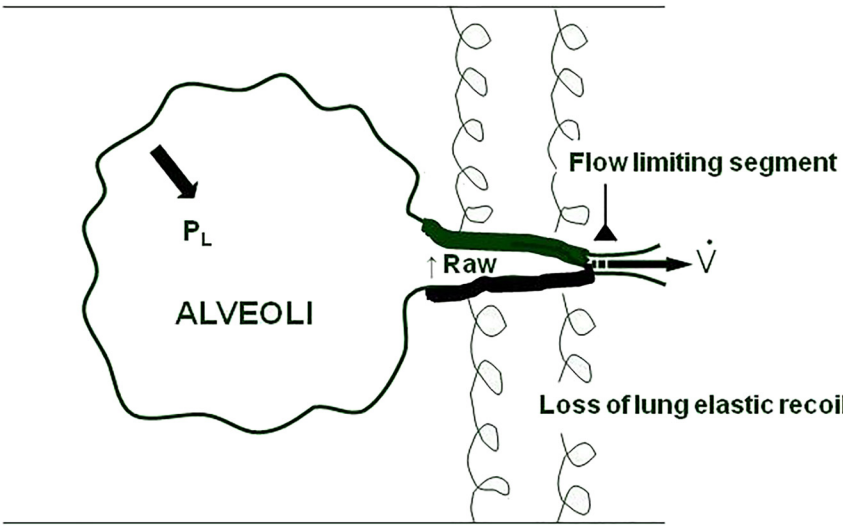

Figure 1: The "waterfall" model and the contribution of airway and lung remodeling to airflow limitation. PL, transpulmonary pressure; Raw, airway resistance.

Data collected during autopsies of adult nonsmoking patients who succumbed to an acute asthma attack showed alveolar abnormalities including cleavage of terminal bronchiolar-alveolar tethering attachments with fragmented and decreased abnormal elastic fibers and localized destruction with emphysema [53]. Research based on post-mortem lung resection and transbronchial biopsies from SA patients uncovered neutrophilic, eosinophilic and lymphocytic inflammation and remodeling extending from the peripheral airways to the adventitia and lung parenchyma [54]. In the light of these observations, it appears that the inflammatory milieu associated to alveolar injury [55] may represent a potential trigger for AEC2 regeneration and lung repair processes in SA patients.

The pathophysiologic mechanisms underlying parenchymal remodeling and the loss of lung elastic recoil in asthmatic patients are not entirely understood. Some investigators have hypothesized that activation of proteolytic cascades affecting the parenchymal tissue may be the mechanism underlying parenchymal damage and FAO development [56]. Indeed, airway inflammation in asthma affects the entirety of airway wall thickness including the outer airway wall and the surrounding alveolar tissue [57]. Moreover, the outer walls of the small airways are important sites of ECM remodeling and they may be involved in functional changes and in the loss of airway-parenchyma interdependence that has been observed in patients with fatal asthma [58]. Finally, there have been reports that proteolytic enzymes such as MMPs are increased in the airway wall and peribronchiolar parenchyma of patients who succumbed to fatal asthma [59].

In summary, many studies have reported finding structural changes in the lung parenchyma of severe asthmatics with FAO demonstrating that parenchyma inflammation and remodeling leading to functional impairments may affect clinical outcomes. In the light of these considerations, reproducible, reliable biomarkers of lung parenchymal remodeling would greatly aid physicians to identify and manage SA patients.

KL-6: a possible biomarker of lung parenchymal remodeling?

Krebs von den Lungen-6 (KL-6), a mucinous extracellular high-molecular-weight (200 kDa) glycoprotein, is mainly distributed on the apical surface of normal glandular epithelial cells of the lung, stomach, pancreas and breast. The results of some studies have suggested that the glycoprotein could be a biomarker for lung, breast, and pancreatic cancer, although it appears to have lower diagnostic accuracy with respect to others, such as carcinoembryonic antigen (CEA) [60, 61]. KL-6 is expressed in the lung by AEC2, the bronchiolar epithelial cells and by the serous bronchial gland cells [62]. Cleaved at the cysteine bond near the epithelial membrane surface, KL-6 is released into lung lining fluid and can be measured in bronchoalveolar lavage (BAL) fluid [50]. Reflecting alveolar damage and/or regeneration, KL-6 diffuses into the bloodstream when AEC2 are injured and/or the permeability of the alveolar-capillary barrier is altered [63, 64]. Clinical and experimental studies have shown that there is an association between circulating KL-6 levels and epithelial/endothelial permeability parameters, suggesting an association between higher serum KL-6 levels and air-blood barrier dysfunction [64, 65]. Serious damage to both the pulmonary epithelium and the interstitium seems to be required to

