

Matrix Metalloproteinase-1 Mediated Extra-Cellular Matrix Remodelling Contributes to Airway Smooth Muscle Growth and Asthma Severity

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Abstract

Introduction

Airway remodelling describes the histopathological changes in tissue architecture observed in obstructive lung diseases such as asthma and may have a negative impact on lung function. These changes do not appear to be treated by current asthma treatments. Changes observed during airway remodelling include increased thickness of airway smooth muscle (ASM) layer and enhanced extracellular matrix (ECM) deposition. Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes, which facilitate tissue remodelling via ECM protein degradation. MMP expression is highly regulated in lungs and their expression is increased in disease states. My project aimed to assess MMP-1, -2 and -9 expression and activity in asthma airways. Furthermore, the underlying mechanism of MMP-1 activation and subsequently its role in airway remodelling and worsening asthma severity was investigated in the context of asthma exacerbation, which is thought to be an exaggerated lower airway inflammatory response to an environmental exposure such as respiratory virus infection.

Methods

Patients with stable asthma and healthy controls underwent spirometry, methacholine (PC_{20}) challenge, exhaled nitric oxide (FeNO) test, bronchoscopy/bronchial washings and primary ASM cell cultures. A second asthma group (mild to moderate severity) and controls had symptom scores, spirometry and bronchoalveolar lavage (BAL) before and after rhinovirus inoculation. ECM was prepared from decellularised primary ASM cultures. MMP-1 protein levels and activity were assessed in bronchial fluid samples by enzyme-linked immunosorbent assay (ELISA), western blotting and fluorescent activity assay. ASM cell growth

was measured by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reduction assay and cell counts. Bronchial fluid gelatinase (MMP-2 and -9) expression and activity was assessed by gelatin zymography.

Results

MMP-1 and MMP-9 expression was enhanced in both stable asthma and during asthma exacerbations, whilst MMP-2 expression was only increased during asthma exacerbations. MMP-1 can be activated by tryptase, which is an inflammatory product of mast cell degranulation. Activated (degranulated) mast cells enhanced proliferation of both control and asthma ASM cells via the production of a pro-proliferative ECM in vitro and the proliferative effect was dependent on MMP-1. In patients with asthma, mast cells numbers within ASM bundles were associated with ASM growth. MMP-1 protein levels were related to bronchial reactivity and MMP-1 activity increased during asthma exacerbations, where its levels were related to exacerbation severity.

Conclusion

This study suggests that MMP-1 plays an important role in asthma pathophysiology and that ASM/mast cell interactions contribute to asthma severity by transiently increasing MMP-1 activation, ASM growth and airway responsiveness. Moreover, there is increased expression of MMP-2 and -9 during asthma exacerbations compared with stable asthma. As both MMP-2 and -9 act as mediators of inflammation (Okada, S. et al., 1997) (Elkington, P.T.G., 2006) and tissue remodelling (Oshita, Y. et al., 2003), an increase in gelatinolytic activity linked to MMP-2 and MMP-9 is likely to play a significant role in the pathophysiology of asthma exacerbations.

Publications and abstracts

Publication arising from this thesis

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Abstracts arising from this thesis

1. Naveed, S. et al., 2016. C98 Mast cell tryptase activates matrix metalloproteinase-1 causing matrix remodelling, airway smooth muscle growth and airway obstruction during asthma exacerbations. American Journal of Respiratory and Critical Care Medicine 2016;193: A6174

2. Naveed, S. et al., 2015. S92 Matrix metalloproteinase-1 activation by mast cell tryptase causes airway remodelling and is associated with bronchial hyper-responsiveness in patients with asthma. Thorax, 70 (3), pp. A52–A52.

3. Naveed, S. et al., 2015. D29 Matrix Metalloproteinase-1 can modulate extra-cellular matrix proteins to increase airway smooth muscle proliferation, thereby contributing to airway remodelling in asthma. American Journal of Respiratory and Critical Care Medicine 2015;191: A5583

4. Naveed, S. et al., 2014. A66 A study of mechanism of airway remodelling in asthma: Mast cell proteases stimulate matrix metalloproteinase (MMP-1) activation and human airway smooth muscle growth. American Journal of Respiratory and Critical Care Medicine 2014;189: A2096

5. Naveed, S., Shaw, D. & Johnson, S.R., 2013. P186 Mast cell mediators stimulate human airway smooth muscle growth, a feature of airway remodelling in asthma via matrix metalloproteinase (MMP-1) activity. Thorax, 68 (3), pp. A159–A160

Statement of personal contribution to this work

My research required collaboration with other research scientists based in Imperial College, London and some of the data (asthma exacerbation study) was drawn from their projects to which they contributed the majority of their work. Therefore, a small part of data in chapter 5 of this thesis has been utilized in their previous publications but has never been analysed or interpreted in the manner I undertook for this project.

