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An Investigation of Mucus Plugging in Severe Asthma

Submitted to the National University of Ireland, Galway for the degree Doctor of Philosophy (PhD)

By

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May 2018
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Declaration

This thesis describes work I undertook between 2013 and 2018 at the University of California San Francisco. This work was supervised and mentored by Professor John Fahy at University of California San Francisco and Professor Anthony O’Regan at National University of Ireland, Galway.

I, Eleanor Marguerite Dunican, declare that the results presented in this thesis have not been submitted for degree, diploma or other qualification at any other university. I conducted the experiments presented and wrote the thesis under the supervision of Professors Anthony O’Regan and John Fahy. There were a few occasions when the experimental work was performed in collaboration with other investigators and these are clearly indicated in the relevant figure legends. Specifically, five radiologists were involved in the development of the mucus score and scored the CT scans.

I published several of the methodologies and results presented in this thesis in the Journal of Clinical Investigation under the title “Mucus plugs in patients with asthma linked to eosinophilia and airflow obstruction” (February 5th 2018). I am the first author on this publication.

Competing financial interests: I am listed as an inventor on a patent (WO2017197360 A1) related to the development and application of the mucus score (“Dunican Score”) as a biomarker and companion diagnostic tool in pulmonary disease.

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Abbreviations

ABPA    Allergic bronchopulmonary aspergillosis
ACQ     Asthma control questionnaire
ACT     Asthma control test
AQLQ    Asthma quality of life questionnaire
ATS     American thoracic society
BALF    Bronchoalveolar lavage fluid
BE      Brett Elicker (radiologist)
BMI     Body mass index
BODIPY FL Boron-dipyrromethene fluorophore
CF      Cystic fibrosis
CMH     Chronic mucus hypersecretion
CONA    Clinical Trial of NAC in Asthma
COPD    Chronic obstructive pulmonary disease
CRTH2   Chemoattractant receptor-homologous molecule expressed on TH2 cells
CS      Corticosteroids
CT      Computerized tomography
CTDIvol CT Dose Index, per kg of tissue
CXR     Chest X ray
DCC     Data coordinating centre
DG      David Gierada (radiologist)
DNA     Deoxyribonucleic acid
DTT     Dithiothreitol
ED      Eleanor Duncan (author)
EPO     Eosinophil peroxidase
ERS     European respiratory society
FeNO    Fractional expired nitric oxide
FEV1    Forced expiratory volume in first second
FoxA2   Forkhead box A2
FRC     Functional residual capacity
FVC     Forced vital capacity
<table>
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<td>GABAAR</td>
<td>γ-aminobutyric acid a receptor</td>
</tr>
<tr>
<td>GuHCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>³He</td>
<td>Helium-3</td>
</tr>
<tr>
<td>HRCT</td>
<td>High resolution computerized tomography</td>
</tr>
<tr>
<td>HU</td>
<td>Hounsfield unit</td>
</tr>
<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled corticosteroids</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILC2</td>
<td>Type 2 innate lymphoid (cell)</td>
</tr>
<tr>
<td>JF</td>
<td>John Fahy (author)</td>
</tr>
<tr>
<td>JN</td>
<td>John Newell (radiologist)</td>
</tr>
<tr>
<td>LA</td>
<td>Lumen area</td>
</tr>
<tr>
<td>KSCN</td>
<td>Potassium thiocyanate</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>MBRT</td>
<td>Maximum bronchodilator reversibility test</td>
</tr>
<tr>
<td>MDCT</td>
<td>Multidetector computerized tomography</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Mark Schiebler (radiologist)</td>
</tr>
<tr>
<td>MUC</td>
<td>Mucin gene</td>
</tr>
<tr>
<td>NaBr</td>
<td>Sodium bromide</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC20</td>
<td>Concentration of methacholine needed to produce a 20% fall in FEV1 from baseline</td>
</tr>
<tr>
<td>PEFR</td>
<td>Peak expiratory flow rate</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RIN</td>
<td>RNA integrity number</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonuclease acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RV</td>
<td>Residual volume</td>
</tr>
<tr>
<td>Acronym</td>
<td>Term</td>
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<tr>
<td>SARP</td>
<td>Severe asthma research program</td>
</tr>
<tr>
<td>SCRT</td>
<td>Systemic corticosteroid responsiveness test</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SH</td>
<td>Thiol group</td>
</tr>
<tr>
<td>SN</td>
<td>Scott Nagle (radiologist)</td>
</tr>
<tr>
<td>SPDEF</td>
<td>SAM pointed domain containing ETS transcription factor</td>
</tr>
<tr>
<td>S-S</td>
<td>Disulfide bond</td>
</tr>
<tr>
<td>TA</td>
<td>Total area</td>
</tr>
<tr>
<td>TCEP</td>
<td>Tris (2-carboxyethyl) phosphine hydrochloride</td>
</tr>
<tr>
<td>Th2</td>
<td>Type 2 T helper (cell)</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>TTF-1</td>
<td>Thyroid transcription factor-1</td>
</tr>
<tr>
<td>WA</td>
<td>Wall area</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wall thickness</td>
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Acknowledgements

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My time in San Francisco during these past 5 years has changes my life forever and I will never forget all the people I have met and all the experiences I have lived. To all of you, many thanks.
Abstract

The classic pathologic finding in patients with fatal asthma is of markedly hyperinflated lungs. This hyperinflation is the consequence of widespread plugging of the segmental and sub-segmental airways. In contrast to fatal asthma, the link between mucus plugs and airflow obstruction is not established in chronic asthma, and the role of eosinophils and their products in mucus plug formation is unknown. I hypothesize that patients with chronic asthma who have a significant number of airway mucus plugs are characterized by a low forced expiratory volume in 1s (FEV1), airway type 2 inflammation and abnormal mucin gene expression. Furthermore, I hypothesize that eosinophils play a role in mucus plugs formation.

To test these hypotheses, I developed a novel bronchopulmonary segment-based scoring system to quantify mucus plugs on multidetector computed tomography (MDCT) lung scans. I applied the score to the MDCT lung scans of 146 subjects with asthma and 22 healthy controls in the Severe Asthma Research Program, and analysed relationships between mucus plug scores, FEV1, symptoms of chronic mucus hypersecretion (CMH), treatment responses, and measures of airway inflammation. In bench studies, I used model systems of airway mucus gels to explore whether oxidants generated by eosinophil peroxidase (EPO) oxidize cysteine thiol groups promote mucus plug formation.

Mucus plugs were seen in 58% of subjects with asthma and in 4.5% of controls, and plugs in subjects with asthma were seen in the same segment for years. A high mucus score (plugs in ≥ 4 segments) occurred in 67% of subjects with asthma FEV1 < 60% of predicted volume, 19% with FEV1 60-80%, and 6% with FEV1 > 80% (p<0.001) and also predicted residual abnormalities in FEV1 after albuterol and corticosteroid treatment. Symptoms of CMH were absent in the majority of patients with a high mucus score. A high mucus score was associated with markedly higher sputum eosinophils and EPO, and moderately higher type 2 cytokines. A high mucus score was associated with altered mucin gene expression with moderate overlap between groups. Ex vivo experimental models of mucin crosslinking demonstrated that EPO catalyses oxidation of thiocyanate by H₂O₂ to generate oxidants that crosslink cysteine thiol groups and stiffen thiolated hydrogels.
In conclusion, MDCT reveals an asthma phenotype characterised by mucus plugs in subsegmental airways, low FEV1, and marked type 2 inflammation, that is largely refractory to corticosteroid treatment. Overlap in mucin gene expression between groups limits their predictive value. Mucus plugs are a plausible mechanism of chronic airflow obstruction in severe asthma, and EPO-generated oxidants may mediate mucus plug formation. I propose a novel approach for quantifying airway mucus plugging using MDCT lung scans and suggest that treating mucus plugs with mucolytics or inhibitors of type 2 inflammation may improve airflow in chronic severe asthma.
Chapter 1

Chapter 1 Introduction and Research Questions

1.0 Introduction

Asthma affects 235 million people worldwide and represents a growing public health problem internationally (1). In the United States, 1 in 12 (approximately 25 million) had asthma in 2009, compared to 1 in 14 (approximately 20 million) in 2001 (2). In Ireland, 7.1% of the adult population have asthma, giving Ireland the 4\textsuperscript{th} highest prevalence of asthma in the world (3).

Severe asthma is asthma that requires high doses of corticosteroids and other asthma medications in order to maintain symptom control. Patients with severe asthma experience greater morbidity and disproportionate health care needs compared to patients with non-severe asthma. Severe asthma affects approximately 10% of individuals with asthma in the United States and accounts for more than half of the annual costs ($20 billion) of asthma (4). In 2003, approximately 6,300 Irish patients met the GINA criteria for severe asthma and the annual cost of emergency care and hospitalisation for asthma (3) was in the region of €227 million (5). Treatment-resistant asthma is a frequent feature of severe asthma where adequate asthma control cannot be achieved using standard asthma treatments. This group of asthma has been termed ‘severe refractory’ or ‘difficult-to-treat’ asthma. These patients are often treated with high-dose systemic corticosteroids for extended periods in an attempt to gain asthma control and experience significant side effects from this treatment.

Current asthma guidelines emphasize categorizing asthma patients into mild, moderate, and severe based upon an assessment of asthma symptoms (6, 7). Correspondingly, treatment algorithms for asthma recommend escalating asthma medications in response to increasing asthma symptoms (6, 7). This step-wise and symptom-based approach has been effective at decreasing asthma morbidity in some asthma patients (8, 9), but the relative impact of these guidelines on decreasing asthma
hospitalizations or improving asthma control has been small (8, 10). These symptom-based approaches originate from a simplified view of asthma and do not recognize asthma as a heterogeneous disease both at the clinical and molecular levels. A one-size-fits-all paradigm continues to pervade asthma management with few opportunities to match differences in underlying disease biology to specific treatments.

1.1 Clinical and Molecular Phenotypes in Severe Asthma

One of the great challenges of severe asthma is that it is a heterogeneous disease with distinct clinical phenotypes characterized by differences in susceptibility to exacerbation, loss of lung function, chronic mucus hypersecretion, and refractoriness to anti-inflammatory therapy. To further understand severe asthma, investigators in the Severe Asthma Research Program (SARP)-2 used cluster analysis to identify five phenotypes of asthma that differed in multiple ways, including age of asthma onset, gender, body weight, degree of airflow limitation, reversibility of airflow limitation, and asthma exacerbation frequency (11). This work has reinforced the concept that asthma is a disease that affects a diverse array of individuals and has provided a basis for researchers to associate complex phenotypic profiles with disease in asthma (11-14). However, using phenotypic traits to understand the pathophysiology of asthma has limitations. The main limitation is that phenotypic characteristics can be caused by multiple biologic processes. For example, identifying subjects with lower FEV1 measurements does little to support a specific biologic cause since airflow obstruction can be caused by coinciding disease processes (e.g., smooth muscle contraction, mucus impaction, and sub-epithelial fibrosis). Consequently, identifying these patients does little to determine an appropriate treatment target.

An alternative approach to studying heterogeneity focuses on identifying the molecular pathways that drive asthma with the objective of identifying disease “endotypes,” defined as disease subtypes with distinct functional or pathobiological mechanisms (15, 16). Examples of endotypes within asthma include aspirin-exacerbated respiratory disease (17), severe asthma with fungal sensitization (18, 19) and “Type-2 high” asthma (15). To date, the most well-established disease endotype
in asthma is type-2 high asthma or asthma that is driven by increased airway type 2 inflammation (15). Type 2 inflammation describes an inflammatory pathway involving a subpopulation of CD4+ T cells known as Th2 cells that secrete IL-4, IL-5, and IL-13 and stimulate type 2 immunity, which is characterized by high IgE antibody titres and eosinophilia (23). Upstream events in the airway epithelium involving master regulators such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) regulate maturation of CD4+ T cells into Th2 cells and overproduction of type 2 cytokines (IL-4, IL-5, and IL-13). Type 2 cytokines drive a cascade of downstream events, including activation of airway epithelial cells, chemoattraction of effector cells (mast cells, eosinophils, and basophils), and remodelling of the epithelium and subepithelial matrix (24).

Although type 2 inflammation plays an important role in asthma pathophysiology, not all patients with asthma have type 2 inflammation in their airways. Gene expression levels of type 2 cytokines (IL-4, IL-5, and IL-13) and of epithelial cell genes that are upregulated by IL-13 (periostin, CLCA1, and SERPINB2) range from low to high in airway biospecimens from patients with asthma (25, 26), and there is a threshold level of expression in the airway that, when exceeded, defines a distinct, “type-2 high” endotype of asthma. Type-2 high asthma is characterized by greater airway hyperresponsiveness, airway and systemic eosinophilia, and responsiveness to glucocorticoids and inhibitors of type 2 inflammation. Only 50% patients with asthma have the type-2 high endotype (25, 26), and alternative mechanisms must operate to drive disease in “type-2 low” asthma. The pathways operating in type-2 low asthma have not been elucidated and are a subject of ongoing research.

This molecular approach to better understand the pathophysiology of asthma has relied on the development of biomarkers, defined as objectively measured characteristics that indicate a biological process. Because biomarkers quantify molecular pathways, they can then be used to understand how molecular abnormalities lead to disease phenotypes or significant clinical outcomes, such as asthma exacerbations. Currently, the most prominent biomarkers in the field of asthma have been indicators of airway type 2 inflammation: blood eosinophil cell counts (20), airway type 2 gene expression profiles (21), and blood periostin levels (22).
strength of this approach is its potential to advance a “precision medicine” treatment paradigm in asthma. Specifically, treatments can be developed to target dysfunctional molecular pathways identified by biomarkers.

Given the relative refractoriness to standard asthma therapies seen in severe asthma, there is unmet need among patients with severe asthma for new treatments and approaches to asthma care. For progress to be made it will be necessary to have a better understanding of the molecular underpinnings of asthma phenotypes so that 1) specific treatments can be targeted to subgroups who will benefit, and 2) biomarkers to identify them can be validated. In this thesis, I propose and explore a new phenotype of asthma characterized by airway mucus plugging. Pathologists have recognized the central role of diffuse mucus plugging of the airways in the pathophysiology of asthma for more than 100 years (23). However, clinicians often underappreciate mucus dysfunction in asthma, possibly because cough infrequently results in expectoration or because the unavailability of therapies to clear mucus plugs has diverted attention exclusively toward reversing bronchoconstriction and inflammation (24). Understanding the biology and pathophysiology of mucus dysfunction in asthma is an important step towards identifying novel targets for mucus directed therapy.

1.2 Mucus Plugging as a Phenotype in Asthma

1.21 Mucus plugging and fatal asthma

Mucus dysfunction has been underappreciated as a cause of respiratory failure in severe asthma. Indeed, there is strong evidence that it may be the principal cause. Airway obstruction with partially formed or complete mucus plugs has been documented in the lungs of patients with fatal asthma for over 100 years but it was Huber and Koessler’s report in 1922 provided one of the earliest systematic studies of the morbid anatomy of asthma (25). Their report emphasized the presence of intraluminal mucus secretion, airway epithelial desquamation and goblet cell hyperplasia. They extended on previous descriptions by showing that the tenacious mucus plugs seen in the airways consisted of an exudate of plasma containing
inflammatory cells. Dunnill provided graphic descriptions in 20 cases of fatal asthma, noting “a striking picture with numerous grey, glistening, mucous plugs scattered throughout the airway passages” (26). The consequences of these mucus plugs were manifestly apparent at autopsy. The lungs were markedly hyperinflated, ballooning to occupy the whole thoracic cavity, “failing to collapse once the negative intrathoracic pressure is released”. Dunnill summarized that "pathologically, the outstanding feature of the asthmatic lung lies in the failure of clearance of the bronchial secretions". Although there have been isolated reports since the 1960s of deaths without airway mucus impaction, studies have since found that cases of fatal asthma in the absence of mucus plugging are very rare and likely represent an artefact of tissue processing and sectioning since subsequent studies confirm that “empty airways” are rare occurrence in fatal asthma (27). Despite this prominence of mucus in the pathophysiology of acute fatal asthma, it was unknown if mucus occlusion of the airways occurs in chronic asthma.

Kuyper et al. provided insight into the pathophysiology of mucus plugging in fatal asthma with the finding that cells made up a higher proportion of the exudate in the small airways. This may reflect more intense inflammation in these airways or greater airway wall mechanical forces inducing disruption of the abnormal epithelial layer (27). The same group also found that while airway narrowing was less marked in younger patients with fatal asthma, luminal mucus was greater. This illustrates the catastrophic effects luminal mucus and cells with even minor degrees of smooth muscle constriction. A reduction in airway luminal cross-sectional area will amplify any increase in airflow resistance due to bronchoconstriction. Even small increases in luminal liquid lead to marked airflow limitation with bronchoconstriction. This exaggerated response is known as airway hyperresponsiveness and is characteristic of asthma.

Determining the gel-forming constituents in these mucus plugs has been challenging due to difficulty in solubilizing these “abnormally solid” plugs. Sheehan et al. used serial solutions of high concentration (6M) guanidine hydrochloride (GuHCl) to solubilize the mucus plugs of a patient that died in status asthmaticus (28). The authors found that the major constituents of the plugs were mucins, present at 50 times the
concentration of heathy subjects and 9 times the concentration of chronic asthmatics. There were low quantities of proteoglycans and DNA accounted for <1% of the plug solids. There were large quantities of non-mucin proteins, which is consistent with other histopathological studies of mucus plugs. These non-mucin proteins were removed from the gel in the first extraction with GuHCl, leaving a pure mucin gel. The authors concluded that although solutes other than mucins are present in mucus plugs in varying quantities, mucins are the principle component of gel structure in fatal asthma mucus plugs.

1.22 Mucus plugging and non-fatal asthma

Evidence has emerged that mucus plugging occurs in chronic asthma as well as fatal asthma, from examining silicone casts of the airways post mortem (29, 30). In subjects without asthma, silicone casts delineate the airways all the way to the alveolar duct level, whereas in subjects with fatal asthma, there is widespread truncation and loss of airways due to mucus plugging (30). In subjects with asthma that died of non-pulmonary causes (non-fatal asthma), we also find significant loss of airways, though not as extreme a phenotype as fatal asthma (30). Furthermore, smaller distal airways tended to be lost in non-fatal asthma, rather than the larger proximal airways lost in fatal asthma. These data strongly suggest that mucus plugs are not limited to fatal asthma but occlude the subsegmental airways in chronic severe asthma.

1.3 Pathophysiology of Mucus Dysfunction in Asthma

1.31 Mucociliary clearance

Maintaining airway patency is essential to life; consequently, sophisticated mucociliary machinery has evolved in the airway to balance mucus secretion, transport and clearance to keep the airways healthy (31). Mucociliary clearance relies both on normal mucus composition, periciliary fluid and ciliary activity. Airway mucus is the product of secretory cells (club, goblet and serous cells) in the airway epithelium. Mucus is a complex three-dimensional structure, comprising of an upper gel layer and a lower periciliary fluid (sol) layer (32, 33). Extending through the
periciliary fluid layer into the mucus layer are hair-like structures called cilia that line the surface of bronchial epithelial cells and beat in a coordinated and wavelike rhythm to transport mucus caudally. Inhaled particles become trapped in the gel layer and are transported caudally by the cilia, to be expectorated or swallowed (33) (Figure 1.1). The mucus gel layer is separated from the airway epithelium by the sol layer. Because of its low viscosity, the sol layer allows the cilia to beat rapidly, and prevents the glycoproteins of the mucus layer from adhering to the glycocalyx at the surface of the epithelial apical membrane. This enables the mucus layer to float over the epithelium and be propelled by the cilia (Figure 1.1) (31).

**Figure 1.1 Mucociliary clearance in health and disease**

Schematic of the distal bronchiole showing secretory cells (goblet cells) and ciliated epithelial cells. Goblet cells contain secretory granules and ciliated cells show cilia extending apically into the periciliary fluid. Floating above the periciliary layer is the mucus gel layer, which traps particles and pathogens and carry them apically through the coordinated beat of the cilia. Alterations in volume, composition or viscoelastic properties of mucus combine to form “pathologic mucus”. This pathologic mucus is not as easily cleared by the cilia and can accumulate in the airway leading to mucus plugging and airflow obstruction.
Normal clearance of airway mucus requires a balance between the volume and composition of the mucus, which can be altered by inflammatory conditions. Alterations in the volume, composition and viscoelastic properties of mucus, can combine in varying degrees to form abnormal or “pathologic mucus”. Airway mucus hypersecretion is pathognomonic of asthma and reflects increased output from airway secretory cells. Submucosal gland hypertrophy is a feature common to many patients with asthma, with the size of glands increasing 2 to 4-fold compared to controls (34, 35). Even in the absence of gland hypertrophy, patients with asthma may have excess luminal mucus as glands of normal size can hypersecrete under the influence of inflammatory mediators in asthma (36).

Goblet cells contribute to mucus hypersecretion in the large and small airways through metaplasia and hyperplasia (37). Epidermal growth factor and IL-13 induce both club and ciliated cells to transition into goblet cells through the coordinated actions of FoxA2, TTF-1, SPDEF and GABAAR (38). Activation of these pathways lead to upregulation of mucin gene expression (in particular MUC5AC), increased mucin production and release.

Normal clearance may be further limited by mucin tethering. Mucins have been found tethered to the apical surface of goblet cells in fatal asthma (39). This phenomenon has not been seen in chronic bronchitis or healthy controls. The absence of tethering seen in chronic bronchitis has been attributed to the abundance of neutrophils as the predominant inflammatory cells in COPD, which secrete proteases that cleave mucins. Eosinophils, which are the predominant inflammatory cells in asthma, do not have the capacity to cleave mucins (40). Mucus hypersecretion and impaired mucociliary transport has been shown to occur in cultured airway epithelial cells in response to stimulation with interleukin-13, a key mediator in asthma (39). Impaired mucociliary transport was not associated with defects in ciliary function, but instead was related to tethering of MUC5AC-prominent mucus gels to the mucus-secreting cells in the epithelium. This indicates that tethering of MUC5AC-prominent mucus gels to the epithelium causes mucostasis and likely represents a major cause of mucus plugging in fatal asthma.