produce elevated circulating KL-6 levels [66]. As KL-6 functions as a chemotactic factor for human fibroblasts, the high concentrations of the glycoprotein found in epithelial lining fluid may trigger intra-alveolar fibrosis; this suggests that it may have a critical effect on the development and evolution of pulmonary fibrosis [67]. KL-6 has been exhaustively studied as a diagnostic and prognostic biomarker in patients with different types of fibrotic interstitial lung disease (ILD), and in fact significantly higher serum levels with respect to those in control subjects [67, 68] and a significant association with the severity of clinical symptoms and gas exchange abnormalities have been demonstrated [69, 70]. Just as ILD, KL-6 has been shown to be a specific, sensitive indicator of parenchymal lung damage objectively reflecting the severity of pulmonary injury in young patients with bronchopulmonary dysplasia [71]. Increased serum KL-6 levels have also been reported in clinically stable COPD patients with respect to non-COPD control counterparts [72]. Peng et al. recently reported finding significantly higher serum KL-6 concentrations in patients with severe COVID-19 at the time of hospital admission with respect to patients with milder disease [73]. Conditions that can potentially elevate KL-6 levels are outlined in Table 2.

Since KL-6 seems to reflect alveolar damage and regeneration in connection to various respiratory diseases and important structural changes have been reported in the lung parenchyma of severe asthmatics, including loss of elastic recoil due to localized emphysema and alveolar inflammation, we hypothesize that the glycoprotein may be able to identify asthmatics with lung remodeling leading to FAO and to predict their response to treatment. To date, only a few investigators have examined the link between KL-6 levels and asthma diagnosis and severity. One of these was Imai et al. who conducted an observational multiple case-control study investigating KL-6 levels in pediatric patients with or without respiratory diseases. The KL-6 levels in the 101 samples from 86 patients in the bronchial asthma group were significantly higher with respect to those from the healthy controls. Moreover, they were abnormally elevated at the time of acute exacerbation and recovery periods, but normal during clinically stable ones. Those investigators concluded that there had been some alveolar damage in the exacerbated cases and that an increase in alveolar pressure and/or alveolar hyperinflation might be linked to the alveolar damage [74]. These conclusions can however be considered arguable. First and foremost, the upper-normal cut-off value of plasma KL-6 was set at 250 U/mL, despite the fact that it is commonly agreed that KL-6 is normally lower than 500 U/mL even in newborn and children [71]. Moreover, the highest plasma KL-6 value during acute exacerbation was below 400 U/mL, that is, within the range of normality. Furthermore, only 16 out of the 45 patients (35.6%) with acute exacerbation of asthma studied by Imai et al. had relatively higher levels of plasma KL-6. Thus, no significant correlation between plasma KL-6 levels and the severity of the asthma attack was noted.

Table 2: Conditions potentially increasing the serum level of KL-6. CTD-ILD, Connective tissue disease-interstitial lung disease.

- Interstitial lung diseases [67, 68]
  - CTD-ILD
  - Idiopathic pulmonary fibrosis
  - Acute hypersensitivity pneumonitis
  - Pleuroparenchymal fibroelastosis
- Bronchopulmonary dysplasia [71]
- Clinically stable COPD [72]
- Acute asthma exacerbation (pediatric population) [74]
- Severe eosinophilic asthma (adult population) [75]
- Severe COVID-19 [73]

Bergantini et al. recently investigated 28 patients with severe eosinophilic asthma who were being treated with mepolizumab or benralizumab, new anti-IL-5 antibodies, to evaluate KL-6's potential as a bioindicator of airway hyperresponsiveness and remodeling. The patients were stratified into "early responder" and "partial responder" groups. Data analysis uncovered that higher levels of peripheral eosinophils, sL-selectin, and KL-6 concentrations at baseline were associated with early (within 1 month) response to mepolizumab treatment. They concluded that together with peripheral eosinophils and sL-selectin, KL-6 can be considered a biomarker that can identify "early responders" to mepolizumab [75]. The presence of an eosinophilic infiltrate extending from the peripheral airways to lung parenchyma [55], subsequently attenuated by mepolizumab treatment, could explain the increased KL-6 levels reported in these subjects. It is important to point out, however, that the levels of KL-6 found in the asthmatic patients studied fell, as in the case above, within the normal range.

In summary, only a few heterogeneous studies examining this subject are available and the results collected until now do not irrefutably demonstrate that KL-6 may be a helpful diagnostic and prognostic tool in identifying SA patients with lung remodeling leading to FAO and predicting their clinical course and response to therapy.

Conclusions

Evidence is growing that SA should not be considered a single disease entity but rather a syndrome comprising

several pheno/endotypes characterized by different underlying molecular mechanisms. Our review highlights the importance of airway and lung parenchymal remodeling as the pathological basis for the development of a SA phenotype that features FAO, does not respond to standard treatment, and is frequently accompanied by an accelerated decline in lung function. It also discusses currently available serum biomarkers that are able to identify subjects at risk of developing FAO. Finally, it examines the hypothesis that KL-6 may be able to predict the clinical course and response to biologic therapy in this particular asthmatic phenotype.

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