Stable asthma study

This study was not possible without the assistance of several scientific and clinical research staff within the Nottingham Respiratory Research Unit (NRRU) at different stages of this PhD. However, the work presented in this thesis is my own unless stated otherwise. I personally assessed suitability for volunteers for this study at screening visits, was present at all of the 35 individual study visits for eligible volunteers, performed each of the 27 bronchoscopies and processed all bronchial biopsy and fluid samples generated from these study visits. I carried out lung functions tests, airway challenge tests, FeNO measurements and asthma questionnaire data collection with support from respiratory research nurses. I personally performed all the cell cultures, ECM derivation, ASM proliferation experiments, MMPs protein/activity analyses and immunohistochemistry. I was also responsible for all data collation, statistical analyses and led with our publication arising from this work.

Asthma exacerbation study

Professor Sebastian Johnston (SJ), Dr David Jackson (DJ) of Imperial College, London and their research team were responsible for the recruitment of patients and their demographic data collection for the asthma exacerbation study; these patients were not recruited specifically for my project. DJ carried out all bronchoscopies,

viral inoculations, lung function tests, nasal lavages and symptom scores. I undertook MMPs analyses of BAL fluid, data collation, statistical analyses/interpretation and finally led with our publication arising from this work.

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Finally, to **my family** who had to endure this chapter in my life a lot more than I did, thank you for keeping your faith in me.

List of abbreviations

ACQ Asthma control questionnaire

AHR Airway hyperresponsiveness

ANOVA Analysis of variance

ASM Airway smooth muscle

ATS American Thoracic Society

BAL Bronchoalveolar lavage

BHR Bronchial hyperresponsiveness

BPTs Bronchial provocation tests

bFGF basic Fibroblast growth factor

BO Bronchiolitis obliterans

BSA Bovine serum albumin

BTS British Thoracic Society

CF Cystic fibrosis

COPD Chronic obstructive pulmonary disease

CSF Colony stimulating factor

CT Computed tomography

CysLT-1 Cysteinyl leukotriene-1

DAPI *4'*,6-diamidino-2-phenylindole

DMEM Dulbecco's modified eagle's medium

DNA/RNA Deoxyribonucleic acid/Ribonucleic acid

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

ERK Extracellular signal-regulated kinase

ERS European Respiratory Society

FAK Focal adhesion kinase

FBS Foetal bovine serum

FEV_1 Forced Expiratory Volume in the first second

FGF Fibroblast growth factor

FVC Forced vital capacity

GINA Global initiative for asthma

GMCSF Granulocyte monocyte colony stimulating factor

GOLD Global initiative for chronic obstructive disease

HRP Horseradish peroxidase

ICS Inhaled Corticosteroids

IFN-δ Interferon-gamma

IgE Immunoglobulin E

IGF Insulin-like growth factor

IGFBP Insulin-like growth factor binding protein

IL Interleukin

IL-1β Interleukin-1β

IPF Idiopathic pulmonary fibrosis

LABA Long acting β2 agonists

LAM Lymphangioleiomyomatosis

LAMA Long acting antimuscarinics

MCT Mast cell tryptase

MMPs Matrix metalloproteinases

MT-MMPs Membrane type MMPs

MTT 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide

NFκB Nuclear factor kappa B

NGAL Neutrophil gelatinase-associated lipocalin

OD Optical density

OSA Obstructive sleep apnoea

OVA Ovalbumin

PBS Phosphate buffered saline

PDGF Platelet-derived growth factor

PEFR/PEF Peak expiratory flow rate/Peak expiratory flow

PG Prostaglandin

PR3 Serine protease 3

PVDF Polyvinylidene difluoride

RDS Respiratory distress syndrome

RSV Respiratory syncytial virus

RV Rhinovirus

SABA Short acting β2 agonists

SCF Stem cell factor

SDS Sodium dodecyl sulphate

SOP Standard operating procedure

TFPI Tissue factor pathway inhibitor

TGF-β Transforming growth factor-β

TIMPs Tissue inhibitors of matrix metalloproteinases

TNF-α Tumour necrosis factor-alpha

TSLP Thymic stromal lymphopoietin

VEGF Vascular endothelial growth factor

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Chapter 1: General Introduction