1.32 Mucus composition and mucin structure
Mucus is largely composed of water, with only 3% solids. Mucins are large high molecular weight glycoproteins and make up the principal solids in mucus (Figure 1.2). One of the key characteristics of mucins is their ability to form gels, making them a key component in most gel-like secretions (41). The ability of mucins to form gels comes from their structure with two distinct regions. The N- and C- terminal regions are cysteine rich and so form disulfide linkages with other mucin monomers to form long mucin polymers (41, 42). The central region is composed of a heavily glycosylated core, which also contains internal cysteine-rich domains (41, 43, 44). Under conditions of oxidation, internal cysteine thiols can participate in disulfide crosslinking between adjacent mucin polymers, altering the biophysical properties of the gel. In cystic fibrosis, high levels of oxidative stress have been shown to promote mucin crosslinking through disulfide bonds, thereby forming highly crosslinked and elastic mucus gels (45).

Figure 1.2 Structure of mucin protein backbone

Schematic representation of the components of a generic mucin glycoprotein. (A) The mucin monomer typically consists of a cysteine-rich NH3- and –COOH termini (red) and one or more central domain(s) (green/blue). The central domains contain heavily glycosylated proteins (green) interspersed with internal cysteine domains (blue). (B) The N- and C- terminal domains form disulfide bonds with other mucin monomers end to-end, to form long polymers. (C) Internal cysteines form disulfide bonds with other mucin polymers side-to-side to form a complex entangled three-dimensional mesh, which is the basis of the elastic properties of mucus gel.
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Mucins are encoded by specific mucin genes (MUCs), with MUC5AC and MUC5B gene products being the major gel-forming glycoproteins in airways (43). In healthy individuals, MUC5AC is produced predominantly in proximal airways by surface goblet cells, whereas MUC5B is produced in surface secretory cells throughout the airways and by submucosal glands (46-48). MUC5AB and MUC5B are homotypic polymers - MUC5B monomers bind only with MUC5B and MUCAC monomers bind only with MUC5AC (24).

Sputum in patients with asthma has significantly more mucin compared to healthy controls because of increases in both MUC5B and MUC5AC mucins (49). As well as quantitative there are also qualitative abnormalities to mucus in asthma. The relative proportions of MUC5AC and MUC5B can change in asthma, with a relative excess of MUC5AC relative to MUC5B (49-51). During allergic mucous metaplasia, the production and secretion of MUC5AC increases dramatically (40-200 times normal levels) with moderate increase in MUC5B (3-10 times normal) resulting in an increase in the ratio of MUC5AC to MUC5B (37, 49). This pattern of mucin gene expression is characteristic of type 2 airway inflammation (51). In a two-step model of goblet cell metaplasia, EGFR inhibited ciliated cell apoptosis and primed these cells to respond to IL-13 (38). IL-13 in turn induces transition from ciliated cells to a goblet cell phenotype and induces the MUC5AC gene expression and release. The relative difference in mucin composition that results may have consequences for differences in biophysical properties seen in asthma compared to other inflammatory conditions (51).

1.33 Biophysical properties of mucus

Efficient mucociliary transport has been shown to be a function of the rheological properties of the viscoelastic mucus (52). The lightly cross-linked nature of mucin polymers gives mucus the necessary properties of a gel. Healthy mucus demonstrates both viscosity (a liquid property) through its resistance to flow and its capacity to absorb energy when in motion, and elasticity (a solid property) through its capacity to store energy that is used to move or deform mass. Elasticity is a necessary property of the mucus gel to transmit the kinetic energy of the cilia through the mucus layer and move it towards the pharynx (53). All of these rheological and physical properties
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enhance the ability mucus to function as a barrier, lining the airway, trapping particles and pathogens and transporting them caudally to be cleared via mucociliary transport and cough \((54)\). These biophysical properties of mucus can be measured with a rheometer. The rheometer measures the response of fluids to strain (fluid displacement) over a range of oscillatory frequencies. The response of the fluid is measured as the elastic \((G')\) and viscous \((G'')\) moduli. The elastic modulus is related to the density of molecular cross-links, whereas the viscous modulus is related to molecular weight.

In asthmatic airways, mucus cell metaplasia results in an increase in mucins compared to healthy airways. However, the dominant rheological abnormality in asthma is more consistent with increased cross-linking of mucin polymers, rather than high concentrations of mucins. On the rheometer, this is evidenced by a markedly increased elastic modulus, with less markedly increased viscous modulus. Efficient mucociliary transport has also been shown to be a function of the rheological properties of the viscoelastic mucus, and in animal models, mucociliary transport decreases with increasing gel elasticity \((52)\). In addition to polymer crosslinking, imbalance between hydration and relative concentration of solids can dramatically influence the viscoelastic properties and clearance of mucus. Mucin hypersecretion or dehydration of airway surface liquid can increase the solid concentration from 3% to as much as 15%, resulting in mucus that is more viscous and elastic, making it difficult to clear \((55,56)\).

1.4 Identification of Mucus Plugging on Imaging

Less than a hundred years ago, mucus plugging in asthma could only be identified in the airways of patients’ post-mortem. ‘Mucoid impaction of the bronchi’ in chronic asthma, was first visualized \textit{in vivo} by bronchography and then by chest radiography (CXR) \((57)\). The radiographic appearance of mucus plugging is variable. In the large airways, the mucus plugs are classically described by tubular or branching opacities, which typically radiate from the hilum toward the periphery of the lung, resembling fingers, so-called “finger-in-glove” \((58)\). When mucus is retained in bronchioles and nondilated airways, the radiographic appearance is typically normal, or subtle enough
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to be overlooked (59). Atypical appearances of mucus plugging, such as ovoid opacities, also are common (59).

Computerized tomography (CT) has now superseded plain film radiography in the imaging of the lungs, owing to the higher degree of anatomic detail. Multi-detector Computerized Tomography (MDCT) of the lungs has emerged as the imaging modality of choice for non-invasive assessment of airway anatomy, regional lung mechanics, and associated lung function (60). MDCT is particularly well suited for distinguishing mucus plugs from other pathologies of the lung. In mucus plugging of the large airways, CT images clearly show low-attenuation mucus inspissated in the bronchi, and clear connection with the central airways (59). When mucus is retained in bronchioles and nondilated airways, they are visible on HRCT as branching, ovoid and “tree-in-bud” opacities. Multiplanar reformatted images also may be of benefit for showing these characteristic findings (59).

In a study comparing CXR and CT in non-smoking patients with asthma, CT identified abnormalities in 71.9% of patients with asthma, whereas CXR identified abnormalities in only 37.6% of the patients (61). Mucoid impaction was present on CT in 21% of patients with asthma, but not identified in any of the patients on CXR. CT scans did not demonstrate mucus plugs in any of the healthy controls. In the same study, mucus plugging was present on CT in 2 of the patients at time of exacerbation. These plugs were no longer present on repeat CT, 2 weeks after treatment with corticosteroids (methylprednisolone 2mg/kg daily). Other reversible changes included acinar pattern and lobar collapse, both likely to have been related to mucus plugging, whereas bronchiectasis, wall thickening, and emphysema were unchanged. These data imply that mucus plugging can be reversible with appropriate treatment and that CT could be used as a tool in management of asthma.

There are some limitations to how mucus plugging has been assessed in CT studies of asthma. Assessment for mucus plugs has largely been qualitative rather than quantitative, and mucoid impaction has usually been assessed as one of many changes on CT. Many studies have given limited description of the criteria used to define mucus plugging (62, 63) or include in the definition non-specific findings of mucoid impaction such as centrilobular “tree-in-bud” nodules. Notwithstanding, the lack of a
systematic approach for quantifying mucus plugging on CT, the presence of mucus plugging on CT has been associated with some clinical indices of asthma severity (64, 65).

1.5 Pharmacotherapy of Mucus in Asthma

1.51 Traditional mucolytic drugs

Many potential therapies that influence the pathway from mucus signalling to secretion have been investigated with a view to addressing mucus pathology in asthma, but none have been adopted in the clinical management of asthma. Mucolytic agents reduce the viscosity and/or elasticity of mucus by lysing the principle polymers within it. The two principal mucolytic agents approved for clinical use are N-acetylcysteine (Mucomyst) and Dornase alfa (Pulmozyme®). N-acetylcysteine (NAC), has been used for many years in Europe as a mucolytic drug. NAC contains sulfhydryl groups and acts through depolymerisation of mucin oligomers by hydrolysing the disulphide bonds that link mucins together. It requires high-dose (61mmol, three times daily) nebulized administration, has an unpleasant ‘rotten-egg’ smell and can provoke idiosyncratic bronchoconstriction in patients with asthma (66-69). When administered orally (600mg daily), NAC is undetectable in the bronchoalveolar lavage (70). Studies in the use of NAC during asthma exacerbation have failed to demonstrate a benefit but were limited to case reports and trials with small sample sizes and used only the oral formulation (71). Dornase alfa is a peptide mucolytic that degrades DNA. Administered by nebulizer, it has demonstrated benefits in cystic fibrosis where DNA is released in large quantities from necrotic neutrophils into sputum. Reducing the length and size of highly polymerized DNA reduces viscosity and elasticity making mucus easier to expectorate (72). Dornase alfa has been employed in severely ill patients in status asthmaticus with the understanding that severe asthma exacerbation is characterized by predominantly neutrophilic inflammation that may have higher DNA levels than stable chronic asthma. Again, the studies in asthma are limited to case reports and small, non-randomized clinical trials.
Currently there is insufficient evidence for use of Dornase alfa in acute asthma.

1.52 Novel mucolytic drugs

Currently available mucolytics have significant limitations in terms of low potency, sort half-life and poor tolerability, that restrict their application in clinical practice. In recent years, there has been renewed interest in developing novel mucolytics that focus on reducing agents, specifically thiols, as an effective mucolytic strategy. NAC is an acetylated amino acid with a thiol group (i.e. amino-thiol) attached but has relatively weak reducing power, perhaps due a chemical tendency of the thiol to retain rather than exchange electrons. DTT has 2 thiol groups and is a potent mucolytic but is too strong a reducing agent to be used clinically. This has led to the exploration of other potential thiol-scaffolds including carbohydrates for novel mucolytics. Monosaccharides have a number of hydroxyl groups at different positions in the carbon ring that can be modified with a thiol group to generate different isomers with different chemical properties. Fahy et al. considered that a thiol-saccharide may be a stronger reducing agent than an amio-thiol and synthesized a galactose structure with a thiol group in the 6-position (45). This thiol-saccharide had a stronger reducing activity than NAC and more potent and fast acting mucolytic activity on CF sputum. In addition, initial toxicological studies in mice did not suggest any safety concerns. This group proposes thiol-modified carbohydrates as novel mucolytic treatments for pathologic mucus characterized by excessive disulfide crosslinking. A number of candidate thiol-saccharide drugs based on different sugar scaffolds with thiols in different positions have been tested and lead compounds are currently in development.

1.6 Conclusion

Mucus dysfunction is central to the pathophysiology of asthma, yet there remains an unmet need for effective treatment of pathologic mucus in asthma. Studies in the use of mucus directed therapies in asthma have been small to-date and have failed to advance our knowledge on the role of mucus therapy in promoting lung health. Understanding the biology of mucus and the mechanisms of pathologic mucus
formation in asthma will help identify novel targets for drug development. The recent finding that oxidation promotes abnormal viscoelastic characteristics in sputum through excess disulphide crosslinking is an exciting start. The future direction of research in this area should focus on identifying the subgroup of patients with asthma that have pathologic mucus as a feature of their disease severity, a so-called “mucus phenotype”. Having a clinical tool that identifies and stratifies this phenotype of asthma will enable selection of appropriate patients for intervention trials directed at treating mucus.

1.7 Aims and Outline of the Thesis

I hypothesize that patients with asthma who have a significant number of mucus plugs on CT would be characterized by a low FEV1, airway type 2 inflammation and abnormal mucin gene expression. Furthermore, I hypothesize that eosinophils play a role in mucus plugs formation. To test these hypotheses, I developed a visual scoring system to quantify mucus plugs on MDCT scans of the lungs and applied it to patients enrolled in the SARP. I investigated the reproducibility of the scores across multiple readers and time. I used this scoring system to identify patients with mucus plugs and to determine how mucus plugs relate to FEV1, symptoms of CMH, measures of type 2 inflammation, and mucin gene expression in induced sputum cells. Finally, I used model systems of airway mucus gels to explore whether oxidants generated by eosinophil peroxidase (EPO) oxidize cysteine thiol groups promote mucus plug formation.

In Chapter 2, I describe the study design used in SARP, the population analysed, study visits and procedures performed. I describe the experimental methodology used to further explore the mechanisms underlying mucus plugging in severe asthma.

In Chapter 3, I report the results of a novel scoring system for mucus plugging on MDCT, investigating whether such a scoring system, based on bronchopulmonary segmental anatomy, can quantify mucus plugging in patients with asthma. The following hypotheses were tested (not stated in the null for ease of interpretation):
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1) The CT mucus scores, applied to scans of patients with and without asthma, are reproducible across multiple raters (reproducibility).
2) Subjects with asthma will have higher CT mucus scores than healthy controls (validity).
3) CT mucus scores will remain relatively stable over time in subjects with asthma (stability).

In Chapter 4, I report the results of the relationship between mucus plugging and disease severity in asthma. The following hypotheses were tested (not stated in the null for ease of interpretation):

1) Patients with high CT mucus scores will report worse asthma control than patients with zero or low mucus scores.
2) Patients with high CT mucus scores will not report more symptoms of cough and sputum production than patients with zero or low mucus scores.
3) Patients with high CT mucus scores will have lower measures of FEV1 and FEV1/FVC than patients with zero or low mucus scores.
4) Patients with high CT mucus scores will have lower post-treatment FEV1 and FEV1/FVC following bronchodilator and steroid administration than patients with zero or low mucus scores.

In Chapter 5, I report the results of the relationship between mucus plugging and airway inflammation in asthma. The following hypotheses were tested (not stated in the null for ease of interpretation)

1) Patients with high CT mucus scores will have higher measures of airway and blood eosinophilia than patients with zero or low mucus scores.
2) Patients with high CT mucus scores will have higher post-treatment sputum eosinophilia following bronchodilator and steroid administration than patients with zero or low mucus scores.
3) Patients with high CT mucus scores will have higher sputum gene expression of airway type 2 cytokines (IL-4, IL-5 and IL-13) than patients with zero or low mucus scores.
4) Patients with high CT mucus scores will have higher post-treatment airway type 2 cytokines following bronchodilator and steroid administration than patients with zero or low mucus scores.

5) Patients with high CT mucus scores will have an altered sputum gene expression of airway mucins (MUC5AC and MUC5B) compared to patients with zero or low mucus scores.

6) Patients will demonstrate heterogeneous inflammation in segments with plugs and those with no mucus plugs.

7) Eosinophils promote mucus plug formation by generating oxidative products that oxidize and crosslink cysteines between mucin polymers.

Finally, in Chapter 6, the results presented in this thesis are summarized and a final discussion and future perspectives on diagnosis and treatment of mucus plugging in asthma are also presented.

1.8 References


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Chapter 2 Design and Research Methods

2.0 Introduction

Severe asthma is a debilitating form of asthma, which affects up to 10% of asthma sufferers(1, 2). Accumulating evidence suggests that severe asthma is complex and phenotypically heterogeneous across individuals and time. Lack of knowledge regarding underlying mechanisms and stability of severe asthma sub-phenotypes impede the development of effective treatments and management strategies. Because of the relatively small number of patients with severe asthma at any one institution, along with differing diagnostic criteria, it has been difficult to obtain meaningful data on the underlying features, causes, and pathophysiology of severe asthma. Thus, the Severe Asthma Research Program (SARP) network was established, to address novel research questions that require more patients, expertise, and resources than can be available at any one institution.

The Severe Asthma Research Program (SARP)-3 is a National Institutes of Health-sponsored multicentre study across 7 asthma university centres in the United States. SARP was designed as a comprehensive longitudinal characterization study to identify the cellular and molecular mechanisms underlying different phenotypes of severe asthma, as well as understand how the stability and expression of these phenotypes over time.

2.1 Study Design

SARP 3 is a 3-year longitudinal cohort study. Asthma patients and healthy controls were recruited as part of the SARP cohort across 7 centres. The clinical centres in the network were Brigham and Women’s Hospital; University of California at San Francisco; University of Pittsburgh; University of Virginia; University of Wisconsin, Madison; Wake Forrest School of Medicine; and Washington University in St Louis (with co-investigators at the University of Iowa). All centres used the same
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characterization procedures, and all assessments adhered to standardized protocols and techniques ensuring uniformity of data and adherence to safety precautions.

2.2 Study Population

The target population recruited at each centre was 75 adults with asthma. SARP mandated that at least 60% of the patients with asthma meet the American Thoracic Society/European Respiratory Society (ATS/ERS) definition of severe asthma (3). Inclusion of at least 50% females and 30% minorities in the target population was encouraged. Data reported here are from patients that had MDCTs as part of their characterization. Furthermore, a healthy cohort was enrolled as part of a sub-study to serve as a control group. Healthy subjects for MDCT scans (n=24) were recruited at a single centre (Washington University in St Louis), and those recruited for sputum cell analyses (n=39) were from all SARP 3 centres.

2.2.1 Asthma patients

658 asthma patients were enrolled to the Severe Asthma Research Program (SARP) from November 1, 2012 to October 1, 2014 by seven clinical research centres across the United States (Figure 2.1). All patients underwent detailed characterization and provided samples of venous blood and induced sputum. In addition, 146 of the 658 subjects underwent MDCT of the lungs. Among 146 asthma patients who had MDCT scans as part of the SARP 3 protocol, 25 patients also had MDCT lung scans available for comparison from their participation in SARP-1 or SARP-2 protocols. These patients were enrolled at three sites (University of Pittsburgh, University of Wisconsin, Madison and Washington University) from November 17, 2004 to January 25, 2012. These patients provided comparison scans ranging from 2-9 (mean 5.2) years prior to their SARP 3 MDCT scans.

**Inclusion criteria:** To be considered eligible for inclusion in SARP, patients with asthma were required to have evidence of bronchial hyper-responsiveness (defined as a PC20 methacholine < 16mg/ml) or reversible airflow obstruction, as evidenced by an increase in FEV1 of ≥12% following albuterol inhalation (up to 720 µg) and/or
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Ipratropium bromide inhalation (136 µg). Historical methacholine data from a previous NIH trial (e.g., SARP 1 or 2, AsthmaNet, ALA-ACRC, KIA ACRN or CARE) was acceptable for eligibility. An exception was made for enrollees whose FEV1 was <50% predicted, precluding methacholine challenge testing. If bronchodilator reversibility was <12% in these patients, a diagnosis of asthma acceptable to the investigator was considered sufficient for inclusion in SARP.

Inclusion criteria for SARP mandated that at least 60% of patients meet the ATS/ERS definition for severe asthma (3).

Exclusion criteria: Patients were excluded if they were pregnant or breastfeeding during the initial characterization period, had a history of premature birth (<35 weeks’ gestation), or had a diagnosis of any other chronic pulmonary disorder, which, in the

Figure 2.1 Consort diagram

Flow chart shows the number of asthma patients who were screened, enrolled, and included in the final analyses.
opinion of the investigator, contributed significantly to the patient’s respiratory symptoms. Patients were excluded if they were smokers within the past year or had a > 10 pack year smoking history if ≥ 30 years of age or > 5 pack year smoking history if < 30 years of age.

2.22 Definition of severe asthma

Asthma severity was established at enrolment using the ATS/ERS guidelines on definition, evaluation and treatment of severe asthma (3). Severe asthma was defined as asthma which requires treatment with high dose inhaled corticosteroid plus a second “controller” for the previous year, or systemic corticosteroids for 50% of the previous year to prevent it from becoming ‘uncontrolled’ or which remains ‘uncontrolled’ despite this therapy.

Uncontrolled asthma is defined as at least one of the following:
1) Poor symptom control: ACQ consistently >1.5, ACT <20 (or “not well controlled” by NAEPP/GINA guidelines)
2) Frequent severe exacerbations: two or more bursts of systemic corticosteroids (CS) (>3 days each) in the previous year
3) Serious exacerbations: at least one hospitalisation, ICU stay or mechanical ventilation in the previous year
4) Airflow limitation: FEV1 <80% predicted after appropriate bronchodilator withhold
5) Controlled asthma that worsens on tapering of these high doses of ICS or systemic CS (or additional biologics).
2.23 Healthy subjects

Healthy adults were enrolled as part of the SARP Healthy Control Sub-Study. The purpose of this sub-study was to generate reference data for outcomes measured in biospecimens collected from subjects with asthma enrolled in the SARP Longitudinal Protocol. The SARP protocol for healthy subjects included 1-2 baseline characterization visits.

**Inclusion criteria:** Healthy patients between the age of 18y and 65y with normal lung function (pre-bronchodilator FEV/FVC >0.7 and <12% increase in FEV1 following 4 puffs of albuterol) were considered eligible for inclusion.

**Exclusion Criteria:** Patients were excluded if they were pregnant or breastfeeding during the initial characterization period, had a history of premature birth (<35 weeks’ gestation), or had a diagnosis of any other chronic pulmonary disorder, which, in the opinion of the investigator, contributed significantly to the patient’s respiratory symptoms. In addition, patients were excluded if they had a history of allergic rhinitis, chronic sinusitis or eczema that persisted into adulthood. Patients were excluded if they were smokers within the past year or had a > 10 pack year smoking history if ≥ 30 years of age, or > 5 pack year smoking history if < 30 years of age.
2.3 Study Protocol and Visits

All centres used the same characterization procedures and all assessments adhered to standardized protocols and techniques ensuring uniformity of data and adherence to safety precautions. The SARP protocol included 2-3 baseline characterization visits and annual follow-up visits for 3 years. Data reported here are from the baseline characterisation visits. At the baseline visits, the diagnosis of asthma was confirmed and severity of illness defined. All patients completed comprehensive phenotypic characterization, including a physician-directed medical history, spirometry, maximum bronchodilator reversibility, blood draw for complete blood count and serum IgE, sputum induction, and FeNO measurement (Figure 2.3 and Supplemental Table 7.1). In addition, subjects with asthma completed extensive questionnaires that characterized asthma symptoms, sputum symptoms, quality of life, medication use, and health care utilization. Patients with asthma underwent steroid responsiveness testing as part of characterization (Section 2.45). The comprehensive phenotypic characterization performed at baseline (pre- and post-steroid) was repeated at the...
annual visits (12, 24 and 36 months). Patients that enrolled in the optional CT sub-study underwent CT as part of baseline characterization (pre-steroid) and at Year 3 (Figure 2.3 and Supplemental Table 7.1).

