EDITORIALS

ⓐ Zooming into the Matrix: Using Nonlinear Optical Microscopy to Visualize Collagen Remodeling in Asthmatic Airways

The extracellular matrix (ECM) is of fundamental importance for the functional capabilities of the lung because it serves to maintain tissue tensile strength, elasticity, and barrier function. Furthermore, it is increasingly apparent that the ECM not only acts as a scaffold for tissue resident cells but also directly orchestrates essential cellular processes, including morphogenesis, signal transduction, migration, proliferation, and wound repair (1). Accordingly, an aberrant ECM, as is frequently seen in chronic lung diseases, is not only an end-stage pathological manifestation that compromises tissue functionality but also likely dictates the development and progression of the disease. Collagen accumulation around the airways of patients with asthma is a hallmark pathological feature of ECM remodeling that is associated with irreversible changes in airway obstruction (2). However, our understanding of these pathological changes is limited to a macroscopic assessment of the gross quantities of collagen as adjudged by historical histological stains, and this tells us very little about how the structural or biochemical properties of the collagen may be aberrant. This oversimplification likely neglects potentially important differences in the organization of the collagen, which could impact its capacity to modulate cellular behaviors and define tissue architecture and functionality.

In a study presented in this issue of the *Journal*, Mostaço-Guidolin and colleagues (pp. 431–443) used nonlinear optical microscopy (NLOM) to assess the biochemical and structural features of collagen and elastin in nontransplantable donor lungs from individuals with and without asthma (3). The nonlinear interactions that are the basis of the images generated by NLOM occur naturally between light and specific biological molecules owing to their intrinsic natural properties, thus allowing these macromolecules to be imaged without the application of exogenous stains. In their elegant study, the authors comprehensively demonstrate that fibrillar collagen was not only increased in mass within the lamina propria of small and large airways of patients with asthma, but also disorganized and fragmented. These changes were apparent in patients with asthma regardless of age or sex, and were comparable between patients with fatal disease and those with nonfatal disease. Rationalizing these changes, the authors demonstrated that fibroblasts isolated from the airways of patients with asthma were defective in collagen I fiber formation relative to those derived from healthy control subjects. They attributed this failing to a reduced expression of decorin (a proteoglycan that is

required for normal spacing of collagen fibrils within fibers) by airway fibroblasts from subjects with asthma. In support of this assertion, the authors demonstrated that packaging of collagen fibrils was indeed disorganized in the airways of patients with asthma.

The study by Mostaço-Guidolin and colleagues represents a step change in the manner in which we should now view the ECM in defining a disease state. It is no longer enough to consider the relative levels of ECM macromolecules—we should also consider the potential implications of more subtle differences in morphology and physiology. However, these studies inevitably raise further questions. At present, the postulated mechanism whereby reduced fibroblast decorin expression drives disorganized airway collagen in patients with asthma remains primarily correlative and needs to be proved experimentally. The authors' assertion that changes in decorin are causal is supported by observations that decorin knockout mice present with collagen fibril disorganization in the skin and tendons (4). Future studies should seek to manipulate decorin levels in murine models of allergic airways disease and primary patient cells to validate that they impact collagen organization and ultimately asthma pathophysiology. Furthermore, it is important to confirm that decorin expression is indeed reduced in fibroblasts proximal to the disorganized airway collagen of patients with asthma, as well as to gain a fuller understanding of the pathways that define its expression and determine whether they can be therapeutically manipulated.