Asthma is the commonest chronic respiratory disease in the western world affecting up to eight million people in the UK alone. Of these, approximately 5.1 million people receive treatment, with the total cost to the UK National Health Service (NHS) being around £850 million/year (www.asthma.org.uk). It is typically defined as reversible airflow obstruction and is often limited in younger patients to a milder disease. However, various degrees of airflow obstruction may persist, and in the long term, asthma may become moderately to fully irreversible. There are four main features fitting the current description of asthma: symptoms, airway inflammation, airway hyperresponsiveness (AHR), and variable airflow obstruction (Shaw, D. et al., 2012) (Benayoun, L. et al., 2003). Persistent airway inflammation and irreversible structural changes of the bronchial wall, collectively termed as airway remodelling, are thought to play an important role in severe asthma and are associated with AHR, airflow obstruction, worsening asthma symptoms, increased β2 agonist use and exacerbations and can persist despite optimal asthma treatment. Although asthma is a very common condition with increasing prevalence, its natural history has not been well described. Recent studies of patients with asthma from the general population have shown increased mortality in subjects with reduced ventilatory function and have therefore underlined the importance of preservation of normal lung function in asthmatics (Lange, P. et al., 1998) (Larsen, G.L., 1999). Treatment with regular long-acting β2 agonists (LABA) has increased in recent years, and their use is recommended as add-on medication to anti-inflammatory treatment in chronic persistent asthma to slow down the decline in lung functions in patients of moderate to severe

asthma. Combining salmeterol or formoterol with a low or medium dose inhaled corticosteroid (ICS) has also been shown to be more effective in controlling asthma symptoms and exacerbation frequency than increasing the dose of ICS alone (Lindqvist, A. et al., 2003).

1.1 Airway remodelling and its clinical relevance

Airway remodelling is a term used to define permanent changes in the tissue architecture of airways, mostly seen in obstructive airway diseases such as asthma. These structural changes result in steady decline of lung function and therefore are thought to be linked to overall disease morbidity. Current evidence suggests that asthma therapies available at present might not be very effective at reducing or preventing these changes (Beckett, P.A. & Howarth, P.H., 2003).

Airway remodelling was first described in 1922 by Hubert and Koesssler in cases of fatal asthma (Redington, A.E. et al., 1997). Airway remodelling has since been regularly observed in other types of obstructive respiratory illnesses, such as COPD, respiratory distress syndrome (RDS), cystic fibrosis (CF), bronchiolitis obliterans (BO) and obstructive sleep apnoea (OSA). These structural changes include thickening of basement membrane, loss of epithelial integrity and sub epithelial fibrosis, increased smooth muscle mass/ sub-epithelial myofibroblasts, goblet cell and mucous gland hyperplasia (Naylor, B. et al., 1962) (Carroll, N. et al., 1993) (Siddiqui, S. et al., 2008), increased bronchial wall vascularity with vasodilatation (LI, X. & Wilson J W. et al., 1997) and abnormal ECM/collagen deposition in the lamina reticularis underneath the epithelial basement membrane (Roche, W. et al., 1989) (Woodruff, P.G. et al., 2004). The increased ASM mass may be due to a combination of both ASM hyperplasia and hypertrophy,

selectively associated with disease severity and fixed airflow obstruction (Benayoun, L. et al., 2003).

For a long time, asthma had been considered to be a condition characterised by reversible airflow obstruction either spontaneously or in response to treatment (Brusasco, V. et al., 1998). However, cross sectional studies have shown that many asthmatics, both symptomatic (Brown, P.J. et al., 1984) and asymptomatic (Cade, J.F. & Pain, M.C.F., 2008) have evidence of persistent irreversible airflow obstruction. Moreover, increasing research in this area involving histopathological or imaging studies have shown structural defects and residual airway narrowing in some patients with asthma that may not be due to inflammation alone (Gibson, P.G., 2007). Structural changes of airway remodelling are associated with worse long-term clinical outcomes and have been attributed to both eosinophilic inflammation and the compressive mechanical forces that are produced during bronchoconstriction which may even induce remodelling independently of inflammation (Grainge, C.L. et al., 2011). Although in the majority of asthma patients, expiratory flows normalise with treatment, an incomplete reversibility of airflow obstruction may be found in a subset of patients without significant history of smoking or other associated respiratory conditions, despite maximum bronchodilator and corticosteroid treatment (Hudson, C. et al., 1997). Additionally, studies have confirmed that asthmatics as a group experience an accelerated rate of deterioration in respiratory function (Peat, J.K. et al., 1987) (Lange, P. et al., 1998).

Although airway remodelling is closely linked to airway inflammation, it has its own consequences in terms of asthma symptoms, exacerbations, decline in lung function and response to treatment. The possibility that the structural changes of

airway remodelling might be responsible for at least some of the clinical manifestations of asthma has only been investigated in detail in the last 10–15 years. However, despite extensive research, a number of important questions remain unanswered. For example, the specific elements of airway remodelling which contribute to the symptoms or progression of asthma are not well understood. Similarly, the factors which initiate and perpetuate remodelling are not clearly known. This may partly be due to the heterogeneity of this disease and the complex reciprocating relationships between numerous structural components within the airways (James, A.L. & Wenzel, S., 2007).