2.4 Study Procedures

2.41 Asthma questionnaires

Questionnaires that assessed the clinical, environmental and medication history were administered and completed by asthma patients at study entry as part of characterization (Figure 2.3). Separate questionnaires that related to asthma quality of life (AQLQ) and asthma control (ACT, ACQ) were completed at baseline and at each subsequent study visit and phone call as an outcome measure of disease control.

Asthma Control Test (ACT)

This is a validated self-administered tool for identifying poorly controlled asthma (4, 5). ACT assesses the frequency of shortness of breath and general asthma symptoms, use of rescue medications, the effect of asthma on daily functioning, and overall self-assessment of asthma control in the previous 4 weeks rated on a scale of 5-25. A score of 5 represents least control of asthma while a score of 25 represents complete control of asthma.

Sputum and cough questions

Chronic mucus hypersecretion (CMH) was defined using the ATS/WHO definition of chronic bronchitis, which assesses chronic cough and sputum production in the preceding 2 years (6, 7). The specific question used was: “Have you had cough and sputum production on most days for at least 3 months a year for at least 2 consecutive years”. The answer options were: ‘Yes’, ‘No’, or ‘Don’t Know’. ‘Don’t Know’ answers were subsequently recoded as ‘No’.
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2.42 Lung function testing

Spirometry, lung volume measurement, and maximum bronchodilation procedures were conducted according to a SARP manual of procedures, which conformed with ATS/ERS guidelines for spirometry (8) and lung volumes measurements (9). Total Lung Capacity (TLC) and Residual Volume (RV) were measured by body plethysmography.

2.43 Procedures for withholding asthma and allergy medications

Subjects were asked to withhold taking their bronchodilator medications prior to spirometry and plethysmography testing. The medication holds for SARP were as follows; short-acting beta agonists - 4 hours; short-acting anticholinergics - 6 hours; long-acting beta agonists - 12 hours; long-acting muscarinic antagonists - 24 hours; and leukotriene modifiers - 24 hours.

2.44 Maximum Bronchodilator Reversibility Test (MBRT)

Following baseline spirometry, 4 puffs of albuterol (360 mcg) were administered. Spirometry was then repeated 15 minutes later. If the change in FEV1 from the spirometry manoeuvre performed after 4 puffs was greater than 5%, an additional 2 puffs of albuterol (180 mcg) were then administered and spirometry was repeated again 15 minutes later. If the change in FEV1 after 6 puffs was greater than 5%, an additional 2 puffs of albuterol were administered with repeat spirometry after an additional 15 minutes. If the change was less than 5% after 4 or 6 puffs of albuterol, the procedure was stopped and the last manoeuvre was taken to be the highest achievable measure. No more than 8 puffs of albuterol were administered as part of the MBRT procedure. MBRT was measured on baseline visits 2 and 3.

2.45 Systemic Corticosteroid Response Test (SCRT)

All asthma patients underwent a SCRT. Subjects were given an intramuscular injection of triamcinolone acetonide (40 mg) following complete characterization on Visit 2. Repeat characterization post steroid injection (excluding MDCT), was carried out on Visit 3 (2-4 weeks later).
2.46 Exhaled nitric oxide

Exhaled nitric oxide is a non-invasive procedure that is considered to be an indirect measure of airway inflammation (10). Subjects were instructed to take a deep breath in and then blow out at a constant pressure as directed by the study coordinator. One measurement was taken and recorded.

2.47 Sputum induction, cell counts and gene expression analyses

Sputum induction was performed on visits 2 and 3 (Figure 2.3). For safety, induced sputum was only performed in patients with an FEV1 > 50% predicted after albuterol pre-treatment (360ug). Sputum was induced using a standard procedure in all subjects, as previously described (11) (Supplemental Appendix). Induced sputum was processed and analysed at two SARP centres. The Wake Forest University centre generated the sputum cell differential counts for SARP, and the University of California, San Francisco centre extracted the RNA and measured gene expression for IL-4, IL-5, IL-13, IL-17, MUC5AC, MUC5B and housekeeping genes for SARP.

Total and differential cell counts were quantified in SARP subjects using methods previously described (11, 12). Gene expression of IL-4, IL-5, IL-13, and for airway gel-forming mucins (MUC5AC, MUC5B) was measured from RNA isolated from induced sputum cell pellets from 77 asthma subjects using previously described methods of real-time Taqman-based quantitative PCR (qPCR)(13). The details of the sputum quality control and specific design of the primers and probes are provided in the appendix (Supplemental Table 7.2).

2.48 Flexible bronchoscopy and bronchoalveolar lavage

A low-dose CT scan was performed one week before bronchoscopy and assessed jointly by both the radiologist and the bronchoscopist. One segment with a mucus plug and one segment without mucus plug were chosen for sampling. Segments with and without mucus plugs were chosen from contralateral lungs. Bronchoscopy was performed under conscious sedation. Bronchoalveolar lavage fluid (BALF) was collected first from the site with mucus, followed by the site without mucus. The bronchoscope was flushed with normal saline between plugged and non-plugged
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segment sampling. In processing the BALF, no straining of fluid through gauze or wire mesh was performed, in order to minimize loss of cells.

2.49 Multidetector CT scanning

MDCT was performed as part of a sub-study within SARP. All patients enrolled in the main SARP study were considered eligible to participate in this sub-study. Patients willing to participate signed a separate consent and underwent MDCT scanning on Visit 1 or 2. MDCT was performed within 2 hours following maximal bronchodilation according to a standard protocol. The basic CT scanning protocol for SARP 3 adults consists of obtaining MDCT images of the entire lung at full inspiration (TLC); and at end expiration, (Functional Residual Capacity, FRC). Details about the MDCT parameters are provided in the appendix (Supplemental Table 7.3 and 7.4). A standard window width of 1200 HU and centre of 600 HU were used for bronchial wall evaluation.

2.50 Automated CT analysis of airway remodelling

Quantitative airway morphology was measured from MDCT scans using automated software that was designed to reliably label and segment the first five to six airway generations, and to allow the accurate measurement of airway walls and lumen diameters obtained perpendicular to the long axis of each airway (Apollo 1.2; VIDA Diagnostics; Iowa City, IA) (14). Airway measurements of RB1, RB4, RB10, LB1, LB4, LB10 (4th generation) were made at each centreline voxel and were averaged over the middle third of the segment. The specific MDCT scan measurements used here included airway wall thickness (WT), percentage of WT (WT%), wall area (WA), percentage of WA (WA%), lumen area (LA) and percentage of LA (LA%) (Figure 2.4).
The calculations are as follows: WT: average outer diameter - average inner diameter; WT\%: (WT/average outer diameter) \times 100; WA: total area (TA) - LA; WA\%: (WA/TA) \times 100; and LA\%: (LA/TA) \times 100. WA\%, LA\% and WT\% were used in analysis for differences in airway size. Airway measurements of RB1, RB4, RB10, LB1, LB4, LB10 were averaged to give a summary estimate for each patient. Averaging measurements in this way has many limitations but is the standard approach for analysing these data. WT\% was reported in results but all 3 measurements gave similar results.

2.5 CT Mucus Score

2.5.1 Criteria for applying the CT mucus score
1. A 2cm peripheral exclusion zone confined to the costal and diaphragmatic pleura was excluded from evaluation as the small calibre of these peripheral airways
makes occlusion by mucus difficult to ascertain. The 2cm peripheral zone adjacent to the mediastinal pleura was *not* excluded from evaluation owing to the larger airways adjacent to the mediastinum.

2. Mucus plugs were defined as complete occlusion of a bronchus, irrespective of generation. When parallel to the scan plane, mucus plugs were recognized as tubular densities with or without branching. When oriented obliquely or perpendicularly to the scan plane, they were identified as oval or rounded opacities seen on sequential slices and differentiated from blood vessels by their continuity with non-impacted portions of the bronchial lumen and their position relative to adjacent blood vessels.

3. The bronchopulmonary segments of each lobe were systematically examined for the presence or absence of mucus plugs and given a score of 1 or 0 accordingly. The segment scores of each lobe were summed to generate a total mucus score for both lungs, yielding an aggregate score ranging from 0-20.

**Figure 2.5 Schematic representation of the CT mucus score**

Airways within the 2-cm peripheral zone on MDCT (shown in red) or airways that were partially occluded were excluded from assessment. Mucus plugs were defined as complete occlusion of an airway. Each bronchopulmonary segment was assessed and scored for the presence or absence of ≥1 mucus plug(s) and the segment scores were summed to generate the mucus score.
2.52 CT bronchiectasis score

Each of the five lung lobes was also systematically examined for the presence or absence of bronchiectasis in addition to the mucus score. Bronchiectasis was defined as an increased broncho-arterial ratio >1.5.

2.53 Measuring stability of the CT mucus score over time

Among the 146 asthma patients who had MDCT scans as part of the SARP 3 protocol, 25 patients also had MDCT lung scans available from their participation in SARP 1 or SARP 2 protocols. These scans were performed 1.6-9.5 (mean 5.2) years prior to the SARP 3 MDCT scans. The characteristics of this subgroup of patients at both time points are listed in Supplemental Table 7.5. CT scans were acquired using the same MDCT scanning protocol as in SARP 3. The web-based survey used for these scans was modified to capture mucus plugging at the level of the bronchopulmonary segments to allow direct comparison of individual segmental involvement within the same subject over time (Supplemental Figure 7.1). Both baseline and follow-up scans of these 25 patients with asthma were read by two radiologists (MS and SN) together to minimize disagreement. The score generated by the raters was used as the CT mucus score for each subject. The SARP 1 and SARP 2 scans were given different ID’s to the SARP 3 scans and readers were blinded to which scans belonged to the same subject.

2.6 Experimental Materials and Methods

2.61 Eosinophil peroxidase assay

Human EPO was measured by sandwich ELISA in supernatant from sputum induction methods adopted from the NHLBI Asthma Clinical Research Network (15). Samples were diluted 1:50 in assay diluent, per manufacturer recommended protocols (Diagnostic Development; Uppsala, Sweden). The detection limit of the assay is less than 0.2 ng/ml.
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2.62 Eosinophil isolation and stimulation of respiratory burst

Eosinophils were purified from the peripheral blood of 4 atopic subjects with asthma (age 48 ± 23 years). Each subject donated 100 mL of blood on one or more occasions. All subjects signed consent forms and usage was approved by the UCSF Committee on Human Research. Eosinophils were isolated from whole blood using a three-step method in which we first pelleted the cells, followed by water lysis to remove red blood cells, and eosinophil purification using immunomagnetic beads (Human Eosinophil Isolation kit; Miltenyi Biotec). Briefly, whole blood was collected in EDTA (purple-top) tubes and pelleted at 1500g for 15 minutes at 4°C. The plasma on top was removed and cell pellet retained for two cycles of water lysis. Water lysis of red blood cells was scaled up based on methods previously described (16). Isolated leucocytes were washed in PBS (Gibco) pH 7.2 containing 2 mM EDTA and 0.5% low-IgG BSA (Gemini) and passed through a 70 µm nylon filter to remove debris. Cells were incubated with biotin-antibody cocktail for 10 minutes at 4°C followed by incubation with anti-biotin microbeads for 15 additional minutes. Eosinophils were purified from labelled granulocytes by passing the solution over a separation column in a magnetic sorting field. Eosinophil purity of ≥99.8% was confirmed by cytospin and staining with Diff-Quik (ThermoFisher Scientific). Eosinophils were resuspended in Iscove's Modified Dulbecco's Medium (Gibco) + 10% foetal calf serum (Gibco), to a density of 1x10^6 cells/mL. Eosinophils were then allowed to rest at 37°C in non-treated 6-well plates (Corning Costar) for at least 20 min before stimulation.

Eosinophils can be induced to undergo respiratory burst by a number of stimuli (17). Phorbol-12-myristate-13-acetate (PMA) (Sigma) was chosen to activate the respiratory burst over physiological activators, such as C5a and Leukotriene B4, because PMA has a slower but more sustained effect. LTB4 and C5a are rapid in onset but transient and their effects could be missed on a kinetic assay (18). Furthermore, PMA stimulates degranulation of all granules rather than piecemeal degranulation of selected granules. Since we wanted to reproduce the respiratory burst, this was considered an advantageous characteristic of PMA. Prior to stimulation, cells were pelleted at 300g for 5 minutes at 4°C and the media was fully aspirated. Cells were resuspended in 1 mL of Tyrode’s salts (Sigma), pelleted again at 300g for 5 minutes,
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and the buffer was fully aspirated to remove any residual FCS. The cells were resuspended in Tyrode’s salts +/- PMA as detailed below.

2.63 Cysteine crosslinking by stimulated versus unstimulated eosinophils

To explore cystine formation generated by eosinophil stimulation, BODIPY FL (Boron-dipyrromethene fluorophore) labelled L-cysteine was generated from 800 mM BODIPY FL L-Cystine (ThermoFisher Scientific) in Tyrode’s Salts by reduction with one quarter volume packed TCEP gel (ThermoFisher Scientific) for 1 hour at 25ºC. The reaction yields an 8 to 10-fold increase in fluorescence at 490 nm/520 nm Ex/Em. This reagent was diluted in 100 μL Tyrode’s Salts to 4 μM with and without 200 nM PMA in 96 well round bottom non-treated black polystyrene plates (Corning Costar). The decrease in fluorescence at 490 nm/520 nm Ex/Em was monitored over 2 hours at 37ºC on a Synergy H1 plate reader (BioTek Instruments) following the addition of 50,000 peripheral blood eosinophils in 100 μL Tyrode’s Salts. The plates were sealed with optical adhesive film (Applied Biosystems) to prevent evaporation. The quenching of BODIPY fluorescence by eosinophil stimulation was shown to reverse to starting values by the addition of DTT to the wells at the end of incubation, indicating that this effect was due to reformation of cystine, and not destruction or bleaching of the fluorophore.

2.64 Cysteine crosslinking with EPO and (pseudo-)halides

To further explore cystine formation generated by constituents of stimulated eosinophils (EPO with H₂O₂) in the presence of chloride, bromide or thiocyanate, BODIPY FL L-cysteine was generated from 800 mM BODIPY FL L-Cystine in Tyrode’s Salts as above. This reagent was diluted in 100 μL Tyrode’s Salts to 4 μM in 96 well round bottom non-treated black polystyrene plates. The decrease in fluorescence at 490 nm/520 nm Ex/Em was monitored over 90 minutes at 37ºC on a Synergy H1 plate reader following the addition of EPO and H₂O₂ with sodium chloride (NaCl), sodium bromide (NaBr) or potassium thiocyanate (KSCN). The plates were sealed with optical adhesive film to prevent evaporation. The quenching of BODIPY fluorescence by EPO was shown to reverse to starting values by the
addition of DTT to the wells at the end of incubation, indicating that this effect was due to reformation of cystine, and not destruction or bleaching of the fluorophore.

2.65 Thiolated hydrogel

Hypothiocyanous acid, the product of the EPO-catalysed reaction of \( \text{H}_2\text{O}_2 \) with KSCN, was examined for its ability to increase the elasticity of thiolated hyaluronic acid. KSCN (2 mM) and \( \text{H}_2\text{O}_2 \) (2 mM) were incubated for 15 minutes at 25ºC with or without EPO (6 nM), in a 0.5% solution of thiolated hyaluronic acid/ phosphate buffer pH 7.4 (Glycosil®; Ascendance Bio, Alameda, CA USA). The elastic moduli (\( G' \)) for the solutions were then measured on a cone and plate rheometer (TA Instruments) oscillating at 1 Hz and 5% strain. The gel solution without reactants was also assessed for background elasticity.

2.7 Statistical Methods

Sample size was based on estimated differences in mean FEV1% across 3 categories of mucus score, as FEV1% was a primary outcome of interest. A sample size calculation was performed using one-way analysis of variance to determine the sample sizes for 3 mucus score groups based on the following assumptions:

- \( \alpha = 0.05 \)
- \( \beta = 0.9 \)
- Number of groups = 3
- Estimated mean FEV1% for group 1 (zero mucus group) = 80
- Estimated mean FEV1% for group 2 (low mucus group) = 70
- Estimated mean FEV1% for group 3 (high mucus group) = 60
- Error variance = 600
- Group weights (group 1, group 2, group 3) = 3, 2, 2

We considered a 10% difference between groups to be a clinically meaningful effect size in FEV1%. We anticipated that a zero mucus score would be more frequent than a low or zero score so weighted the groups to accommodate this. The FEV1% in this population ranged between 13% and 123% and we assumed a within-subject error variance of 600 to accommodate this. This analysis estimated a total sample size of
119 and sample sizes for the three groups of 51 (group 1), 34 patients (group 2) and 34 (group 3). At time of randomization, 146 scans were available for scoring. We elected to score all the available scans, so we could explore secondary outcomes of interest with smaller effect sizes as well as rare events e.g. exacerbations.

Agreement between raters was estimated using the intra-class correlation coefficient (ICC) calculated using a one-way random effects analysis of variance model. Within-rater agreement was calculated using a random subset of 14 scans. Categorical variables are presented as frequencies with percentages and evaluated using the chi-square test. Continuous variables are presented as means ±1 SD or medians with quartiles. Box-and-whisker plots were prepared showing the median (marked by a horizontal line), 25% and 75% quartiles (box) and 1.5 x interquartile range (IQR) (whiskers). Data points outside 1.5 IQR are plotted as outliers. One-way analysis of variance was used for multiple group comparison followed by a Bonferroni correction. Kruskal-Wallis one-way analysis of variance was used for non-parametric multiple group comparison. Correlation between variables was evaluated using Spearman’s correlation. Multivariable analyses were calculated using linear and logistic regression models (with 95% confidence intervals). Statistical significance was accepted for 2-sided p-values of <0.05. Statistical analysis was carried out using Stata 13.1 (StataCorp; College Station, TX, USA).

2.8 Quality Control and Data Management

The use of the SARP Manual of Procedures provided the basis for consistent and reproducible results. The manual consisted of a section to cover each component of the study from initial screening to the final statistical evaluation of the data. Furthermore, data integrity checks were programmed into the data management system, which flagged exceptions to the quality control criteria. Using this system, the Data Coordinating Centre (DCC) at Iowa monitor for potential problems with data interpretability, integrity, or a potential lack of compliance to the manual of procedures. These data were reviewed daily, and centres were contacted directly by the DCC if correction action was required. Quality control and data integrity were also reviewed by the SARP Steering Committee at their monthly meeting. The Steering
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Committee would then decide on any corrective actions that needed to be taken to ensure patient safety or data integrity at these meetings. The Data Safety Monitoring Board was also able to make recommendations about action after review of the data.

2.9 Protection of Human Subjects

Written informed consent approved by each centre’s institutional review board was received from participants prior to inclusion in the study. Study procedures and sample collection were carried out using standardized protocols approved by each centre’s institutional review board. The protocol for the CT Sub-Study was approved by each centre’s Radiation Safety Committee. The Data Coordinating Centre (DCC) and an NHLBI-appointed Data and Safety Monitoring Board (DSMB) monitored all 7 clinical centres and associated partners of SARP for all adverse events.

2.10 References

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Chapter 3 Development and Validation of the CT Mucus Score

3.0 Introduction

Airway mucus plugging is an underappreciated pathology in asthma. Mucus plugging represents a failure of mucus homeostasis that is central to the pathophysiology of fatal asthma and has long been believed to play a major role in acute asthma (1). However, the clinical significance of mucus plugging in chronic asthma is unknown and efforts to answer this question have been hindered by an inability to measure intraluminal mucus.

MDCT has emerged as the imaging modality of choice for non-invasive comprehensive assessment of airway anatomy (2). Airway remodelling in chronic asthma has been studied using MDCT (3, 4). Measurements of airway wall thickness and wall area on CT correlate with pathologic changes on airway wall biopsy and with clinical measures of disease severity and airflow obstruction (5). These studies demonstrate that CT is a highly reproducible method for studying airway pathology. However, quantitative assessment of mucoid impaction using CT has never been attempted in asthma. CT studies in asthma have primarily focused on airway remodelling and air trapping (5-8). Assessment of mucoid impaction by CT has been qualitative, with limited description of the criteria used to define it (9-12). Grenier et al. developed a comprehensive definition of ‘mucoid impaction’ on CT, but only reported absence or presence of ‘mucoid impaction’ at a patient level. Therefore, CT has not been validated as a clinical tool to specifically quantify mucus plugging in respiratory disease or health.

The objectives of this study were to test the hypothesis that mucus plugging on MDCT lung scans is more prevalent in asthmatic patients than healthy controls; to test the hypothesis that radiologists can independently identify and score mucus plugging with a high degree of reproducibility; and to test the hypothesis that CT mucus scores remain relatively stable over time in patients with asthma.
To test these hypotheses, we developed a mucus score to quantify intraluminal mucus plugging on MDCT scans. To validate the mucus score, we tested the inter-rater and intra-rater agreement of the radiologists applying the score. Finally, we examined the stability of the CT mucus score in a subgroup of these patients with lung imaging from earlier iterations of SARP, to demonstrate that the CT mucus phenotype is stable over many years.