It is interesting that disorganized collagen is observed in the airways of patients with asthma regardless of sex and age, and whether they have fatal or nonfatal disease, but this ultimately raises questions about the role that changes in collagen organization play in driving disease pathology and severity. Analysis of cadavers, although clearly advantageous for whole-lung analysis of ECM by NLOM, has limitations with regard to the availability of paired patient histories. In the future, it would be of interest to correlate these observed changes in collagen organization with underlying inflammation, lung function, disease severity, and, more broadly, patient endotype. This ultimately raises a more generic question as to the physiological significance of disorganized collagen. The abundance of collagen fibers correlates with the resistance and elasticity of parenchymal tissues (5), and the organized assembly of collagen fibrils is of central importance in defining the tensile strength of the collagen (6). However, there is clearly a need to more fully understand the effects of disorganized collagen on airway mechanics and airway hyperresponsiveness. More broadly, given the capacity of ECM macromolecules to regulate pleiotropic cellular activities, it will be intriguing to determine how structural changes in collagen impact its signaling capacity. A more in-depth understanding of the processes that drive disorganized airway collagen in individuals with asthma, and ensuing implications of these changes in regulating airway mechanics and
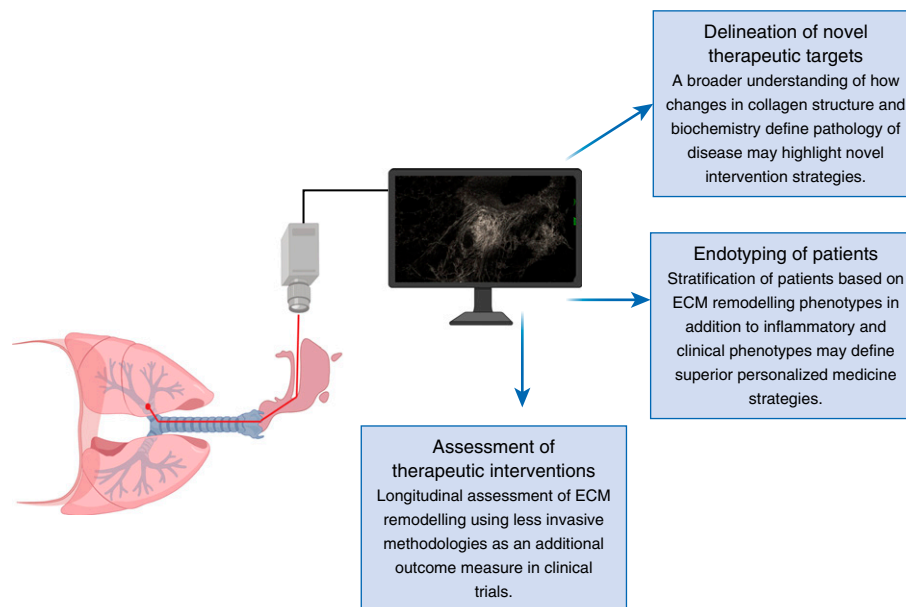
Figure 1. Future clinical applications for nonlinear optical microscopy. The ability to combine nonlinear optical microscopy with bronchoscopy to generate images of extracellular matrix (ECM) macromolecular structure without the use of exogenous stains makes this approach an exciting, less invasive way to longitudinally assess airway remodeling in patients.

behavior of proximal cells, could ultimately lead to identification of new therapeutic targets.

The chronic inflammation observed in asthmatic airways was classically believed to be the driver of airway remodeling, but it is now acknowledged that both processes can occur in parallel and independently, and are of comparable importance in defining the clinical course of disease (7). Given the importance of both airway inflammation and remodeling in defining asthma pathogenesis, it is noteworthy that there is significant disparity in the assessment of these dual processes. The vast majority of clinical studies have focused on assessment of the inflammatory rather than airway remodeling profile of patients owing to the challenges of assessing airway remodeling noninvasively, with analysis generally necessitating bronchial biopsies. It is intriguing, therefore, that NLOM has successfully been coupled with endoscopy techniques for the assessment of airways *in vivo* (8), and given that visualization of collagens and elastin by NLOM does not necessitate the addition of exogenous stains, it is clear that this represents a potentially exciting, less invasive technology with clear clinical applications (Figure 1). Ideally, unbiased statistical approaches to define asthma endotypes should incorporate a remodeling phenotype together with routinely assessed clinical and inflammatory parameters, as the contributions and interactions of each factor will ultimately dictate disease outcomes. It is tantalizing to consider that the combination of endoscopy with NLOM technology could lead to a more routine assessment of airway collagen and elastin deposition in individuals with asthma, which could be tracked longitudinally with inflammation and clinical parameters to delineate the evolution of the disease. Therapeutic strategies for asthma are entering an age of personalized medicine (9, 10), and incorporating remodeling phenotypes to stratify patients will be fundamentally important for identifying optimal treatment regimens. Furthermore, assessment of airway remodeling is rarely included as an outcome measure in clinical trials owing to the invasive nature of tissue biopsies, but the

use of NLOM could facilitate a longitudinal interrogation of the effects of therapeutic interventions on changes to the ECM and ensuing disease control or remission. ■

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Modern Inflammatory Phenotyping of Asthma
Breathomics Is Here to Stay

The key progress in the management of asthma has been the recognition that individual phenotyping of patients, rather than standard assessment of the diagnosis and asthma severity, is a prerequisite for optimal disease outcome (1). This is predominantly based on the concept that inflammatory profiling enables us to improve the efficacy of steroids and modern biologicals targeting type 2 mechanisms (2). Hence, sputum eosinophils, and in some circumstances circulating eosinophils, have definitively shown their clinical value in the stratification of patients for interventions aimed to at least suppress asthma exacerbations.