3.1 Development and Validation of the CT Mucus Score

3.11 Appearance of mucus plugs on MDCT in asthma

In preliminary studies, we discovered that we could discern mucus plugs in the lungs of patients with asthma using MDCT scans. Specifically, we could identify mucus plugs as areas of opacification within the airway lumen, contiguous with patent airway lumen across sequential CT slices. These opacities were less radiodense than adjacent blood vessels, and occlusion of the lumen by these opacities could be partial or complete. These mucus plugs were predominantly seen in sub-segmental airways, appearing as focal or branching opacities (Figures 3.1A, 3.1B, and 3.1C) and usually occurred in the absence of bronchial dilatation. Based on these findings, we went on to develop a visual scoring system to formally quantify mucus plugs in MDCT scans (Figure 3.2).
Figure 3.1 Appearance of mucus plugging on MDCT

(A) Intraluminal mucus plug with branching seen in longitudinal section (coronal plane). Mucus is identified as a tubular opacification (yellow arrow) that bifurcates distal to a patent airway. (B) Intraluminal mucus plug with extensive branching seen in longitudinal section (yellow arrow) extending to the lung periphery (transverse plane). The mucus impaction is not associated with bronchial wall dilatation. (C) Intraluminal mucus plug seen in cross-section (transverse plane). Mucus is identified as rounded opacification (yellow arrow) that is visible on sequential MDCT slices.
The CT mucus score was developed sequentially in 3 versions by consensus. The final version of the score used in this study excluded the peripheral lung to the mediastinal interface and required complete occlusion of segmental and sub-segmental bronchi. The CT mucus score was developed and tested by ED, BE, DG, SN, MS, and JN.
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3.12 Development of the CT mucus score

A scoring system to quantify mucus plugs in lung images generated using multi-detector computerized tomography was developed by a mucus score team (ED, JF, BE, DG, SN, MS, and JN). The scoring system was based on bronchopulmonary segmental anatomy. Each bronchopulmonary segment was given a score of 1 (mucus plug(s) present) or 0 (mucus plug(s) absent). The segment scores of each lobe were summed to generate a total mucus score for both lungs, yielding a mucus score ranging from 0-20. The score was initially tested and refined using 10 scans from patients with severe asthma recruited at UCSF for SARP. The initial version of the score (Version 1) was modified twice to yield the final version (Version 3), developed to maximize agreement and biologic plausibility. The final version was tested in 25 scans before applying the score to the final cohort.

Version 1. The initial mucus score counted mucus plugs in both central and peripheral lung regions and defined a mucus plug as either partial or complete occlusion of an airway by mucus. Peripheral lung was defined as the portion of lung within 2 cm of the mediastinal, costal or diaphragmatic pleura (Figure 3.2). The ICC for agreement between 5 radiologists independently scoring 10 scans was 0.3 (95% CI 0.0 to 0.64). This ICC data was reviewed by the mucus score team, and the relatively poor inter-rater agreement was judged to result from inconsistent reads arising from two factors; (i) scoring mucus plugs in the outer 2 cm of the lungs where airways are small and mucus plugs can be hard to identify reliably and consistently; (ii) inconsistency among radiologists in scoring mucus plugs in airways partially occluded by mucus.

Version 2. This version was revised by consensus in two ways (Figure 3.2):
- Mucus plugs were not evaluated in airways in the outer 2 cm of the lungs.
- Mucus plugs were defined as complete or partial occlusion of a segmental bronchus or complete occlusion of a sub-segmental bronchus.

The revised score (version 2) was applied to 10 scans by the 5 radiologists and the ICC was 0.67 (95% CI 0.43 to 0.92). This data was reviewed and discussed by the mucus score team. Based on this review and discussion, additional modifications were suggested for a version 3 mucus score (below).
Version 3. This version was revised by consensus in three ways (Figure 3.2):
- Mucus plugs were defined as complete occlusion of a bronchus by mucus, irrespective of generation.
- The 2 cm peripheral exclusion zone was confined to the costal and diaphragmatic pleura so as not to exclude the larger airways adjacent to the mediastinum.
- Use of a standard window width of 1200 HU and level -600 HU for bronchial wall evaluation.

3.13 CT mucus score
Version 3 of the mucus score was agreed as the final version to be used in the study (Figure 3.2). First, we defined the area of evaluation. Peripheral airways within 2 cm of the diaphragmatic pleura and costal pleura were excluded from evaluation as the small calibre of these peripheral airways makes occlusion by mucus difficult to ascertain. Second, we defined mucus plugs as complete occlusion of a bronchus, irrespective of generation or size. When parallel to the scan plane, we identified mucus plugs as tubular densities with or without branching. When oriented obliquely or perpendicularly to the scan plane, we identified mucus plugs as oval or rounded opacities. We differentiated mucus plugs from blood vessels by their continuity with patent portions of the bronchial lumen and their position relative to adjacent blood vessels on sequential slices (Supplemental Figure 7.2 and Supplemental video 1). Finally, we systematically examined the segments of each lobe for the presence or absence of mucus plugs and given a score of 1 or 0 accordingly. The segment scores of each lobe were summed to generate a total mucus score for both lungs, yielding an aggregate score ranging from 0-20.

Each of the five lung lobes was also systematically examined for the presence or absence of bronchiectasis, defined as a bronchoarterial ratio >1.5. Five radiologists with sub-specialty training in thoracic radiology reviewed the MDCT scans. Two radiologists were randomly assigned to score each scan, and the scores of both raters were averaged to generate the CT mucus score of each subject. A schematic representation showing how MDCT’s were evaluated to generate the mucus score is presented in Figure 3.2.
3.14 Validation of the CT mucus score

Each scan was scored by two raters randomly drawn from the group of five raters. We tested the validity of the mucus score by analysing for inter-rater bias followed by inter-rater and intra-rater agreement. Bias between raters, where one rater consistently over- or underscores relative to the other rater, was evaluated using paired analyses. We found no significant bias between any of the pairs of raters (p>0.05). Once we confirmed absence of bias, inter-rater agreement of the CT mucus score was assessed by ICC. We made an initial check of inter-rater agreement after half of the scans were scored, with a plan to recalibrate any rater(s) with outlying scores to the group mean. The ICC at interim analysis was 0.69 and we provided retraining for one rater. At the end of the study, the ICC for agreement between readers (i.e., inter-rater) was 0.80 (95% CI 0.74 to 0.85) for all 171 scans and 0.79 (95% CI 0.72 to 0.85) for the 146 asthma scans alone. The intra-rater agreement for a random subset of 14 scans (3 healthy, 11 asthma) that were scored twice by each of the five radiologists was 0.99 (95% CI 0.99 to 1.00).

3.15 Application of the CT mucus score

Before application of the scoring system to the SARP cohort, we held a teleconference, which included a slide presentation with a detailed description of the final scoring system, followed by a 1-hour consensus reading session using a training set of 3 CT scans. To generate the mucus score, two radiologists were randomly assigned to independently score each scan. Each radiologist was provided with their individual set of scans in digital format. The radiologists entered the mucus score data in real-time into a secure online survey (Research Electronic Data Capture) (Supplemental Figure 7.1). The average score of both raters was used to calculate the CT mucus score for each subject. This generated a continuous score ranging from 0 to 20 in increments of 0.5.
3.2 Mucus Plugging Prevalence in Asthma

To test the hypothesis that mucus plugging would be present in a subgroup of patients with asthma, we analysed the CT mucus scores from patients with asthma as well as healthy controls. CT scans from 146 asthma subjects and 25 healthy controls were analysed as part of this study. Ninety-seven (66.4%) subjects had asthma that qualified as severe asthma using the ATS/ERS criteria (13), and the pre-bronchodilator FEV1 was less than 80% predicted in 85 patients (58%) and less than 60% predicted in 35 patients. Compared to the healthy subjects, subjects with asthma were older, had lower baseline lung function and had a higher prevalence of atopy (Table 3.1).

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<th>Asthma (n=146)</th>
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<td>46.8 ± 16.0</td>
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<td>0 (0)</td>
</tr>
<tr>
<td>Spirometry data†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 (% predicted) *</td>
<td>98.2 ± 9.3</td>
<td>75.5 ± 21.8</td>
</tr>
<tr>
<td>FVC (% predicted) *</td>
<td>100.1 ± 10.3</td>
<td>90.0 ± 19.0</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>0.84 ± 0.03</td>
<td>0.83 ± 0.13</td>
</tr>
<tr>
<td>History of atopy*</td>
<td>4 (16)</td>
<td>110 (75.3)</td>
</tr>
</tbody>
</table>

Data reported as mean and standard deviation unless otherwise indicated. CT scans of healthy controls from SARP II and SARP III. CT scans of asthma subjects from SARP III.

* p<0.05 comparing asthma to healthy subjects

†Predicted values could not be calculated in one healthy male subject for sputum analysis
Mucus plugging was present in at least 1 of 20 lung segments in 58% of patients with asthma and in only 4.5% of healthy controls (p=0.0001) (Figure 3.3). There was no difference in the distribution of mucus plugging within the lung (Supplemental Figure 7.3). Among patients with asthma, the median value of the mucus score in the “mucus present” group was 3.5, and we used this value to divide patients with asthma into three mucus groups based on mucus score. Asthma patients with a mucus score of 0 were assigned to the “zero mucus group”, while those with mucus scores between 0.5 and 3.5, and 4 and 20, were assigned to the “low mucus group” and “high mucus group”, respectively (Figure 3.4).

Figure 3.3 CT mucus score in health and asthma

Segment score in healthy patients and patients with asthma. *** Indicates significantly different to the data in health, p<0.001.
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3.3 Stability of Mucus Plugging and the CT Mucus Score

Longitudinal CT data for comparison were available in 25 patients with asthma from SARP 1 or 2. These SARP-1 and SARP-2 scans were obtained 2-9 years prior to the SARP 3 MDCT scans. Two radiologists at the University of Wisconsin, Madison centre read the 50 scans together to identify and score the mucus plugs. In a score-based analysis, we compared mucus scores assigned to the first and second scans; mucus scores were unchanged in 7 patients (28%), increased in 10 patients (40%), and decreased in 8 patients (32%) over an average of 5.2 years (SD 2.5). We found that 90% of subjects with a high mucus score (≥4) on the first scan had a high score on the second scan (Figure 3.5).

Figure 3.4 Distribution of the mucus score in asthma

Frequency distribution of segment score in patients with asthma. The colour code above the x-axis defines three mucus groups: the green colour indicates patients with a mucus score of 0 (“zero mucus group”); the blue colour indicates patients with mucus scores between 0.5 and 3.5 (“low mucus group”) and the orange colour indicates patients with mucus scores ≥4.0 (“high mucus group”).

3.3 Stability of Mucus Plugging and the CT Mucus Score

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In a segment-based analysis, we compared individual lung segments in the first and second scans and found that 80% of lung segments with no mucus plug found on the first scan had no mucus plug found in the same segment on the second scan (Figure 3.6A). Furthermore, we found that 65% of lung segments that had a mucus plug on the first scan, had a mucus plug in the same segment on the second scan (Figure 3.6A). In fact, in many instances the mucus plug(s) visible in the first scan could still be seen on the second scan in the same bronchopulmonary segment airway (Figure 3.6B). Persistent presence or absence of mucus plugs from first to second scan was seen with similar frequency across all bronchopulmonary segments.
At the same time that the radiologists scored the scans for mucus plugging, they also systematically examined each of the five lung lobes for the presence or absence of bronchiectasis, defined as a bronchoarterial ratio >1.5. This approach generated a mean bronchiectasis score ranging from 0 to 5, as well as a binary outcome for presence or absence of bronchiectasis. We found that only 20% of the subjects with asthma had bronchiectasis (Figure 3.7).

**3.4 Airway Mucus Plugs and Bronchiectasis**

At the same time that the radiologists scored the scans for mucus plugging, they also systematically examined each of the five lung lobes for the presence or absence of bronchiectasis, defined as a bronchoarterial ratio >1.5. This approach generated a mean bronchiectasis score ranging from 0 to 5, as well as a binary outcome for presence or absence of bronchiectasis. We found that only 20% of the subjects with asthma had bronchiectasis (Figure 3.7).
The prevalence of bronchiectasis or mucus plugging did not differ among the five lung lobes, but the prevalence of mucus plugging was 4 to 5 times higher than that of bronchiectasis in any given lung lobe (Figure 3.8A). Bronchiectasis was weakly but significantly associated with mucus plugging at a lobar level – mainly because most lobes with bronchiectasis had mucus plugging (Figure 3.8B) – but relatively few lobes with mucus plugging also had bronchiectasis (Figure 3.8C).
In an analysis of the 25 subjects with repeat MDCT scans for comparison, there was a low prevalence of mucus plugging on the initial CT scan (2 out of 25 subjects) and the prevalence was unchanged in the second scan. In a lobe-based analysis, we compared individual lung lobes in the first and second scans and found that 83% of

Figure 3.8 Prevalence of bronchiectasis versus mucus plugging in each lung lobe

(A) The prevalence of mucus plugging is 4-5 times higher than the prevalence of bronchiectasis in each lobe. There is no significant difference in prevalence of bronchiectasis or mucus plugging across individual lobes. (B) Mucus plugging is present in 35% of lobes that have no bronchiectasis present and 58% of lobes that have bronchiectasis present (p=0.001). (C) Bronchiectasis is present in 5% of lobes that have no mucus plugging present and only 12% of segments that have mucus plugging present (p=0.001). There is a positive association between mucus plugging and bronchiectasis but mucus plugging usually occurs in the absence of bronchiectasis.
l lung lobes (5/6 lobes) that had a bronchiectasis on the first scan had bronchiectasis in the same lobe on the second scan (Figure 3.9A). We also found that 99% of lung lobes (118/119 lobes) with no bronchiectasis on the first scan had no bronchiectasis in the same lobe on the second scan (Figure 3.9B).

![Figure 3.9 Lobar based analysis of bronchiectasis in subjects with repeat CT scans](image)

**Figure 3.9 Lobar based analysis of bronchiectasis in subjects with repeat CT scans**

Pie-charts illustrating the prevalence of bronchiectasis in repeat CT scans in 25 patients. (A) The data show that 83% of lung lobes with mucus plugs visible on the first scan had mucus plugging visible on the second scan; in contrast, (B) 99% of lung lobes with no mucus plugs visible in the first scan also had no mucus plugs visible on the second scan.

### 3.5 Discussion

This is the first study, to our knowledge, to propose criteria for identifying mucus plugging on CT and apply them in a score to quantify mucus burden in chronic asthma. In this study, we developed a semi-quantitative measure of airway mucus plugging on CT over multiple iterations. The systematic approach of quantifying mucus burden at the segmental level is analogous to sampling lungs 20 times for the presence or absence of intraluminal mucus. Agreement was optimized by defining mucus plugging as complete opacification of an airway. As a result, partially occluded airways were not included in the score. Partially occluded airways can be difficult to distinguish from airway wall thickening and contribute to disagreement among readers.

Furthermore, partial occlusion with mucus may be a transient finding on CT and is less likely to contribute to airflow obstruction than complete occlusion of an airway.
by mucus. Airways within 2 cm of the peripheries were excluded from scoring due to the difficulty in reliably identifying occlusion in these small calibre airways. While these small airways may contribute to airflow obstruction secondary to mucus plugging, the airways in this region are too small to accurately differentiate airway tapering from plugging on CT. Exclusion of the peripheral 2 cm enabled consensus among multiple readers to be achieved, increasing the reproducibility of the score but may also have introduced a bias towards underestimation of mucus burden by excluding the smaller peripheral airways. Peripheral centrilobular opacities in a tree-in-bud pattern, are occasionally included in definitions of mucus plugging on CT, as they can represent impaction of the small bronchioles by mucus (14, 15). Peripheral centrilobular opacities were not included in the definition of mucus plugging for this study, as they are not specific to mucoid impaction (16). The final inter-rater agreement achieved was 0.8 and this represents “excellent” agreement (17) (Supplemental Table 7.6).

We found that mucus plugs, as assessed by MDCT, occur commonly in patients with asthma. The CT mucus score identified mucus plugging in 58.2% of subjects with chronic asthma in this study, compared to previous estimates of 16.5 to 21% (9, 10, 12) in the published literature. These differences in estimates may in part reflect differences in the study populations investigated. In the SARP cohort, approximately 60% of the patients had severe asthma. Notwithstanding these differences, this study suggests that the prevalence of mucus plugging in chronic asthma has been underestimated.

We found that mucus plugs in asthma occurred predominantly in subsegmental airways that were usually not dilated. This suggests that that the plugs are occurring in airways that are structurally normal. Since bronchial dilatation was rarely seen in association with mucus plugging, inclusion of this feature in the definition would have greatly underestimated the prevalence of plugging in this population.

It is notable that mucus plugs persisted in some patients over periods as long as 9.5 years and occurred in the same lung segments (within an individual subject) over time. Conversely, lung segments that were plug-free in SARP 1 and SARP 2 tended to remain plug-free over time. In the absence of structural differences (bronchiectasis) in
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these airways, predisposing them to plug formation, we speculate that there is regional heterogeneity among lung segments in susceptibility to mucus plug formation. Regional heterogeneity in ventilation defects that persist over time has previously been noted in asthma on MRI (18).

Although previous asthma studies have included qualitative assessments of mucus pathology in MDCT-based assessment of lung pathology (19), I believe this is the first study to focus on quantification of mucus plugs using a novel CT-based scoring system and show stability of a mucus phenotype over a period of almost 10 years.

The objectives of this study were to test whether mucus plugging on MDCT lung scans is more prevalent in asthmatic patients than healthy controls; to test if radiologists can independently identify and score mucus plugging with a high degree of reproducibility; and to test if CT mucus scores remain relatively stable over time in patients with asthma. In light of the findings in this study, we can accept that mucus plugging on MDCT is more prevalent in patients with asthma than healthy controls. We can accept that radiologists can apply a CT mucus score with good inter-rater and intra-rater reproducibility. Finally, we can accept that the mucus score groups are stable over many years.

3.6 References

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Chapter 4 Relationship Between Mucus Plugging and Airflow Obstruction in Asthma

4.0 Introduction

While importance of mucus plugging in the pathophysiology of severe fatal asthma has been demonstrated repeatedly in pathology studies, the role of mucus plugging in chronic severe asthma remains poorly understood. The degree to which mucus plugs are associated with airflow obstruction, independent of airway remodelling, in severe asthma is unknown. The objective of this study was to test the hypothesis that mucus plugs are associated with asthma symptoms, disease severity and airflow obstruction, as well as treatment-resistant asthma.

To test the hypothesis that mucus plugging is associated with severe asthma, we examined the association between mucus plugs and different clinical markers of disease severity (asthma control, medication use and exacerbation history). We tested whether mucus plugging is associated with symptoms of chronic mucus hypersecretion. Finally, we tested whether mucus plugging is associated with persistent airflow obstruction following maximal bronchodilation and systemic steroid treatment.

4.1 Mucus Plugging is Associated with Severe Asthma

Sixty-one (43%) patients with asthma had a zero mucus score, 45 (30%) patients had a low mucus score and 40 (27%) patients had a high mucus score (Table 4.1). Patients in the high mucus group were older than those in the zero mucus group at baseline and were older at time of symptom onset and asthma diagnosis, but the duration of asthma did not differ between the groups (Table 4.1). No significant association was seen between mucus group and gender, race or BMI.
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### TABLE 4.1 CHARACTERISTICS OF SUBJECTS WITH ASTHMA ACROSS MUCUS SCORE CATEGORIES

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n=146)</th>
<th>Mucus Score</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Zero (n=61)</td>
<td>Low (n=45)</td>
<td>High (n=40)</td>
<td></td>
</tr>
<tr>
<td>Mucus score</td>
<td>0.5 (0-4.5)</td>
<td>0 (0)</td>
<td>1.5 (0.5-2.5)</td>
<td>9.5 (6-12)</td>
<td></td>
</tr>
<tr>
<td>Mean age (years) †</td>
<td>46.8 ± 16.0</td>
<td>43.2 ± 15.4</td>
<td>46.7 ± 15.6</td>
<td>52.3 ± 16.3</td>
<td></td>
</tr>
<tr>
<td>Female sex - no. (%)</td>
<td>91 (62.3)</td>
<td>43 (70.5)</td>
<td>26 (57.8)</td>
<td>22 (55.0)</td>
<td></td>
</tr>
<tr>
<td>Body Mass Index (kg/m²)</td>
<td>32.7 ± 9.3</td>
<td>34.3 ± 9.9</td>
<td>32.5 ± 10.5</td>
<td>30.7 ± 6.3</td>
<td></td>
</tr>
<tr>
<td>Maintenance corticosteroid use - no. (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhaled - any dose</td>
<td>141 (96.6)</td>
<td>56 (91.8)</td>
<td>45 (100.0)</td>
<td>40 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Inhaled - high dose †</td>
<td>102 (69.9)</td>
<td>36 (59.0)</td>
<td>30 (66.7)</td>
<td>36 (90.0)</td>
<td></td>
</tr>
<tr>
<td>Systemic†</td>
<td>15 (10.3)</td>
<td>3 (4.9)</td>
<td>3 (6.7)</td>
<td>9 (22.5)</td>
<td></td>
</tr>
<tr>
<td>Severe Asthma - no. (%) ‡</td>
<td>96 (65.8)</td>
<td>31 (50.8)</td>
<td>29 (64.4)</td>
<td>36 (90.0)</td>
<td></td>
</tr>
<tr>
<td>Asthma Control Test†</td>
<td>18 (14-21)</td>
<td>19 (15-21)</td>
<td>18 (14-22)</td>
<td>16.5 (13-19)</td>
<td></td>
</tr>
<tr>
<td>Spirometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV1 (% predicted) ††</td>
<td>72.2 ± 20.6</td>
<td>81.0 ± 16.2</td>
<td>74.5 ± 20.8</td>
<td>56.1 ± 17.4</td>
<td></td>
</tr>
<tr>
<td>FVC (% predicted) ††</td>
<td>85.5 ± 17.9</td>
<td>89.3 ± 14.0</td>
<td>88.3 ± 19.4</td>
<td>76.7 ± 19.0</td>
<td></td>
</tr>
<tr>
<td>FEV1/FVC (predicted)* ††</td>
<td>0.83 ± 0.13</td>
<td>0.90 ± 0.10</td>
<td>0.83 ± 0.11</td>
<td>0.72 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Exacerbations in last 12 months - no. (%)</td>
<td>74 (50.7)</td>
<td>29 (47.5)</td>
<td>23 (51.1)</td>
<td>22 (55.0)</td>
<td></td>
</tr>
<tr>
<td>Chronic mucus hypersecretion - no. (%) ††</td>
<td>41 (34.0)</td>
<td>18 (29.5)</td>
<td>10 (22.2)</td>
<td>13 (32.5)</td>
<td></td>
</tr>
<tr>
<td>Bronchiectasis on CT - no. (%)</td>
<td>29 (19.9)</td>
<td>7 (11.5)</td>
<td>11 (24.4)</td>
<td>11 (27.5)</td>
<td></td>
</tr>
<tr>
<td>ABPA - no. (%) ‡‡</td>
<td>3 (2.1)</td>
<td>0 (0)</td>
<td>2 (1.4)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Ex-smokers - no. (%) §§</td>
<td>22 (15)</td>
<td>9 (14.8)</td>
<td>5 (11.1)</td>
<td>8 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Environmental smoke exposure - no. (%) †§§</td>
<td>18 (12.3)</td>
<td>5 (8.2)</td>
<td>4 (8.9)</td>
<td>9 (22.5)</td>
<td></td>
</tr>
</tbody>
</table>

Data reported as mean ± standard deviation or median (interquartile range). Zero represents the “mucus absent” group (mucus score=0). Low represents the group with mucus scores 0.5-3.5 and high represents the group with mucus scores ≥4, based on the median score of 3.5 in the “mucus present” group.

† p<0.05 for comparison of zero and high scores
‡ p<0.05 for comparison of low and high scores
§ The classification of asthma severity was determined using ATS/ERS criteria
†† Diagnosed using elevated total IgE, specific IgE to Aspergillus fumigatus, systemic eosinophilia, and radiographic changes consistent with ABPA.
§§ Smoking history data missing in 5 patients. All ex-smokers had <10 pack year smoking history.
We next explored whether the CT mucus score is associated with markers of asthma severity. We found that patients in the high mucus group were more likely to be treated with high dose inhaled corticosteroids, and more likely to be on systemic corticosteroids (oral or injectable) than patients in the zero-mucus group (Table 4.1). Patients in the high mucus group had significantly lower median Asthma Control Test (ACT) scores compared to the zero mucus group (p=0.04) (Table 4.1). Specifically, 78% of the high mucus group were classified as uncontrolled (ACT <20) compared to 50% of the zero mucus group. In addition, the percentage of patients in the high mucus group who had experienced at least one asthma exacerbation in the previous year was higher than in the zero mucus group, but this difference was not statistically significant. Finally, only two asthma subjects in the cohort met criteria for a diagnosis of allergic bronchopulmonary aspergillosis (ABPA) (1), and both had mucus scores in the low (0.5 - 3.5) range (Table 4.1); sensitivity to other moulds and aeroallergens did not differ significantly among mucus groups (Supplemental Table 7.7). There was no association between mucus group and history of previous smoking but there was an association between mucus group and current second-hand smoke exposure (Table 4.1). There was no association found between mucus group and socioeconomic status measured by education level, occupation of annual income.

4.11 Chronic mucus hypersecretion is not sensitive or specific for mucus plugs

To determine whether subjects with asthma could have mucus plugs without CMH symptoms, we examined the frequency of symptoms of CMH in the three mucus plug groups. Among 121 subjects who completed the cough and sputum questionnaire, 41 (34%) satisfied World Health Organization criteria for CMH (cough and sputum production on most days for at least 3 months a year for at least 2 consecutive years) (2). We found that 16 (40%) subjects in the high mucus group did not have symptoms of CMH (Table 4.1). Conversely, we found that 18 (30%) of the subjects in the zero mucus group had symptoms of CMH. Although the group of subjects with CMH did not have higher mucus scores than subjects without CMH, the subjects with CMH were characterized by other clinical differences, such as older age, higher BMI and evidence of more severe asthma (Supplemental Table 7.8).
4.2 Mucus Plugging is Associated with Persistent Airflow Obstruction

4.21 Mucus plugging is associated with impaired lung function

To determine the association between airway mucus plugs and airflow obstruction in the subjects with asthma, we examined the relationship between the mucus score and measures of airflow obstruction by spirometry. We found that the mucus scores were inversely correlated with pre-bronchodilator measures of FEV1% predicted (Spearman’s rho = -0.51, p<0.001), forced vital capacity (FVC) % predicted (Spearman’s rho = -0.32, p<0.001), and FEV1/FVC predicted (Spearman’s rho = -0.54, p<0.001). These associations remained significant after controlling for age, gender, and measures of airway wall thickness in regression analyses (Table 4.2).

<table>
<thead>
<tr>
<th>Asthma outcome*</th>
<th>Unadjusted</th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEV1, % predicted</td>
<td>-2.2 (-2.8, -1.5)</td>
<td>-1.9 (-2.6, -1.3)</td>
<td>-1.9 (-2.5, -1.2)</td>
<td>-1.5 (-2.2, 0.8)</td>
</tr>
<tr>
<td></td>
<td>R²=0.24,</td>
<td>R²=0.28,</td>
<td>R²=0.34,</td>
<td>R²=0.38,</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>FVC, % predicted</td>
<td>-1.3 (-1.8 to -0.7)</td>
<td>-0.9 (-1.5, -0.3)</td>
<td>-0.9 (-1.4, -0.3)</td>
<td>-0.7 (-1.3, -0.04)</td>
</tr>
<tr>
<td></td>
<td>R²=0.11,</td>
<td>R²=0.25,</td>
<td>R²=0.27,</td>
<td>R²=0.29,</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p=0.002</td>
<td>p=0.003</td>
<td>p=0.04</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>-1.4 (-1.8 to -1.0)</td>
<td>-1.5 (-1.9, -1.1)</td>
<td>-1.4 (-1.8, -1.0)</td>
<td>-1.3 (-1.7, -0.8)</td>
</tr>
<tr>
<td></td>
<td>R²=0.26,</td>
<td>R²=0.29,</td>
<td>R²=0.34,</td>
<td>R²=0.38,</td>
</tr>
<tr>
<td></td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

* Linear Regression model reports β coefficient (95% confidence interval) for asthma outcomes.

Predictor variable is mucus score ranging from 0-20

Model 1 adjusts for the covariate of age at screening

Model 2 adjusts for the covariates of age and gender

Model 3 adjusts for the covariates of age, gender and wall thickness %

Models were not adjusted for concurrent corticosteroid therapy as there was a very low number of subjects not on corticosteroids (inhaled or systemic).
The mean FEV1 was 25% lower in the high mucus group than the zero mucus group (Table 4.1 and Figure 4.1), and the values for the FVC and FEV1/FVC ratio were also significantly lower in the high mucus group (Figure 4.1). In addition, 66.7% of subjects with a pre-bronchodilator FEV1 <60% predicted had high mucus scores compared to 19% of subjects with FEV1 60-80% predicted and 6.1% of subjects with FEV1 >80% predicted. The low FVC in subjects with high mucus scores suggested air trapping in these subjects (3), and we confirmed this in a subset of subjects (n=43) who had undergone body plethysmography as part of their baseline characterization studies. Specifically, we found that the ratio of residual volume to total lung capacity (RV/TLC) was higher in the high mucus group than the low mucus group indicating more air trapping in the high mucus group (p=0.04) (Figure 4.2)

Figure 4.1 Mucus plugging is associated with low lung function

Spirometric measures of lung function (FEV1, FVC and FEV1/FVC) in the subjects with a high mucus score were significantly lower than in subjects with a low mucus score and subjects with a zero mucus score. *** Indicates p<0.001. ** Indicates p<0.01. P value was determined using a Kruskal-Wallis test with Dunn’s correction.
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4.22 Mucus plugging is associated with suboptimal response in FEV1 to standard asthma treatments

To explore if airway mucus plugs influence treatment responses, we first examined responses to inhaled albuterol (540-720 mcg) using data from the MBRT. Although there was no significant difference in the absolute change in FEV1 following albuterol treatment among the 3 mucus groups (Figure 4.3A), we found that the mean post-bronchodilator FEV1 in the high mucus group was 23% lower than in the zero mucus group (Figure 4.3B), and a persistently low FEV1 (FEV1 < 80% predicted) following MBRT was common in subjects with high mucus scores, but uncommon in subjects with zero mucus scores (Figure 4.3C).

Figure 4.2 Mucus plugging is associated with air trapping

The RV/TLC % was higher in patients with a high mucus score than patients with a zero mucus score. Data were generated by body plethysmography and represents post-bronchodilator values. * indicates p<0.05.
Figure 4.3 Persistent airflow obstruction in high mucus group after bronchodilators

(A) The absolute change in FEV1% predicted post bronchodilator treatment did not differ across mucus groups. (B) The median FEV1% predicted post bronchodilator treatment was significantly lower in the high mucus group than the zero mucus group. (C) Residual post bronchodilator abnormalities in FEV1 (FEV1<80%) occur more commonly in subjects with a high mucus score than those with a zero mucus score. Inhaled albuterol used as bronchodilator treatment. *** Indicates p<0.001 determined by Kruskal-Wallis test with Dunn’s correction.

Figure 4.4 Persistent airflow obstruction in high mucus group after steroids

(A) The absolute change in FEV1% predicted post steroid treatment did not differ across mucus groups. (B) The median FEV1% predicted post steroid treatment was significantly lower in the high and low mucus groups than the zero mucus group. (C) Residual post steroid abnormalities in FEV1 (FEV1<80%) occurred more commonly in subjects with a high mucus score than those with a zero mucus score. Intramuscular triamcinolone acetonide used as steroid treatment. *** Indicates p<0.001. ** Indicates p<0.01 determined by Kruskal-Wallis test with Dunn’s correction.
We next examined responses to intramuscular triamcinolone acetonide (40 mg) using data from the SCRT. Although there was no significant difference in the absolute change in FEV1 following corticosteroid treatment among the three mucus groups (Figure 4.4A), we found that the mean post steroid FEV1 was 20% lower in the high mucus group than in the zero mucus group (Figure 4.4B). As with the data for albuterol treatment, we noted that a persistently low FEV1 following SCRT was common in subjects with high mucus scores but uncommon in subjects with zero mucus scores (Figure 4.4C). In addition, we found that the CT mucus score was an independent predictor of residual abnormalities in FEV1 after systemic corticosteroids in logistic regression models (Figure 4.5).

**Figure 4.5 Logistic regression of the effects of mucus score on lung function**

Forrest plot of the association between mucus plugging and lung function outcomes in asthma. Associations were derived from multivariable logistic regression models. Shown in the figure are the adjusted odds ratios (aOR) for subjects having FEV1 <80%, FVC <80% and FEV1/FVC <0.07, predicted by the mucus score (ranging 0-20). Intramuscular triamcinolone acetonide used as steroid treatment. Age, gender, and wall thickness (surrogate for airway remodelling) were included in the model as covariates. Models were not adjusted for concurrent corticosteroid therapy as there was a very low number of subjects not on corticosteroids (inhaled or systemic).
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Finally, we examined responses to combined treatment with albuterol and systemic corticosteroid in the 3 mucus groups. Here, we found that the absolute change in FEV1 following MBRT and SCRT was significantly higher in the high mucus group than in the zero and low mucus groups (Figure 4.6A), but the mean post bronchodilator/post steroid FEV1 was still significantly lower in the high mucus group than in the zero mucus group (Figure 4.6B). Half of the subjects with high mucus scores had persistently low FEV1 following SCRT and MBRT, whereas only a small group of subjects with zero mucus scores had persistently low FEV1 (Figure 4.6C). Thus, aggressive treatment with beta adrenergic agonists and corticosteroids frequently does not normalize lung function in subjects with airway mucus plugs, and additional treatments need to be considered for these subjects.

Figure 4.6 Persistent airflow obstruction in high mucus group after bronchodilators and steroids

(A) The absolute change in FEV1% predicted post bronchodilator and steroid treatment was significantly higher in the high mucus group than the zero mucus group. (B) The median FEV1% predicted post bronchodilator and steroid treatment was significantly lower on the high mucus group than the zero mucus group. (C) Residual post bronchodilator and post steroid abnormalities in FEV1 (FEV1<80%) occurred more commonly in subjects with a high mucus score than in those with a zero mucus score. Inhaled albuterol used as bronchodilator treatment and intramuscular triamcinolone acetonide used as steroid treatment. ** Indicates p<0.01 determined by Kruskal-Wallis test with Dunn’s correction.
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4.23 Mucus plugging is associated with airflow obstruction independent of airway remodelling

We then assessed for a relationship between mucus score, airway remodelling and mucus plugging using automated measures of airway dimensions generated from quantitative CT analysis. We found a positive correlation between mucus score and both percent wall thickness (WT%) and an inverse correlation between mucus score and lumen area (LA%). In a mucus group-based analysis, there was a significant difference between the zero and high mucus scores for WT% and LA% (Figure 4.7).

To test the potential confounding effects of airway remodelling in the relationship between mucus plugging and airflow obstruction, we performed linear regression, with mucus score predicting FEV1%, FVC% or FEV1/FVC, with WT% as the confounding variable in Model 3 (Table 4.2). We included age and gender as covariates in the models. Although the β coefficient (effect size) of mucus score predicting FEV1 decreased with addition of WT% in the linear model, the association between mucus score and obstruction remained significant (p=0.001). We also performed logistic regression models predicting FEV1 <80%, FVC <80% and FEV1/FVC <0.7 (pre and post steroid) by mucus score using the same covariates. Both FEV1 and FEV1/FVC remained significant after controlling for age, gender and wall thickness (p=0.007 and p<0.001 respectively), but FVC was no longer significant in this model (p=0.4) (Figure 4.5).
4.3 Discussion

We used MDCT imaging of the lungs to explore the role of airway occlusion with mucus in the pathophysiology of lung dysfunction in chronic severe asthma. On average, the patients with airway mucus occlusion in four or more bronchopulmonary segments (high mucus group) had much lower FEV1 and FEV1/FVC values than patients with no airway mucus occlusion representing significant airflow obstruction, though there was some overlap in FEV1% between the three mucus groups. FVC was also lower in the high mucus group and may represent air trapping. Reductions in FEV1 and FVC are known physiologic characteristics of severe asthma (3). The mechanisms of obstruction and air trapping in severe asthma include airway smooth muscle contraction (4), airway remodelling (5-8), or loss of lung elastic recoil (9, 10), but our data reveal and highlight the importance of airway occlusion with mucus. Patients with mucus plugs had much lower FEV1 values than patients without mucus plugs, and this combination of mucus plugs and low FEV1 occurred despite treatment.
with high doses of asthma controller medications. In addition, low FEV1 values persisted following protocol-directed treatment with high doses of bronchodilators and intramuscular injections of triamcinolone acetonide. These data show that conventional therapies result in suboptimal responses in patients with mucus plugging and provide a strong rationale to use mucolytics as a strategy to improve FEV1 abnormalities that are not fully responsive to standard asthma treatments.

Although our data cannot prove a causal relationship between the presence of mucus plugs and low FEV1, such a causal relationship is highly plausible, because the plugs that were scored were ones that completely occluded airways. Since mucus gland hypertrophy is a feature of airway remodelling in severe asthma, mucus plugging could be marking airway remodelling. This raises the question whether the relationship between mucus plugging and airflow obstruction is causal, or the result of confounding by airway remodelling. Using automated CT measures of percent wall thickness and percent lumen area, we demonstrated that association between mucus plugging and FEV1 remained significant after controlling for wall thickness (%). Therefore, we have shown that the relationship between mucus plugging and airflow obstruction is not the result of confounding by airway remodelling.

The observation that mucus plugging was more frequent in patients with environmental smoke exposure is interesting and novel. In a recent study, we showed that oxidation promotes mucin crosslinking, which in turn makes sputum more elastic (11). Elastic sputum is more difficult to clear from the airway and may constitute a mechanism for formation of mucus plugs. We were not able to examine for an association between active smoking and mucus plugging, as active smokers were excluded from the healthy and asthma populations as part of the study design. An association between smoke exposure and mucus plugging warrants further investigation.

The majority of patients with mucus plugs did not have symptoms of CMH, and patients with mucus symptoms often had no mucus plugs. Atopy was an exclusion criterion in the design of the healthy control group, which may also have excluded healthy controls with CMH. Analysis was therefore restricted to asthmatic subjects
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with and without CMH. Patients with mucus hypersecretion have features of severe asthma and are exacerbation-prone, but their clinical, pathological and imaging characteristics are otherwise not as specific as those of patients with mucus plugs. These findings reveal that reliance on symptoms to identify patients with lung mucus pathology will underestimate the number of patients who have mucus plugs that completely occlude their airways. Clinical trials that use MDCT to identify patients with mucus plugs could test whether treatments that lyse mucus plugs or decrease their formation can improve airflow obstruction in asthma.

Thus, our data suggest a rationale to decrease or alter airway mucus as a strategy to improve FEV1 in patients with severe asthma. Because symptoms of mucus were frequently absent in patients with mucus plugs on MDCT, we propose that MDCT is required to stratify patients for inclusion in clinical trials of mucolytic treatments for low FEV1 in severe asthma. NAC is currently available as a mucolytic that could be used to lyse mucus plugs identified by CT. Novel mucolytics such as thiol-saccharides are also in development. These compounds are more stable and more efficient at disrupting mucin crosslinking in vivo than NAC (11). Using chest imaging to manage asthma represents a novel and feasible approach to personalize treatment, especially with the recent advent of ultra-low-dose MDCT protocols (12).

The objective of this study was to test the hypothesis that mucus plugs are associated with asthma symptoms, disease severity and airflow obstruction, as well as treatment-resistant asthma. In light of the findings in this study, we can accept that mucus plugging is associated with the different clinical markers of disease severity (asthma control, medication use and exacerbation history). We can accept a lack of association between mucus plugging and symptoms of chronic mucus hypersecretion. Finally, we can accept that mucus plugging is associated with persistent airflow obstruction following maximal bronchodilation and systemic steroid treatment.
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4.4 References


Chapter 5 Relationship Between Mucus Plugging and Inflammation in Asthma

5.0 Introduction

Asthma is one of the most common chronic immunological diseases in humans, affecting people from childhood to old age (1). An important molecular mechanism of asthma is type 2 inflammation, which occurs in many but not all asthma patients. Type 2 inflammation has a number of downstream effects that promote mucus dysfunction, including but not limited to, goblet cell hyperplasia (2), mucus hypersecretion (2, 3) and impaired airway clearance (4).

While many of the pathways that result in mucus hypersecretion have been elucidated, the mechanisms that lead to mucus plug formation remain poorly understood. Mucus plugs are a feature of many pulmonary diseases with disparate inflammatory profiles (e.g., COPD and cystic fibrosis), and mucin hypersecretion may be stimulated through inflammatory and non-inflammatory mechanisms. The objective of this study was to test the hypothesis that mucus plugs are associated with type 2 airway inflammation in asthma.

To test this hypothesis, we examined different markers of airway inflammation (blood cell counts, sputum cell counts, FeNO and IgE), as well as gene expression of inflammatory cytokines and mucin genes for association with mucus score. We tested whether mucus plugging was associated with persistent type 2 inflammation following systemic steroid treatment. Furthermore, we performed directed bronchoscopy testing for regional heterogeneity in inflammation between mucus plugged and non-plugged segments. Finally, we explored whether eosinophils and their products promote mucin polymer crosslinking using fluorescent assays and a thiolated hydrogel model of mucus.
5.1 Mucus Plugging is Associated with Type 2 Inflammation

5.11 Mucus plugging is associated with airway eosinophilia

To explore if eosinophils play a pathophysiologic role in the formation of mucus plugs, we analysed multiple outcomes related to type 2 inflammation in blood and biospecimens in the three mucus groups. We found that eosinophils in blood and sputum, and nitric oxide levels in exhaled breath were significantly higher in the high mucus group than in the low and zero mucus groups (Table 5.1 and Figure 5.1A). Among subjects with high mucus scores, 71% had sputum eosinophilia (sputum eosinophils > 2%) and 66% had systemic eosinophilia (blood eosinophils > 300 x 10^9/L). Airway mucus scores were positively and significantly associated with the sputum eosinophils (Spearman’s rho = 0.51, p<0.001) (Figure 5.1B).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All (n=146)</th>
<th>Mucus Score</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucus score</td>
<td>0.5 (0-4.5)</td>
<td>Zero (n=61)</td>
<td>Low (n=45)</td>
<td>High (n=40)</td>
<td></td>
</tr>
<tr>
<td>Sputum cell counts (%) §</td>
<td></td>
<td>0 (0)</td>
<td>1.5 (0.5-2.5)</td>
<td>9.5 (6-12)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils †‡</td>
<td>0.7 (0.4.4)</td>
<td>0.2 (0,9)</td>
<td>0.5 (0.2,1.6)</td>
<td>7.3 (1.5,21.4)</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>58 (35,78)</td>
<td>62 (37,83)</td>
<td>60 (35,79)</td>
<td>47 (31,70)</td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>4.7 (2,11.5)</td>
<td>4.3 (2.3,11.5)</td>
<td>4.3 (2.3,5.9)</td>
<td>6.9 (1.9,17)</td>
<td></td>
</tr>
<tr>
<td>FeNO (ppm) ‡†</td>
<td>22 (12,33)</td>
<td>18 (10,27)</td>
<td>24 (13,38)</td>
<td>28 (19,40)</td>
<td></td>
</tr>
<tr>
<td>Blood cell counts (x10⁶/L) *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils †‡</td>
<td>306 ± 276</td>
<td>209 ± 153</td>
<td>309 ± 282</td>
<td>459 ± 349</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4286 ± 2350</td>
<td>4569 ± 2951</td>
<td>4030 ± 1934</td>
<td>4134 ± 1592</td>
<td></td>
</tr>
<tr>
<td>Total white blood cells</td>
<td>7279 ± 2548</td>
<td>7534 ± 3149</td>
<td>6953 ± 2138</td>
<td>7255 ± 1827</td>
<td></td>
</tr>
<tr>
<td>Nasal polypectomy - no. (%) †</td>
<td>21 (14.4)</td>
<td>1 (1.6)</td>
<td>8 (17.8)</td>
<td>12 (30.0)</td>
<td></td>
</tr>
<tr>
<td>Sinus surgery - no. (%) †</td>
<td>19 (13.0)</td>
<td>3 (4.9)</td>
<td>8 (17.8)</td>
<td>8 (20.0)</td>
<td></td>
</tr>
<tr>
<td>Specific IgE positivity - no. (%)‡</td>
<td>112 (76.7)</td>
<td>45 (73.8)</td>
<td>39 (86.7)</td>
<td>28 (70.0)</td>
<td></td>
</tr>
<tr>
<td>Total IgE (IU/ml) *</td>
<td>150 (52,363)</td>
<td>126 (32,482)</td>
<td>150 (74,335)</td>
<td>181 (79,363)</td>
<td></td>
</tr>
</tbody>
</table>

Data reported as mean ± standard deviation or median (interquartile range). Zero represents the “mucus absent” group (mucus score=0). Low represents the group with mucus scores 0.5-3.5 and high represents the group with mucus scores ≥4, based on the median score of 3.5 in the “mucus present” group.