This paradigm shift has been around for at least a decade, but is it catching on in the clinic? Remarkably, it is not. The main reason is not any dispute on the (highest) levels of evidence for this, but most likely the understandable hesitation to embark on complex (sputum) or invasive (blood) procedures for repeated assessment during the monitoring of patients with asthma.

Modern medicine is showing three developments that are changing the scene. First, capturing information on operational biological networks can now be done far beyond the mere presence of inflammatory cells. For instance, sputum eosinophilia or neutrophilia in asthma appear to be each associated with multiple, distinct molecular networks, as shown by high-throughput molecular analysis using transcriptomics (3) or proteomics (4) in sputum. Second, the information technology to accurately validate these "big data" for clinical precision medicine has been shaped (5). Third, it is now feasible to identify molecular fingerprints by noninvasive technologies, using metabolomics in exhaled air (6). This so-called "breathomics" is widely being validated and can also be used for inflammatory phenotyping of patients with asthma.

In this issue of the *Journal*, Schleich and colleagues (pp. 444–453) present a very solid study on the inflammatory phenotyping of patients with asthma by identification of exhaled volatile organic compounds (VOCs), using two different versions of well-standardized gas chromatography and mass spectrometry with varying resolutions (gas chromatography–mass spectrometry (GC-MS) and two-dimensional gas chromatography coupled with high-resolution time-of-flight mass spectrometry) (7). The authors recruited more than 500 patients from two separate cohorts (discovery and replication), each split into training and test sets, thereby establishing the largest GC-MS study in asthma that is meeting the international Standards for Reporting of Diagnostic Accuracy (STARD). This is exactly what is needed in the field, as sufficient statistical power and external validation are essential for establishing exhaled biomarker profiles for asthma. The authors used the presence or absence of sputum eosinophilia and/or neutrophilia as the gold standard of the inflammatory profile of the patients, and applied appropriate statistics for examining the discriminatory power and classification accuracies, as obtained by the VOCs.

The results show that eosinophilia and neutrophilia in sputum can be captured and discriminated by using various combinations of merely two to four exhaled VOCs. This included compounds such as hexane, 2-hexanone, and an unknown VOC for identification of eosinophilia, and 3-tetradecene with pentadecane for establishing neutrophilia. The two inflammatory profiles could be mutually discriminated by using 3,7-dimethylnonane, nonanal, and 1-propanol. In general, the areas under the receiver operating characteristic curves were excellent in the discovery study (0.85–0.99), whereas these areas under the curve reached more modest values in the replication study (0.68–0.73). Such loss in accuracy is generally to be expected, particularly in this project, as the discovery and replication studies were performed using different technologies. However, this has also a meaningful up side; namely, that the authors are providing true validation by using independent cohorts and methodologies.

Interestingly, the VOCs reached similar accuracies against sputum eosinophilia as exhaled nitric oxide ($F_{E_{NO}}$) and blood eosinophil counts, whereas the combination of these three biomarkers performed best by far. Even though combining three biomarkers systems may not be appealing for clinical practice, this observation suggests that VOCs, $F_{E_{NO}}$, and circulating eosinophil counts provide complementary information in relation to estimating sputum eosinophilia. One could even question whether sputum eosinophilia remains the appropriate gold standard for validating exhaled VOCs. The biological complexity of asthma is likely to be more comprehensively captured by composite molecular fingerprints than the mere presence of a granulocyte in sputum. Therefore, the limited accuracies of VOCs against sputum eosinophilia is not discouraging at all, as it may well be that the VOCs themselves will eventually do a better job in stratifying patients than cell counts.

Will GC-MS qualify for application in the clinic? Not at the moment, so the present study should be regarded as proof of principle. The value of the study is represented by the identification of particular VOCs in relation to inflammatory profiles. It should be emphasized that the present selections of discriminative VOCs were data-driven. Hence, the present high-throughput GC-MS platforms represent breathomics, not being based on *a priori* selection. This approach is empirical and hypothesis generating, providing a set of VOCs apparently being reproducibly discriminative for eosinophilia