† p<0.05 for comparison of zero and high scores
‡ p<0.05 for comparison of low and high scores
§ Sputum cell counts were not available in 40 subjects due to ineligibility for sputum induction or because the induced sputum not meet quality metrics.
‡§ Fraction of nitric oxide in exhaled breath (FeNO) was not measured in 4 subjects.
* Blood measurements were not available for 2 subjects.
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This relationship between mucus score and eosinophilia remained significant in linear regression models that controlled for age and gender (Table 5.2). In addition, sputum eosinophils, and FeNO remained high in many subjects with high mucus scores following SCRT, and the CT mucus score was an independent predictor of residual sputum eosinophilia and elevated FeNO after systemic corticosteroids in logistic regression models (Figure 5.2). Notably, patients in the high mucus group were more likely to report a history of nasal polyposis and to have undergone surgery for removal of nasal polyps and treatment of chronic sinusitis (Table 5.1).

There was no association between mucus plugging and other sputum cell types (Table 5.1, Supplemental Figure 7.4). Patients in the high mucus group were equally likely to be atopic by specific IgE positivity and had similar total IgE levels to the zero and low mucus groups (Table 5.1).
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#### TABLE 5.2. RELATIONSHIP BETWEEN MUCUS SCORE AND EOSINOPHILS

<table>
<thead>
<tr>
<th>Asthma outcome*</th>
<th>Unadjusted</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum Eosinophils %‡</td>
<td>0.96 (0.66, 1.3)</td>
<td>0.93 (0.61, 1.3)</td>
<td>0.94 (0.62, 1.3)</td>
</tr>
<tr>
<td>R²=0.28, p&lt;0.001</td>
<td>R²=0.29, p&lt;0.001</td>
<td>R²=0.3, p&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Blood Eosinophils count</td>
<td>24.5 (15.5, 33.4)</td>
<td>24.8 (15.2, 34.4)</td>
<td>24.6 (15.0, 34.2)</td>
</tr>
<tr>
<td>R²=0.17, p&lt;0.001</td>
<td>R²=0.17, p&lt;0.001</td>
<td>R²=0.18, p&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

* Linear Regression model reports β coefficient (95% confidence interval) for asthma outcome.

‡ Sputum cell counts were not measured in 40 subjects.

Model one adjusts for the covariate of age at screening

Model two adjusts for the covariates of age and gender

Models were not adjusted for concurrent inhaled corticosteroid therapy as there was a very low number of subjects not on inhaled corticosteroids.

#### Figure 5.2 Logistic regression of mucus score on markers of type 2 inflammation

Forrest plot of the association between mucus plugging and markers of type 2 inflammation pre and post steroid treatment. Shown in the figure are the adjusted odds ratios (aOR) for subjects having sputum eosinophilia (>2%), blood eosinophilia (>300/ml) or high FeNO (>50ppm), predicted by the mucus score (ranging 0-20). Age and gender were included in the models as covariates. Analyses were confined to subjects that had paired pre and post steroid data.
5.12 Mucus plugging is associated with gene markers of type 2 inflammation

We next explored gene expression outcomes related to type 2 inflammation and mucins in sputum cells from the three mucus groups. We found that gene expression for IL-13 and IL-5 in sputum cells was significantly higher in the high mucus group than in the low and zero mucus groups and remained high following SCRT (Figure 5.3A and 5.3B). In addition, we found that the ratio of gene expression of MUC5AC to MUC5B in sputum cells was significantly higher in the high mucus group than in the low and zero mucus groups and normalizes following SCRT (Figure 5.3C). The pre-steroid pattern of a high expression of MUC5AC relative to MUC5B in the high mucus group is typical of the activation effects of IL-13 on mucin expression by airway epithelial cells (5).
Figure 5.3 Mucus score is associated with sputum cell gene expression

(A) Gene expression for interleukin-13 is significantly increased in patients with a high mucus score and remains significantly increased following treatment with intramuscular steroid. (B) Gene expression for interleukin-5 is significantly increased in patients with a high mucus score and remains significantly increased following treatment with intramuscular steroid. (C) The MUC5AC/MUC5B ratio is significantly increased in patients with high mucus scores.

***Indicates p<0.001. **Indicates p<0.01. *Indicates p<0.05. Gene expression data generated by Michael Peters.
5.13 Marked eosinophilia found in a bronchial subsegment with mucus plug

The finding that patients with mucus plugs have much higher sputum eosinophil levels than patients without mucus plugs (Figure 5.1), coupled with the data in Chapter 3 showing that mucus plugs can persist in the same bronchial subsegment for many years (Figure 3.6), led us to consider the possibility that regional heterogeneity in mucus plugs reflects regional heterogeneity in type 2 inflammation. We examined this possibility in an asthma patient whose MDCT scan showed a mucus plug in the anterior segment of the left upper lobe (proximally in the medial sub-segment LB3b), and no mucus in the superior segment of the right lower lobe (RB6). We performed bronchoscopy in this patient to separately lavage the LB3b and RB6b sub-segments. We found that the eosinophil percentage in the plugged segment was much higher than in the non-plugged segment (11.8% vs. 2.4%). Notably, staining of the lavage cell cytospin from the plugged segment showed mucus that was densely infiltrated with intact eosinophils (Figures 5.4A and 5.4B), despite the fact that this patient was taking high doses of inhaled corticosteroids.

![Figure 5.4 Bronchopulmonary segment with mucus plugs is eosinophilic](image)

(A) H&E stain of bronchoalveolar lavage (BAL) from the segment with mucus plug at 20X magnification. BAL contains dense area of highly cellular mucus. Asterisk marks the area magnified in panel C. (B) H&E stain of bronchoalveolar lavage (BAL) from the segment with mucus plug at higher magnification. This shows mucus that is densely packed with intact eosinophils.
5.2 Eosinophils Promote Mucin Crosslinking

5.21 Eosinophils generate oxidants that crosslink cysteines

The dense infiltration of mucus by eosinophils led us to consider mechanisms by which eosinophils could promote mucus plug formation. We recently reported that oxidant-rich neutrophils participate in mechanisms of mucus plug formation in cystic fibrosis (6), and this led us to consider here whether oxidant-rich eosinophils might oxidize cysteine domains in mucins to generate covalent disulfide bridges that stiffen the mucus gel and induce mucus plugs to form (Figure 5.5). Activated eosinophils undergo a respiratory burst that releases high concentrations of reactive oxygen species (ROS) that promote crosslinking of mucins and mucus gel stiffening through cysteine oxidation and disulfide bond formation.

Figure 5.5 Conceptual model for eosinophils promoting mucus plug formation in asthma

Epithelium, stimulated by IL-13, secretes high concentrations of mucin, particularly cysteine-rich MUC5AC mucin, into the airway lumen. High levels of IL-5 promote survival of these eosinophils. Upon activation, eosinophils release reactive oxygen species (ROS) that promote crosslinking of mucins and mucus gel stiffening through cysteine oxidation and disulfide bond formation. Crosslinking of mucins increase mucus elasticity, impeding effective mucus clearance.
species such as superoxide anion ($O_2^-$), $H_2O_2$ and $HO^-$ as well as eosinophil peroxidase (EPO) (7). To test whether activated eosinophils convert cysteines to their oxidized cysteine product (cystine), we used a BODIPY-labelled cysteine reagent that fluoresces when cysteine is in its monomeric but quenches completely when cysteine is oxidized to form cystine dimers (Fig. 5.6A). We exposed cysteine-BODIPY to eosinophils from asthma donors. We found that cysteine-BODIPY undergoes a minor amount of time dependent oxidation when maintained in buffer solution alone, but this effect is significantly larger when cysteine is exposed to eosinophils, and much larger still when exposed to eosinophils that are activated with PMA (Fig. 5.6B).

Figure 5.6 Eosinophils promote crosslinking of cysteines through sulphydryl oxidation and disulfide bond formation

(A) Schematic showing two cysteine monomers labelled with BODIPY FL, which fluoresces green when bound to a cysteine monomer. Under oxidative conditions, two cysteines crosslink by disulfide bond to form a cystine dimer. BODIPY FL becomes virtually non-fluorescent due to interactions between the two fluorophores in the dimer. Quenching of fluorescence is a measure of cysteine crosslinking in response to oxidation. (B) Eosinophils drive crosslinking of cysteine residues following respiratory burst. Fold change in crosslinking of cysteines under three conditions; eosinophils in cysteine-BODIPY with PMA, to stimulate the respiratory burst; eosinophils in cysteine-BODIPY with no PMA; and cysteine-BODIPY containing no eosinophils and no PMA as a negative control. The cysteine-BODIPY assay designed by Wilfred Raymond and data generated by Wilfred Raymond and Eleanor Duncanan.
The quenching of BODIPY fluorescence by eosinophil stimulation was shown to reverse to starting values by the addition of DTT to the wells at the end of incubation, indicating that this effect was due to formation of cystine, and not destruction or bleaching of the fluorophore.

5.22 EPO with thiocyanate crosslink cysteines to stiffen thiolated hydrogels

Having shown that eosinophil-driven oxidation might be a mechanism of mucus plug formation in asthma, we explored which components of the respiratory burst are important in oxidation-related cysteine crosslinking. The activity of EPO can generate products that target thiol groups as a cytotoxic mechanism (8), and we considered the possibility that EPO-generated products oxidize the thiol groups of cysteine residues in mucin polymers to cause mucus plugs to form. As a first step, we measured EPO in sputum from the zero and high mucus groups, as well as healthy controls. We found a positive correlation between sputum EPO levels and sputum eosinophils (Spearman’s rho=0.59, p<0.001) (Figure 5.7A) and that EPO levels in the high mucus plug group are markedly higher than in the zero mucus group and in healthy controls (Figure 5.7B).
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We next tested the effect of EPO activity on cysteine crosslinking using two model systems of the airway mucus gel. The first model used the cysteine-BODIPY reagent that quenches when cysteine forms its oxidized disulfide product (cystine) (Figure 5.6A). The second model was a synthetic thiolated hyaluronan reagent that increases in elasticity to form a hydrogel when oxidized (9) (Figure 5.8).

Figure 5.7 Eosinophil peroxidase is associated with mucus plugging

(A) The sputum eosinophil % is positively associated with sputum EPO levels. (B) Sputum EPO is higher in the high mucus group (n=32) than the zero mucus group (n=45) and healthy controls (n=39). ** indicates p<0.01. *** indicates p<0.001. Data in (A) and (B) generated by Marrah Lachowicz-Scroggins and Eleanor Duncan.

We next tested the effect of EPO activity on cysteine crosslinking using two model systems of the airway mucus gel. The first model used the cysteine-BODIPY reagent that quenches when cysteine forms its oxidized disulfide product (cystine) (Figure 5.6A). The second model was a synthetic thiolated hyaluronan reagent that increases in elasticity to form a hydrogel when oxidized (9) (Figure 5.8).
EPO catalyses the oxidation of chloride, bromide and thiocyanate by H$_2$O$_2$ to HOCl (hypochlorous acid), HOBr (hypobromous acid) and HOSCN (hypothiocyanous acid), respectively. Specificity constants indicate that thiocyanate is a major substrate for EPO, and HOSCN is known to be a more thiol specific oxidant than HOBr or HOCl (8). To test if EPO catalyses the disulphide crosslinking of cysteine monomers to form cystine dimers, we exposed cysteine-BODIPY to EPO in the presence of H$_2$O$_2$ and either NaCl, NaBr or KSCN. We found no significant cystine dimer formation when cysteine-BODIPY was exposed to EPO, H$_2$O$_2$ and chloride, but dimer formation was significantly greater when the cysteine-BODIPY was exposed to EPO, H$_2$O$_2$ and bromide, and greatest when the cysteine-BODIPY was exposed to EPO, H$_2$O$_2$ and thiocyanate (Figure 5.9A). Thus, HOBr and HOSCN can both oxidize cysteines, but HOSCN is more potent, while HOCl has no effect in this system. The quenching of BODIPY fluorescence by EPO was shown to reverse to starting values by the addition of DTT to the wells at the end of incubation, indicating that this effect was due to formation of cystine, and not destruction or bleaching of the fluorophore.
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We next tested whether HOSCN can crosslink the thiolated hyaluronan reagent. Here we used a cone and plate rheometer to measure changes in the elasticity of the hydrogel under different conditions. We found that the combination of EPO, H₂O₂ and KSCN markedly increases the elasticity of the thiolated hyaluronan gel, and that EPO is required for this effect (Figure 5.9B). Thus, HOSCN can convert a thiolated hydrogel from a liquid form to a solid form leading us to propose that HOSCN is an oxidant product of EPO activity that may mediate mucus plug formation in asthma (Figure 5.10).

Figure 5.9 Effect of EPO and H₂O₂ on cysteine crosslinking in the presence of chloride, bromide or thiocyanate

(A) Cysteines do not undergo significant cross-linking with EPO and H₂O₂ in the presence of chloride, but cysteines exposed to EPO and H₂O₂ in the presence of bromide, and especially thiocyanate, undergo much more oxidation and cross-linking. (B) Effect of EPO, H₂O₂ and thiocyanate, on the viscoelastic properties of a thiolated hydrogel measured by rheology. A large increase in the elastic modulus (G') of the hydrogel was seen following exposure to EPO with H₂O₂ and KSCN. There was no significant increase in G' in the hydrogel in the absence of EPO. Data presented as means ±SD of 3 replicates in (A) and 4 replicates in (B). *** and ‡ indicates p<0.001. ** and † indicates p<0.01. * indicates p<0.05. P value determined by ANOVA with Bonferroni correction. Data generated by Wilfred Raymond.
5.3 Chronic Mucus Hypersecretion is not Associated with Type 2 Inflammation

Finally, we tested whether similar associations between type 2 inflammation and symptoms of CMH would be found. In contrast to the mucus plugging phenotype, patients with CMH did not differ in measures of type 2 inflammation compared to those without CMH. Patients with CMH did not differ in their sputum or blood eosinophil values (Figure 5.11A, 5.11B). They did not differ in sputum gene expression for IL-13 and other type 2 cytokines (Figure 5.11C, Supplemental Figure 7.5). Furthermore, patients with CMH did not differ in their MUC5AC and MUC5B gene expression (Figure 5.11D); patients with CMH did not differ in measures of non-type 2 inflammation; and patients with CMH did not differ in their sputum neutrophils.
or sputum cell IL-17 expression (Supplemental Table 7.8, Supplemental Figure 7.5).

5.4 Discussion

In this chapter, we examined the relationship between mucus plugging and type 2 inflammation. We found that asthma patients with mucus plugs had profoundly high numbers of airway eosinophils, higher concentrations of nitric oxide in exhaled breath, and higher gene expression levels for type 2 cytokines (IL-4, IL-5 IL-13) in their sputum cells compared to patients without mucus plugs. These are biologically plausible associations, because IL-13 regulates eosinophil accumulation in the airway, nitric oxide production, and mucin gene expression (10-12). Type 2 inflammation could promote mucus plug formation by altering mucin composition and reducing
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mucus clearance, either by a) stiffening the mucus gel, b) decreasing mucus clearance through the function of cilia on airway epithelial cells, or c) by mucin tethering (4-6).

Indeed, we found that patients with mucus plugs had an increase in the ratio of MUC5AC to MUC5B, which represents a molecular signature of IL-13 activation of airway epithelial cells (5). Changes in the relative proportions of MUC5B to MUC5AC could promote eosinophil survival in the airway mucus gel as glycoepitopes present in the MUC5B glycan coat interact with Siglec-8 on eosinophils to induce apoptosis (13). Therefore, a relative deficiency in MUC5B as suggested by the increase in the ratio of MUC5AC to MUC5B could create an environment that promotes eosinophil survival. It is worth noting that although there was a significant difference in medians between the 3 mucus groups, there was also overlap in MUC5B:MUC5AC and IL-5 gene expression levels between the three mucus groups, limiting the specificity of mucus group for expression of these genes. One explanation for the overlap seen in sputum cell gene expression is that sputum has a mixed cell population including epithelial cells, neutrophils, eosinophils, mast cells, macrophages, lymphocytes and squamous cells. For example, gene expression levels for IL-5 reflects expression by Th2 cells, eosinophils, and mast cells, and non-haematopoietic cells including epithelial cells and natural helper cells (14), which can differ slightly in their proportions from subject to subject.

It is notable that patients with mucus plugs had prominent type 2 inflammation and altered mucin gene expression, despite use of high doses of inhaled corticosteroids at enrolment and after protocol-mandated intramuscular triamcinolone acetonide treatment. Corticosteroid treatments should suppress airway type 2 inflammation (15) and, if type 2 inflammation is indeed causal in mucus plugging, in turn decrease mucus plugs. IL-33 initiates type 2 airway inflammation in mouse models of asthma (16). However, much less is known about the mechanisms that regulate persistent type 2 inflammation in chronic stable type 2-high asthma. Mucus plugs may play a role in maintaining persistent and treatment-resistant type 2 inflammation by providing a niche in which eosinophils can survive for longer periods, protected from apoptosis signals or the effects of inhaled or systemic steroids. It may be that protein therapeutics
that target type 2 cytokines are necessary to combat the highly eosinophilic mucus plugs that occur in these patients.

As shown in Chapter 3, mucus plugs persisted in the same lung segments in some patients over periods as long as 9.5 years. Conversely, lung segments that were plug-free tended to remain plug-free over time. Therefore, although the mucus plugs were heterogeneously distributed in the 20 bronchopulmonary segments among patients, they tended to recur in individual patients in the same bronchopulmonary segment. We also consider it remarkable that in our case example, the mucus-positive bronchopulmonary segment had mucus that was intensely infiltrated with eosinophils and a high eosinophil percentage in BALF, whereas the mucus-negative segment had a markedly lower eosinophil percentage in the lavage fluid. Taken together, these data suggest that type 2 inflammation is heterogeneously distributed among lung segments in an individual’s lungs, and that mucus plugs on MDCT scans can mark segments with and without type 2 inflammation. The reasons for heterogeneously distributed type 2 inflammation among lung segments are not revealed by our study, but it is known that many children develop asthma following a viral airway infection and subsequently experience lifelong asthma (17-20). Clues for the mechanisms underlying persistence of childhood-acquired asthma is provided by recent murine studies which show that infectious stressors no longer present in the host can cause localized immune damage (“immunological scarring”) that permanently alter tissue-specific immunity to cause chronic disease (21, 22). These data, combined with our own, lead us to speculate that childhood airway insults, such as viral infections, may damage airway epithelial cells in individual lung segments resulting in permanent immune dysfunction, persistent type 2 inflammation, and long-lasting mucus pathology in these segments. In human lungs, each of the 20 bronchopulmonary segments develop and operate as discrete anatomical and functional units with little collateral communication between them (23). Therefore, each of these segments could emerge from childhood with its own distinct injury history and type 2-associated pathology reflecting previously sustained immunological scars.

Airway mucus is normally a lightly cross-linked mucus gel that is transported easily by the mucociliary escalator, and mucus does not normally form mucus plugs (12, 24).
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Chronic mucus plugs in lung disease are usually found in infected, distal, and dilated airways. The mucus plugs that we find on MDCT in asthma are in sub-segmental non-dilated airways and so cannot be attributed to anatomical changes. We provide insights into the pathophysiology of these mucus plugs. First, the mucus plugs occurred in the context of marked increases in the airway expression of IL-13 and in the expression of MUC5AC relative to MUC5B. IL-13 is known to alter the expression of MUC5AC and MUC5B in airway epithelial cells and to cause tethering of MUC5AC-rich mucus to airway epithelial cells to impair mucociliary transport. Second, the mucus plugs occurred in the context of marked airway eosinophilia and mucus plugs heavily infiltrated with eosinophils.

These findings prompted us to consider mechanisms by which eosinophils might promote mucus plug formation. Previously, we have shown that neutrophils have a role in mucus gel pathology in cystic fibrosis, because they release reactive oxygen species (ROS) that oxidize mucins in mucus to increase its elasticity (6). Given the prominence of airway eosinophilia in asthma patients with mucus plugs, we considered the possibility that eosinophil-generated ROS could play a role in oxidizing cysteine rich mucins to stiffen airway mucus gels. Eosinophils generate up to 3-4-fold more extracellular O₂⁻ than other granulocytes, including neutrophils (25) and eosinophils from patients with asthma generate higher levels of O₂⁻ and ROS than eosinophils from healthy controls (26, 27). We found that activated eosinophils from asthma donors are highly effective in catalysing the conversion of cysteine to its oxidized disulfide product, and that this results from the oxidizing activities of respiratory burst ROS; specifically, oxidants generated by the activity of eosinophil peroxidase.

A potential limitation of this experimental design was in proving the effect of eosinophil products on cysteine crosslinking was due to oxidation. Ideally, an investigator would show either prevention of cysteine crosslinking by co-treating with an antioxidant specific to free radicals, or reversal of cysteine crosslinking by treating with a similarly specific antioxidant. Antioxidants used clinically include NAC, glutathione, erdosteine and carbocysteine. Common to the chemical structure of all these compounds is presence of a thiol (sulfhydryl) group that scavenges free radicals.
by redox/reduction reaction, giving these compounds the characteristics of both antioxidants and reducing agents. DTT and TCEP similarly contain thiol groups and are used primarily as reducing agents but also as antioxidants in research. Because all of these compounds are reducing agents, all would cross-reacted with BODIPY in a non-specific manner rather than specifically scavenge free radicals. The non-specificity of the effect would render this experimental design uninterpretable. The alternative approach of reversing the effects of eosinophils products could be achieved with any of these agents, but the same non-specificity of antioxidant versus reducing effect remains. To this end, I elected to use DTT in preference to the other antioxidants to show reversal of the oxidising effects of eosinophil products because DTT is more potent than NAC or glutathione at reversing an oxidation effect. DTT showed rapid reversal of the crosslinking that followed treatment with stimulated eosinophils or eosinophil products. Taken together, our data lead us to conclude that the pathophysiology of sub-segmental mucus plugs in asthma involves airway type 2 inflammation, which initiates a cascade of events resulting in eosinophil-mediated crosslinking of cysteine-rich mucins. Because cross-linking mucins increases their elasticity (6, 24), this mechanism can explain the pathogenesis of mucus plug formation in eosinophilic airway disease (Figure 5.11).

In Chapter 4, we found that the symptoms of CMH were not sensitive or specific enough to be useful as biomarkers of the mucus plug phenotype. This may be because the mucus plugs occur in sub-segmental airways that lack large numbers of cough receptors (28-30). We also show that the inflammatory profile of the CMH phenotype is different from the profile of mucus plug phenotype. Unlike the mucus phenotype, which is strongly associated with eosinophilia and other markers of type 2 inflammation, the CMH phenotype was not associated with any markers of airway inflammation (type 1 or type 2). This shows that CMH is phenotypically and biologically a distinct from airway mucus plugging. Analysis was restricted to asthmatic subjects with and without CMH and did not include healthy controls due to the exclusion criteria in this group.

In conclusion, we provide a mechanism of airflow obstruction in corticosteroid- and bronchodilator-resistant asthma that involves eosinophil-driven mucus plugging of
sub-segmental airways. Our findings raise the possibility that MDCT scans could be used to identify patients with mucus plugs to facilitate clinical trials to test whether biologic therapies inhibiting type 2 inflammation may decrease mucus plugging in asthma.

The objective of this study was to test the hypothesis that mucus plugs are associated with type 2 airway inflammation. We tested for association between mucus plugging and different markers of airway inflammation, including sputum gene expression for type 2 cytokines and mucin genes, before and after systemic steroid treatment. In addition, we tested for regional heterogeneity of inflammation marked by mucus plugs in a single patient. Finally, we tested whether eosinophils promote mucus plug formation through generation of oxidative products by generating oxidative products that oxidize and crosslink cysteines between mucin polymers. In light of the findings in this study, we can accept that mucus plugging is associated with blood and sputum eosinophilia, FeNO, and sputum gene expression of type 2 cytokines. Furthermore, we can accept that mucus plugging is associated with persistent type 2 inflammation following systemic steroid treatment. We cannot accept that mucus plugging is associated with altered mucin ratio as there is too much overlap between groups. In addition, we cannot accept that there is regional heterogeneity of type 2 inflammation marked by mucus plugs as a larger sample size is required. Finally, we can accept that eosinophils and their products are associated with promotion of cysteine crosslinking that is reversible with DTT treatment. Together, these data are sufficient to accept an association between mucus plugging and type 2 inflammation.

5.5 References

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Chapter 6 Discussion, Summary and Conclusion

6.0 General Discussion

The aim of this thesis was to test the overarching hypotheses that patients with asthma who have a significant number of mucus plugs on CT would be characterized by a low FEV1, airway type 2 inflammation and abnormal mucin gene expression. Furthermore, that eosinophils play a role in mucus plugs formation. To test these hypotheses, I developed a visual scoring system to quantify mucus plugs in MDCT scans of the lungs and applied it to patients enrolled in the SARP. I investigated the reproducibility of the scores across multiple readers and time. I used this scoring system to identify patients with mucus plugs and to determine how mucus plugs relate to FEV1, symptoms of CMH, measures of type 2 inflammation, and mucin gene expression in induced sputum cells. Finally, I used model systems of airway mucus gels to explore whether oxidants generated by eosinophil peroxidase (EPO) oxidize cysteine thiol groups promote mucus plug formation.

The experimental data presented in this thesis provide a detailed profile of airway mucus plugging in chronic severe asthma. It is clear that mucus plugging is associated with airflow limitation and disease severity in asthma. Airflow limitation in asthma is the combined effect of a variety of changes in the airway, including bronchoconstriction, airway oedema, mucus hypersecretion and airway remodelling. Airway remodelling describes structural changes in the airway that contribute to airflow obstruction, including thickening of the basement membrane, subepithelial fibrosis, airway smooth muscle hypertrophy and hyperplasia, blood vessel proliferation and dilation, and mucous gland hyperplasia (1). Although airway remodelling has been considered a mechanism of airflow obstruction in these patients (2, 3), the relative contribution of each of these changes to overall airflow limitation is unknown. Data presented in this thesis represent a body of evidence that mucus plugging is strongly and independently associated with airflow obstruction in asthma, with a substantial effect size. Adjusted multivariate analysis shows that this
association is independent of age, gender and airway wall thickness, with the latter used as a measure of airway remodelling. These data provide compelling evidence that treatment of patients with low FEV1 and mucus plugging on CT (score of ≥4) with mucolytics could improve FEV1.

Another finding was that mucus plugging persisted or reoccurred in the same segment over many years. Conversely, segments without mucus plugs tended to remain plug-free. This heterogeneity between segments was further demonstrated on bronchoalveolar lavage, which showed 5-fold more eosinophils in the airway segment with mucus plug than in the segment without mucus plug on CT. The composition of mucus plugs was previously believed to comprise of varying amounts of mucins, plasma proteins, DNA and cellular debris (epithelial cells and eosinophils). In a detailed analysis of the polymers and proteins within fatal asthma mucus plugs, mucins were the dominant polymer, with proteoglycans, DNA and non-mucin proteins making up a small part of the gel structure of mucus plugs in fatal asthma. Unfortunately, no such analysis has been performed on the mucus plugs in chronic non-fatal asthma to confirm that mucus plugs seen in the subsegmental airways on MDCT are predominantly composed of mucins and eosinophils.

These data support a number of novel hypotheses. First, that there is heterogeneity of type 2 inflammation in the airway, with segments (or foci within segments) that have “ultra”-type 2 inflammation. Second, that high levels of type 2 inflammation in these areas promotes mucus plug formation. Finally, that mucus plugs themselves provide favourable conditions for the survival of eosinophils and even type 2 cytokines in the airway lumen, promoting the persistence of type 2 inflammation. Together, these would create a feed-forward loop by which type 2 inflammation promotes mucus plug formation, which in turn promotes persistent type 2 inflammation and may explain why mucus plugs are found in the same segments many years apart.

Data in Chapter 5 support the hypothesis that activated eosinophils can alter the biophysical properties of mucus, making it more elastic. As described previously, an increase in sputum elasticity has been shown to decrease mucociliary clearance (4), which in turn could lead to mucus plug formation. In patients with the persistent mucus high phenotype, treatment with corticosteroids appears to be insufficient and
may even be harmful. Combined investigation with CT mucus scores and blood eosinophil counts can be used to identify this population of patients with asthma, to enrol them in trials of mucolytic or type 2 inhibitor therapy. I postulate that eradication of mucus plugs would improve overall lung health independent of improving airflow limitation, via disruption of persistent type 2 inflammation.

Testing the overall hypothesis of this thesis relied heavily on the ability to measure mucus plugging in a way that was both accurate and reproducible. These data provide a novel methodology and a refined approach to accurately profiling mucus plugging within the airways using MCDT imaging, which may have future applications for diagnosis of mucus plugging and clinical surveillance. A robust, standardized CT scoring system was developed to measure the extent and site of mucus plugging in the airways. This scoring system demonstrated excellent reproducibility by epidemiologic standards. This CT mucus score, with comprehensive patient characterisation and targeted profiling of the airway at a cellular and molecular level, combined to allow robust modelling of the relationship between mucus plugging and asthma severity, airflow limitation and airway inflammation. Using these techniques, a number of interesting findings emerged relating to the study cohort and mucus groups. The cohort was designed to have at least 60% patients with severe asthma by ATS/ERS criteria and, consequently, the majority of patients were taking high dose corticosteroids (inhaled and/or oral) at enrolment. A significant proportion of these patients demonstrated prominent type 2 inflammation, despite high dose corticosteroid use, which persisted after protocol-mandated intramuscular triamcinolone acetonide treatment. Further analysis revealed that the high mucus group was enriched with patients with this persistent type 2 inflammation.

An interesting observation was the lack of association between mucus score and symptoms of CMH. Detailed phenotyping and endotyping of this cohort provide new evidence that mucus plugging and CMH represent distinct phenotypes within asthma. While both are associated with lower FEV1, mucus plugging displays strong associations with airway inflammation; specifically, type 2 inflammation, whereas CMH was a non-specific finding that was not associated with airway or systemic inflammation. This is particularly relevant, since prior studies of mucus dysfunction
in asthma have focused exclusively on symptoms of CMH to stratify patients. The data presented herein indicate that using a symptom-based approach to identify patients with mucus plugging will result in misclassification of many patients. This highlights the importance of MDCT in identification and classification of patients with mucus plugging for the purpose of future clinical trials of therapies in asthma.

6.1 Summary

The aim of this thesis was to test the hypothesis that patients with asthma who have a significant number of mucus plugs on CT are characterized by a low FEV1, airway type 2 inflammation and abnormal mucin gene expression. Furthermore, to test that eosinophils play a role in mucus plugs formation.

In summary, I present the following key novel findings within this thesis:

1) A CT mucus score based on bronchopulmonary segmental anatomy is a valid and reproducible approach to quantifying burden of mucus plugging on MDCT in chronic severe asthma. Furthermore, mucus plugging was a stable phenotype within individual segments over many years.

2) Mucus plugging, as quantified by CT mucus score, is highly prevalent in patients with severe asthma, and is associated with clinical markers of asthma severity, but not with symptoms of CMH.

3) Using multivariate models of analysis, we demonstrated that mucus plugging was associated with airflow obstruction, independent of airway remodelling, that persists after treatment with maximal bronchodilation and systemic corticosteroids.

4) Mucus plugging is strongly associated with airway type 2 inflammation at a cellular and gene expression level. Additionally, type 2 inflammation in patients with a high mucus score is resistant to treatment with inhaled or systemic corticosteroid therapy.

5) There is regional heterogeneity of type 2 inflammation at a segmental level within asthma patients, wherein segments with mucus plugs visible on MDCT, have a markedly higher percent eosinophils in bronchoalveolar lavage than segments without mucus plugs visible on MDCT.
6) Eosinophils can increase mucin crosslinking through disulphide interactions between cysteines in mucin polymers. This increases the elasticity of mucus making it more difficult to clear from the airways.

In light of the findings above, we can accept that a CT mucus score is a valid and reproducible approach to quantifying mucus plugging on MDCT and that mucus plugging on CT is highly prevalent in patients with asthma, especially in patients with severe disease. We can accept that patients with a high mucus score are characterized by a low FEV1 but not by symptoms of CMH. We can accept that mucus plugging is associated with type 2 inflammation. We do not have enough evidence to accept that mucus plugging is associated with regional heterogeneity of type 2 inflammation as this has only been shown in 1 patient. Finally, we can accept that eosinophils play a role in promoting mucus plug formation by increasing mucin crosslinking through disulphide interactions between cysteines in mucin polymers.

6.2 Conclusion

The experimental work presented herein represents an important step in defining the pathological links between chronic mucus plugging, inflammation and asthma severity. By detailed characterising and endotyping of asthma patients with mucus plugging, we have identified a novel phenotype of asthma characterised by severe, eosinophilic, treatment-resistant disease. Our data provide a strong rationale to decrease or alter airway mucus as a strategy to improve FEV1 in patients with severe asthma. Because symptoms of mucus are frequently absent in patients with mucus plugs on MDCT, I propose that MDCT represents a valid and reproducible biomarker of mucus pathology, that can be used to stratify patients for inclusion in clinical trials of mucolytic or type 2 inhibition as treatments for low FEV1 in severe asthma. Using chest imaging to manage asthma represents a novel and feasible approach to precision treatment, especially with the recent advent of ultra-low-dose MDCT protocols.
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6.3 Future Directions

The findings of this thesis raise a number of important questions that are currently the subject of further investigation.

1) What factors predict the development, persistence and resolution of mucus plugs in asthma?

In Chapter 3, I show that in 25 patients with serial CT; mucus scores increased in 40% of patients, decreased in 32% of patients and remained unchanged in 28% of patients with asthma. Currently, I am investigating the factors that predict these three different disease trajectories by analysing serial imaging in a larger cohort of patients over a defined time period. I have recruited 4 radiologists to score the Year 3 scans in SARP 3 to compare to the baseline data presented here and we are near the completion of this project. Like the original study, each scan is scored by 2 radiologists and the mean scores of both readers are used for analyses. The same radiologists are assigned to score the subjects at both time points to reduce variability. A proportion of subjects will be rescored at both time points to assess for variation in scoring across time. The Year 3 data include the same characterization investigations that was used at baseline (without the steroid injection). The longitudinal study design will allow me to better explore causal associations between airway inflammation, mucus plugging and airflow obstruction in asthma. The same sample size calculated for the baseline analysis will be used in this longitudinal study.

2) Does reduction in mucus plugs result in improved airflow in severe asthma?

In Chapter 4, I assert that the relationship between mucus plugging and airflow obstruction is likely to be causal but cannot prove causation due to the cross-sectional design of the current study. One way to establish causality is to demonstrate that FEV1% increases when mucus plugs are removed. Since mucus plugs in asthma are predominantly composed of mucins (5) and the biochemical and rheological signature is that of cysteine crosslinking, a thiolated mucolytic, such as NAC, should lyse the mucus plugs. NAC has been trialled unsuccessfully as a therapeutic agent in asthma
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exacerbation, but previous trials administered NAC by oral route rather than nebulized route. Given orally, NAC is undetectable in BAL. Negative trials using the oral formulation may just reflect the failure of this drug to reach the airway compartment at a therapeutic dose rather than the inherent effectiveness of the drug itself.

I have designed a clinical study called the ‘Clinical Trial of NAC in Asthma’ (CONA) (ClinicalTrials.gov Identifier: NCT02605824) to examine the causality of relationship between mucus plugging and airflow obstruction. This randomized, double-blind, placebo-controlled study evaluates 20% NAC as a treatment for patients with moderate-to-severe asthma that have mucus plugging on CT. The study is a crossover design, with patients randomized to receive co-treatment with NAC and albuterol (three times daily) in the first 7-day treatment period and then placebo and albuterol (three times daily) in the next 7-day treatment period or placebo and albuterol in the first 7-day treatment period and then NAC with albuterol in the next 7-day treatment period. The crossover design, where each patient acts as their own control, greatly decreases the variance of the estimated treatment effect on FEV1. This permits a much smaller sample size than a parallel group study to meet the same criteria in terms of type I and type II error risks. A sample size of 30 patients will give 80% power to detect a 10% difference (± 19) in FEV1% predicted, with a two-sided alpha of 0.05.

I hypothesize that in patients with asthma, decreasing mucus plugging using a mucolytic, will result in an improvement of lung function. The primary outcome of the study is change in post bronchodilator FEV1 (L) over a seven-day treatment period. The secondary outcome of the study is change in CT mucus score from baseline and from the beginning of each treatment period. This study will achieve two goals; it will test for a causal relationship between mucus plugging and airflow obstruction and, if positive, will support a role for mucolysis in the management of asthma that is complicated by mucus plugging. A dose escalation study has already been performed to determine the dose range that is safe and tolerable in patients with asthma, avoiding the bronchoconstriction that has been reported using NAC in this patient group. To further ensure patient safety, this study will be carried out as an in-patient study.
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3) Does reduction in airway eosinophils lead to a reduction in mucus plugging?

In Chapter 5, I provide evidence that eosinophilia may play a role in mucus plug formation and postulate that anti-eosinophilic agents may have a specific role in the treatment of these patients. In collaboration with Novartis, I designed a study to test this hypothesis in patients treated with Fevipiprant versus placebo. Fevipiprant is an oral treatment for asthma that is currently under investigation in two phase-3 trials. It is a small molecule that competitively and reversibly antagonises the prostaglandin D2 receptor 2 (DP2, also known as CRTh2). DP2 is expressed on inflammatory cells that are important regulators and effectors of Type 2 inflammation; CD4 and CD8 T2 cells (6), ILC2s (7), eosinophils (8), basophils (8), mast cells (9), and monocytes (10). The prostaglandin D2/DP2 axis is implicated in promoting eosinophilic airway inflammation. A phase 2 study of the effect of fevipiprant on airway eosinophils in moderate-to-severe asthma showed a 3.5-fold greater reduction in sputum eosinophils in the fevipiprant group than the control group. This study included pre- and post-treatment CT scans as part of its characterization. I will be analyzing CT mucus scores in these patients with change in mucus score as the primary outcome. Forty-eight patients have serial scans as part of this study. This sample size will give 80% power to detect a 6-point (± 2) change in mucus plug score following treatment, with a two-sided alpha of 0.05.

4) Are there genotypic differences in the airways that form mucus plugs compared to airways that remain plug-free?

In Chapter 5, I showed in one subject that there was a significant difference in sputum eosinophilia in an airway with mucus plug compared to an airway without mucus plug, suggesting that there was heterogeneity in type 2 inflammation between airways in the lung and that mucus plugs mark these areas of high type 2 inflammation. I plan to extend these observations in a larger bronchoscopy study in which BAL from plugged and unplugged airways will be compared endotypically (sputum cell differentials) and genotypically (RNA-sequencing). A sample size of 10 will have 80% power to detect a 5% (± 5) difference in eosinophils between plugged and unplugged airway segments, with a two-sided alpha of 0.05. This study may shed light on this apparent regional
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type 2 inflammation and the interesting theory that these areas may represent immunologic scars.

5) Does mucus plugs cause air trapping or does collateral ventilation develop between segments?

I have previously examined this question using automated measures of air trapping (-856 HU, %) on expiratory CTs, but found this approach limited by only having data at a lobar level, which is not sensitive to show defects secondary to individual mucus plugs. An alternative approach I am currently exploring is to compare the effect of mucus plugs in these subjects with ventilation defects measured on hyperpolarized Helium-3 magnetic resonance imaging ($^3$He MRI). Ventilation defect data are available in 59 subjects from SARP 3 that had $^3$He MRI performed as a sub-study at University of Wisconsin, Madison and Washington University, St. Louis. This sample size will give 80% power to detect a 15% ($\pm$ 20) difference in ventilation defect percent (VDP) between asthma patients with mucus plugging and patients without mucus plugging, with a two-sided alpha of 0.05. In collaboration with investigators at these sites, I plan to compare the presence or absence of mucus plugs with ventilation defects to determine whether mucus plugs result in air trapping in asthma. A recent paper found an association between persistent ventilation defects on $^3$He MRI and sputum eosinophilia and concluded that MRI identifies persistent ventilation defects in patients with severe asthma, ‘which may be the functional consequence of airway inflammation’ (11). My interpretation of these data is that MRI is identifying mucus plugging as ventilation defects and these plugs, in turn, are marking regional areas of high type 2 inflammation.

6) Can airway mucus plugs be quantified using an automated approach?

This thesis set out to develop and refine a method of quantifying mucus plugging on CT scan. Having two radiologists visually identify mucus plugs using defined criteria could be considered a gold standard approach to achieving this as no other approaches are available. I will use the data generated in this study to validate different automated ways of measuring mucus plugs. I am collaborating with FLUIDDA (Kontich,
Belgium), a leading R&D company in the field of quantitative image analysis, to develop machine learning algorithms for measuring mucus plugs by quantifying airway-pruning. Another approach is to utilize artificial intelligence technology that subtracts airways and vessels from CT scans leaving the parenchyma and airspaces. This technology was developed for identifying pulmonary nodules but also identifies mucus plugs, which to date had been viewed as a limitation of the software. I plan to collaborate with Riverain technologies (Ohio, USA) to test whether this technology can improve mucus plug detection and be automated to score CT scans for mucus. Having an automated tool for quantifying mucus plugging would allow mucus plugging to be investigated in larger cohorts of patients with asthma as well as other airway diseases that are complicated by mucus pathology.

6.4 References


7. Xue L, Salimi M, Panse I, Mjöberg JM, McKenzie ANJ, Spits H, et al. Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-


Chapter 7 Supplemental Appendix

7.0 Sputum and Cough Questions

CMH was measured by the question “Have you had cough and sputum production on most days for at least 3 months a year for at least 2 consecutive years?” Data for CMH is missing in 25 patients (17.1%) most of whom were recruited early in SARP. This was due to skip logic in the medical history questionnaire. Initially, the question for CMH was a follow-on to the question “Have you ever had bronchitis?”. Patients who answered “No” to ever having bronchitis were directed to skip the sub-question specific to CMH and these data were therefore missing. This skip logic was removed in October 2013, and CMH became an independent question from then on.

7.1 Sputum Induction and Analyses

Subjects inhaled nebulized 3% saline through a mouthpiece for 12 minutes. Peak expiratory flow rate (PEFR) was measured every 4 minutes to monitor for excessive bronchoconstriction (>20% fall in PEFR), in which the test was suspended, and spirometry performed to confirm a decrease in FEV1% from post-bronchodilator baseline. Induced sputum was collected at these 4-minute intervals after saliva was discarded. Sputum was processed within 1 hour when possible. A 10% solution of Sputolysin (EMD Millipore) was added at a 1:1 ratio (v/w) to the induced sputum, mixed using a serological pipette, and placed in a 37°C shaking water bath for 15 minutes. Samples were removed at 5, 10, and 15-minute intervals for additional mixing with the pipette. A portion of this sample was removed to determine total and differential cell counts, as previously described (1). The sample was then centrifuged at 4°C and 2000 rpm for 10 minutes. The cell pellet was then re-suspended in 1 mL of Qiagen RNAprotect Saliva Reagent. All pellets were stored at -80°C.
7.2 Sputum Quality Systems

A number of metrics were used to determine sputum quality for inclusion in analyses
1) Cell counts: Sputum samples were deemed of sufficient quality if squamous cell count was <80%.
2) qPCR: Only sputum samples with adequate cell counts were analysed for qPCR. RNA quality was measured with the Agilent 2100 Bioanalyzer (Biogen; Weston, MA), which performs electrophoretic separations according to molecular weight. The RNA integrity number (RIN) was measured for each sample (2, 3) and only samples whose RIN value was >5 were considered adequate for gene expression profiling (4).

7.3 Multi Detector Computerized Tomography Protocol

MDCT was performed within 2 hours of maximal bronchodilation according to a standard protocol. The same scanning protocol was used in both asthma patients and healthy controls across all centres. Before beginning the MDCT scans, patients were carefully coached using standardized breathing instructions administered by the technologist, and images of the lungs at Total Lung Capacity (TLC) were obtained from a single breath-hold at full inspiration. The MDCT parameters for each scanner model used are listed in Supplemental Table 7.3. BMI (3 categories), lung volume (e.g., TLC) and scanner model were used to determine the CTDIvol and subsequently the effective mAs or mA settings appropriate for each subject (Supplemental Table 7.4). Scanners at each centre were regularly calibrated with a phantom (COPDgene® Phantom Model CCT162, The Phantom Laboratory - http://www.phantomlab.com/other-catphans/) and all scans were evaluated for protocol adherence by the SARP Imaging Centre at the University of Iowa. De-identified image data (in standard digital format) were distributed to the radiologists for scoring. To blind the readers to the disease status of the subject, healthy subjects were given a SARP identification number, and the scan dates were shifted forward 3 years to match the scanning period of the asthmatic scans. Evaluation of mucus was performed on scans taken at total lung capacity using a standard window width of 1200 HU and level of -600 HU (5).
7.4 Supplemental tables

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<th>TABLE 7.1 SARP 3 VISIT SCHEDULE AND STUDY PROCEDURES</th>
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<td>ImmunoCap (includes IgE)</td>
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### TABLE 7.2 GENE PRIMERS AND PROBES

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<tr>
<td>PPIA-inner reverse</td>
<td>GCAGATGAAAAACTGGGAACCA</td>
</tr>
<tr>
<td>GAPDH-outer forward</td>
<td>CAATGACCCCTTCATTGACCTC</td>
</tr>
<tr>
<td>GAPDH-outer reverse</td>
<td>CTCGCTCTGGAAGATGATGGTA</td>
</tr>
<tr>
<td>GAPDH-inner forward</td>
<td>GATTTCAACATGGCAATTCC</td>
</tr>
<tr>
<td>GAPDH-probe</td>
<td>CGTTCTCAGCCTGGACGTTGCCA</td>
</tr>
<tr>
<td>GAPDH-inner reverse</td>
<td>GGGAATTTCCATGGACAAAGC</td>
</tr>
<tr>
<td>YWHAZ-outer forward</td>
<td>CTTCTGTCTTGTCAACCAACCATTTC</td>
</tr>
<tr>
<td>YWHAZ-outer reverse</td>
<td>CAACTAAGGAGAGATTGTGCTGAG</td>
</tr>
<tr>
<td>YWHAZ-inner forward</td>
<td>TGGAAAAAGGCGCATGAT</td>
</tr>
<tr>
<td>YWHAZ-probe</td>
<td>TGGCTCCACTCAGTGCTAAAGGCACCCCT</td>
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<tr>
<td>YWHAZ-inner reverse</td>
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</tr>
<tr>
<td>PSMB2-outer forward</td>
<td>CCCATATCATGGAACCCCTGCCTTCTC</td>
</tr>
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<td>PSMB2-outer reverse</td>
<td>GTGCGAGTACTGAGAGTCAGGGGA</td>
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<td>PSMB2-inner forward</td>
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<td>PSMB2-probe</td>
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</tr>
<tr>
<td>PSMB2-inner reverse</td>
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<td>IL4-outer forward</td>
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<td>IL4-inner forward</td>
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<tr>
<td>IL4-probe</td>
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<tr>
<td>IL4-inner reverse</td>
<td>GCTCTGTGACAGCTGTTCAAGGTT</td>
</tr>
<tr>
<td>IL5-outer forward</td>
<td>GCCATGAGGATGCTTCTTCTGCA</td>
</tr>
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<td>IL5-outer reverse</td>
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<td>IL5-inner forward</td>
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<td>IL5-probe</td>
<td>CCCACAGAAATTTCCCAACAGTCCAGCA</td>
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<td>IL5-inner reverse</td>
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<tr>
<td>IL13-outer forward</td>
<td>CAACCTGACAGCTGGCATGT</td>
</tr>
<tr>
<td>IL13-outer reverse</td>
<td>CCTTGTCGGCGGAGAATC</td>
</tr>
<tr>
<td>IL13-inner forward</td>
<td>GGCCTGGAATTCCTGATCA</td>
</tr>
<tr>
<td>IL13-probe</td>
<td>TCGATGCGACTGACGCTGAC</td>
</tr>
<tr>
<td>IL13-inner reverse</td>
<td>GCTCAGCATCCTGCTACGTGTT</td>
</tr>
<tr>
<td>Primer Type</td>
<td>Sequence</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>IL17-outer forward</td>
<td>ACTGCTACTGCTGCTGAGCCT</td>
</tr>
<tr>
<td>IL17-outer reverse</td>
<td>GGTGAGGTGGGATCGGGTTGTAGT</td>
</tr>
<tr>
<td>IL17-inner forward</td>
<td>CAATCCCCACGAAAATCCAGGA</td>
</tr>
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<td>IL17-probe</td>
<td>CCCAAATTCTGAGGACAAGAACCTTCCC</td>
</tr>
<tr>
<td>IL17-inner reverse</td>
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</tr>
<tr>
<td>MUC5B-outer forward</td>
<td>TACATCTTGGCCAGGACTACTGT</td>
</tr>
<tr>
<td>MUC5B-outer reverse</td>
<td>AGGATCAGCTCGTAGCTCCAC</td>
</tr>
<tr>
<td>MUC5B-inner forward</td>
<td>CATCGTCACCGAGAACATCC</td>
</tr>
<tr>
<td>MUC5B-probe</td>
<td>CTGTTGGACCACCGGCACACCAC</td>
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<tr>
<td>MUC5B- inner reverse</td>
<td>AAGAGCTTGATGGCCTTGGA</td>
</tr>
<tr>
<td>MUC5AC-outer forward</td>
<td>TGTTGGCGGGAAAGACAGC</td>
</tr>
<tr>
<td>MUC5AC-outer reverse</td>
<td>CCTCCCATGGGCTTAGCTTCAGC</td>
</tr>
<tr>
<td>MUC5AC-inner forward</td>
<td>CGTTGGTCACCGAGAACGT</td>
</tr>
<tr>
<td>MUC5AC-probe</td>
<td>CTGCAGACCCACAGGGACCA</td>
</tr>
<tr>
<td>MUC5AC- inner reverse</td>
<td>ATCTTGATGGCCTTGAGCA</td>
</tr>
</tbody>
</table>
### Supplemental Appendix

#### TABLE 7.3 CT PARAMETERS: TOTAL LUNG CAPACITY (TLC) PROTOCOL

<table>
<thead>
<tr>
<th>Scanner Model</th>
<th>SIEMENS Definition (AS Plus) 128 slice</th>
<th>SIEMENS Definition (DS) 64 slice</th>
<th>SIEMENS Sensation 64 slice</th>
<th>GE VCT 64 slice/Discovery STE</th>
<th>GE Discovery CT 750HD 64 slice</th>
<th>PHILIPS Brilliance 64 slice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan Type</td>
<td>Spiral</td>
<td>Spiral Single Source</td>
<td>Spiral</td>
<td>Helical</td>
<td>Helical - Standard</td>
<td>Spiral Helix</td>
</tr>
<tr>
<td>Scan FOV</td>
<td>No selection</td>
<td>No Selection</td>
<td>No selection</td>
<td>Large</td>
<td>Large</td>
<td>No selection</td>
</tr>
<tr>
<td>Rotation Time (s)</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Det. Configuration</td>
<td>128x0.6</td>
<td>64x0.6</td>
<td>64x0.6</td>
<td>64x0.625</td>
<td>64x0.625</td>
<td>64 x 0.625</td>
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<td>Pitch</td>
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<td>1.0</td>
<td>1.0</td>
<td>0.984</td>
<td>0.984</td>
<td>0.923</td>
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<td>kVp</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Effective mAs</td>
<td>S-90</td>
<td>S-85</td>
<td>S-80</td>
<td>S-145</td>
<td>S-145</td>
<td>S-105</td>
</tr>
<tr>
<td></td>
<td>M-110</td>
<td>M-105</td>
<td>M-100</td>
<td>M-180</td>
<td>M-180</td>
<td>M-130</td>
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<tr>
<td></td>
<td>L-165</td>
<td>L-150</td>
<td>L-145</td>
<td>L-270</td>
<td>L-270</td>
<td>L-190</td>
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<td>Dose modulation</td>
<td>Care Dose OFF</td>
<td>Care Dose OFF</td>
<td>Care Dose OFF</td>
<td>Auto mA OFF</td>
<td>Auto mA OFF</td>
<td>Dose Right (ACS) OFF</td>
</tr>
<tr>
<td>Std. Algorithm</td>
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<td>B35</td>
<td>B35</td>
<td>Standard</td>
<td>Standard</td>
<td>B</td>
</tr>
<tr>
<td>Lung Algorithm</td>
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<td>B31</td>
<td>None</td>
<td>Detail</td>
<td>Detail</td>
<td>YB</td>
</tr>
<tr>
<td>Additional Image filters</td>
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<td>No Selection</td>
<td>No Selection</td>
<td>No Selection</td>
<td>IQ Enhance OFF</td>
<td>Adaptive Filtering OFF</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.625</td>
<td>0.625</td>
<td>0.67</td>
</tr>
<tr>
<td>Interval (mm)</td>
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<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Iterative reconstruction</td>
<td>IRIS OFF</td>
<td>IRIS OFF</td>
<td>No Selection</td>
<td>ASIR</td>
<td>ASIR</td>
<td>iDOSE</td>
</tr>
<tr>
<td>Scan Time (Sec)</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>30cm length</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>Plus</td>
<td>Plus</td>
<td>N/A</td>
</tr>
<tr>
<td>Recon Mode</td>
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<td>N/A</td>
<td>N/A</td>
<td>OFF</td>
<td>OFF</td>
<td>N/A</td>
</tr>
<tr>
<td>Smart mA</td>
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<td>N/A</td>
<td>N/A</td>
<td>OFF</td>
<td>OFF</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* Effective mAs: Siemens = Eff. mAs, GE = mA setting, Philips = mAs. S = small, M = medium, and L = large. BMI categories as defined in supplemental Table 7.4.
### TABLE 7.4 CTDIVOL AS A FUNCTION OF BMI

<table>
<thead>
<tr>
<th>Body Size</th>
<th>BMI Range</th>
<th>CTDIVOL (mGy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small</td>
<td>15 to 19</td>
<td>11.4</td>
</tr>
<tr>
<td>Medium</td>
<td>20 to 30</td>
<td>7.6</td>
</tr>
<tr>
<td>Large</td>
<td>&gt;30</td>
<td>6.1</td>
</tr>
</tbody>
</table>

### Table 7.5 Characteristics of asthma subjects with repeat CT scans

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Time points</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SARP 1/ SARP 2</td>
</tr>
<tr>
<td>Mean age (years)*</td>
<td>44.3 ± 10.3</td>
</tr>
<tr>
<td>Female sex - no. (%)</td>
<td>13 (52)</td>
</tr>
<tr>
<td>Spirometry data*</td>
<td></td>
</tr>
<tr>
<td>FEV1 (% predicted)</td>
<td>67.7 ± 19.5</td>
</tr>
<tr>
<td>FVC (% predicted)</td>
<td>80.4 ± 16.1</td>
</tr>
<tr>
<td>FEV/FVC</td>
<td>0.67 ± 0.11</td>
</tr>
<tr>
<td>Max FEV1 (% predicted)</td>
<td>81.4 ± 21.1</td>
</tr>
<tr>
<td>Max FVC (% predicted)</td>
<td>91.7 ± 15.5</td>
</tr>
<tr>
<td>Sputum cell counts (%)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.3 (0.001, 3.2)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>62 (32.2, 76.3)</td>
</tr>
<tr>
<td>FeNO (ppm)‡</td>
<td>22 (10.3, 39.6)</td>
</tr>
<tr>
<td>Blood cell counts (x10⁶/L)†</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>259 ± 232</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4782 ± 2819</td>
</tr>
<tr>
<td>Mucus Score, segments</td>
<td>2 (0.9)</td>
</tr>
<tr>
<td>Mucus Score, categories</td>
<td></td>
</tr>
<tr>
<td>Zero</td>
<td>10 (40)</td>
</tr>
<tr>
<td>Low</td>
<td>4 (16)</td>
</tr>
<tr>
<td>High</td>
<td>11 (44)</td>
</tr>
</tbody>
</table>

Data reported as mean and standard deviation unless otherwise indicated.

* Age and spirometry data for SARP 1/SARP 2 missing in 1 patient
† Spirometry data for SARP 1/SARP 2 missing in 3 patients
‡ FeNO data for SARP 1/SARP 2 missing in 8 patients
§ Sputum cell count data for SARP 1/SARP 2 missing in 15 patients and for SARP 3 in 5 patients.
### TABLE 7.6 INTERPRETATION OF INTRACLASS CORRELATION COEFFICIENT VALUES

<table>
<thead>
<tr>
<th>Strength of agreement</th>
<th>Agreement value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Almost perfect</td>
<td>&gt;0.8</td>
</tr>
<tr>
<td>Strong</td>
<td>0.7-0.8</td>
</tr>
<tr>
<td>Moderate</td>
<td>0.5-0.6</td>
</tr>
<tr>
<td>Fair</td>
<td>0.3-0.4</td>
</tr>
<tr>
<td>Poor</td>
<td>0-0.2</td>
</tr>
</tbody>
</table>


### TABLE 7.7 AEROALLERGEN SENSITIVITY

<table>
<thead>
<tr>
<th>Allergen</th>
<th>All (n=144)</th>
<th>Zero (n=61)</th>
<th>Low (n=44)</th>
<th>High (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus fumigatus, no. (%)</td>
<td>30 (20.8)</td>
<td>11 (18.0)</td>
<td>11 (25.0)</td>
<td>8 (20.5)</td>
</tr>
<tr>
<td>Cladosporium herbarum, no. (%)</td>
<td>21 (13.9)</td>
<td>8 (13.1)</td>
<td>9 (20.5)</td>
<td>4 (10.3)</td>
</tr>
<tr>
<td>Alternaria alternata, no. (%)</td>
<td>37 (25.7)</td>
<td>15 (24.6)</td>
<td>15 (34.1)</td>
<td>7 (18.0)</td>
</tr>
<tr>
<td><strong>Furred animal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cat dander, no. (%)</td>
<td>82 (56.6)</td>
<td>32 (52.5)</td>
<td>28 (62.2)</td>
<td>32 (56.4)</td>
</tr>
<tr>
<td>Dog dander, no. (%)</td>
<td>78 (53.8)</td>
<td>33 (54.1)</td>
<td>26 (57.8)</td>
<td>19 (48.7)</td>
</tr>
<tr>
<td>Mouse urine proteins, no. (%)</td>
<td>16 (11.0)</td>
<td>6 (9.84)</td>
<td>7 (15.6)</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>Rat urine proteins, no. (%)</td>
<td>21 (14.5)</td>
<td>10 (16.4)</td>
<td>7 (15.6)</td>
<td>4 (10.3)</td>
</tr>
<tr>
<td><strong>Mites and insects</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermatoph pteronyssinus, no. (%)</td>
<td>70 (48.3)</td>
<td>31 (50.8)</td>
<td>23 (51.1)</td>
<td>16 (41.0)</td>
</tr>
<tr>
<td>Dermatoph fariane, no. (%)</td>
<td>71 (49)</td>
<td>32 (52.5)</td>
<td>24 (53.3)</td>
<td>15 (38.5)</td>
</tr>
<tr>
<td>Cockroach, no. (%)</td>
<td>29 (20.1)</td>
<td>16 (26.2)</td>
<td>7 (15.9)</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td><strong>Plant</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ragweed, no. (%) *</td>
<td>44 (30.6)</td>
<td>25 (41.0)</td>
<td>13 (29.6)</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>Weed mix, no. (%)</td>
<td>41 (28.5)</td>
<td>23 (37.7)</td>
<td>12 (27.3)</td>
<td>6 (15.4)</td>
</tr>
<tr>
<td>Grass mix, no. (%)</td>
<td>42 (29.0)</td>
<td>18 (29.5)</td>
<td>13 (29.0)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Tree mix, no. (%)</td>
<td>45 (31.3)</td>
<td>20 (32.8)</td>
<td>14 (31.8)</td>
<td>11 (28.2)</td>
</tr>
</tbody>
</table>

Aeroallergen sanitization defined as specific IgE >0.35 IU on ImmunoCAP test (Phadia; Uppsala Sweden).
Blood measurements were not available for 2 subjects.
* P<0.05
# Supplemental Appendix

## Table 7.8 Inflammatory Markers Stratified by Chronic Mucus Hypersecretion and Mucus Plugging

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Chronic mucus hypersecretion*</th>
<th>Mucus plugging</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent (n=80)</td>
<td>Present (n=41)</td>
<td>Zero (n=61)</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Airway measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeNO (ppm)**</td>
<td>20 (12,35)</td>
<td>20 (11,29)</td>
<td>18 (10,27)</td>
</tr>
<tr>
<td>Sputum eosinophil count (%)††</td>
<td>0.7 (0.2,3.5)</td>
<td>0.6 (0.4,5)</td>
<td>0.2 (0,9)</td>
</tr>
<tr>
<td>Sputum neutrophil count (%)††</td>
<td>59 (33,77)</td>
<td>66 (42,83)</td>
<td>62 (37,83)</td>
</tr>
<tr>
<td><strong>Blood measures‡‡</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood eosinophil count (x10⁶/L)</td>
<td>284 ± 202</td>
<td>338 ± 347</td>
<td>209 ± 153</td>
</tr>
<tr>
<td>Blood neutrophil count (x10⁶/L)</td>
<td>4278 ± 2541</td>
<td>4450 ± 2258</td>
<td>4569 ± 2951</td>
</tr>
<tr>
<td>Total IgE (IU/ml)</td>
<td>138 (46,306)</td>
<td>129 (35,406)</td>
<td>125 (32,482)</td>
</tr>
<tr>
<td>Sputum cell gene expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>20 (17, 21)</td>
<td>20 (18, 21)</td>
<td>19 (17, 21)</td>
</tr>
<tr>
<td>IL-17</td>
<td>18 (18,20)</td>
<td>19 (17,20)</td>
<td>18 (17,20)</td>
</tr>
<tr>
<td>MUC5AC/MUC5B</td>
<td>0.99 (0.9,1.1)</td>
<td>0.99 (0.9,1.1)</td>
<td>0.95 (0.86,1)</td>
</tr>
</tbody>
</table>

Data reported as mean ± standard deviation or median (interquartile range).

* Questionnaire data for chronic bronchitis are available for 121 patients (see Supplemental Appendix)

† p<0.05 for comparison between absent and present or zero and high groups

‡ p<0.01 for comparison between absent and present or zero and high groups

§ p<0.001 for comparison between absent and present or zero and high groups

†† Sputum cell counts were not available in 26 subjects due to ineligibility for sputum induction or because the induced sputum not meet quality metrics.

‡‡ Blood measurements were not available for 1 subject

Sputum cell gene expression data generated by Michael Peters
Figure 7.1 Modified web-based data capture tool used for longitudinal measurements

The figure shows a screen capture of the web-based survey form that was modified from the original data capture tool to measure mucus plugging at a segmental level for comparison within the same patient over time. The same scoring criteria were displayed at the top of the form and the radiologists entered the data into the data fields as shown here. The data capture shown here is for each segment of right upper lobe – additional fields were available in the tool for the segments in other lung lobes.
Figure 7.2 Examples of mucus plugs shown in different planes on MDCT

(A) Transverse plane: Intraluminal mucus plug (red arrow) in longitudinal section on transverse plane. The accompanying bronchopulmonary vessels are indicated with yellow asterisks. (B) Sagittal plane: The mucus plug in (A) is now seen on the sagittal plane (red arrow) with patent airway lumen (green arrow) visible proximally. (C) Transverse plane: Intraluminal mucus plug in cross section appears as a rounded opacification (red arrow) on transverse plane. Adjacent patent airway (green arrow) and bronchopulmonary vessels (yellow asterisks) are also shown. (D) Frontal plane: The plugged airway in (C) is now seen in longitudinal section as a tubular opacification (red arrow), and a patent airway (green arrow) is seen branching off proximally.
Figure 7.3 Distribution of mucus plugging within the lung

Mucus burden in each lobe is shown here as the proportion of segments with mucus plugging (i.e., the segment score) out of the total number of segments in that lobe. There was no significant difference in mucus plugging across the lung lobes.

Figure 7.4 Sputum cell differentials across mucus groups

There is no association between mucus group and (A) % sputum neutrophil, (B) % sputum macrophage or (c) % sputum lymphocyte counts.
Figure 7.5 Sputum cell gene expression of cytokines stratified by mucus score and CMH

(A) Gene expression for interleukin 4 is significantly increased in patients with a high mucus score. (B) Gene expression for interleukin 5 is significantly increased in patients with a high mucus score. (C) Gene expression for interleukin 17 is not significantly increased in patients with a high mucus score. (D) Gene expression for interleukin 4 is not significantly different in patients with sputum symptoms than in patients without sputum symptoms. (E) Gene expression for interleukin 5 is not significantly different in patients with sputum symptoms than in patients without sputum symptoms. (F) Gene expression for interleukin 17 is not significantly different in patients with sputum symptoms than in patients without sputum symptoms. ** signifies p<0.01, *** signifies p<0.001. Sputum cell gene expression data generated by Michael Peters.
7.6 Supplemental Video


https://jci.org/articles/view/95693/sd/3

CT scan demonstrating mucus plugs in relation to anatomical features in the right upper lobe. A patent sub segmental airway and 2 adjacent segmental bronchopulmonary vessels are labelled. Over sequential HRCT slices, airways that have patent lumens proximally (indicated by green arrow heads) are seen to transition into opacified airway lumens (red arrow). These opacified lumens meet the criteria for mucus plugs in the scoring system.

7.7 Peer Reviewed Articles Published Related to Thesis


7.8 References