It is also not entirely known whether airway remodelling is a normal response to an abnormal injury or, alternatively, whether the remodelling/repair response is an abnormal response in itself (Beckett, P.A. & Howarth, P.H., 2003). In addition, while remodelling is generally believed to be disadvantageous, in some ways it may serve a protective function. For example, in a rat model exposure to ovalbumin (OVA) results in increased airway wall thickness suggestive of asthmatic remodelling (Palmans, E. et al., 2000). These rats initially develop AHR but, with prolonged exposure, scarring occurs with increased collagen deposition beneath the basement membrane and a consequent reduction in the thickness of the airway wall. Concurrently, the AHR improves and may even develop into a hypo-responsiveness (Palmans, E. et al., 2000). Likewise, increased collagen deposition around the ASM bundles may interfere with ASM contraction, thus effectively protecting against bronchoconstriction (Bramley, A.M. et al.,1995). It is likely that such fibrosis of the airway wall would replace the variability in peak expiratory flow (PEF), which is characteristic of asthma, with a fixed permanent element of airflow obstruction, and this may be considered a protective response. Therefore,

it is important to be careful to keep these contradictions in mind with regards to relevance of airway remodelling in chronic asthma.

In conclusion, changes observed in airway remodelling have been seen mainly as a consequence of inflammation, but factors other than inflammation (such as airway contraction), in the absence of inflammation, can also drive aspects of remodelling (Grainge, C.L. et al., 2011). Although a number of factors contribute to asthma severity, remodelling is almost certainly important and there is a reproducible relationship between the clinical severity of asthma and AHR (Hargreave, F. et al., 1981). As airway remodelling contributes to AHR, more marked changes of remodelling will result in more severe asthma and gradual decline in lung function (Woolcock, A.J. & Jenkins, C.R., 1990). It is also important to note that the degree to which abnormal lung function and airway remodelling are reversible is unclear. Using imaging modality of computed tomography (CT) scans, one study has shown partial reversibility of airway wall thickness in patients with asthma and suggested that there are steroid-responsive (likely inflammatory) and steroid-unresponsive (likely remodelling) components, contributing to increased airway wall thickness in asthma (Niimi, A. et al., 2004). However, remodelling is generally regarded as an irreversible process, but this may be related to dose and duration of treatment (Ward, C. et al., 2002). As airway structural changes may support inflammation (Carroll, N. et al., 1993), it is likely that airway inflammation, remodelling and AHR interact in a linear, parallel or combined manner to drive the asthma phenotype.

1.1.1 Mechanisms of airway remodelling

1.1.1.1 Alteration in epithelial integrity

Airways and airspaces of lungs are usually lined by different types of epithelial cells. Those lining airways are ciliated pseudo-stratified columnar epithelial cells. Epithelial changes in asthma include increased epithelial shedding, damage to ciliated cells, goblet cell hyperplasia and mucous overproduction as well as over expression of epidermal growth factor receptors (Naylor, B., 1962) (Carroll, N. et al., 1993). Clinically the extent of epithelial injury is correlated with AHR suggesting that the degree of epithelial loss or turnover is related to the development and progression of asthma (Boulet, L.-P. et al., 1997) (Jeffery, P.K. et al., 1989). Disruption of the physical barrier of the bronchial epithelium is implicated in asthma pathophysiology as this may facilitate the passage of allergens to the underlying submucosa, thus contributing to the development of immune responses and rendering airways more susceptible to allergic insult (Xiao, H., 2013). Epithelial changes are also found in other remodelling disease processes alongside sub-epithelial fibrosis, which has been consistently reported in all forms of asthma severities (Boulet, L.-P. et al., 1997).

1.1.1.2 Sub-epithelial fibrosis and ECM deposition

Sub-epithelial fibrosis occurs in lamina reticularis just below the basement membrane resulting in increased basement membrane thickness (Wilson, J.W. & Li, X., 1997). Fibrosis is a result of increased ECM deposition and decreased degradation of ECM proteins by fibroblasts (Roche, W. et al., 1989). Airway fibroblasts in asthmatic airways promote fibrosis by altering/increasing the ratio of tissue inhibitor of metalloproteinase (TIMP)-2 to MMP-2, therefore resulting in less ECM degradation (Bergeron, C.L. et al., 2010). The differential expression of

ECM proteins has also been studied in detail within asthmatic airway biopsies. Increase in ECM expression has been found to be associated with airway hyperresponsiveness and therefore asthma severity (Karjalainen, E.M. et al., 2003). These changes encompass increases in sub-epithelial deposition of collagen I, III, V, fibronectin, laminins, periosten, and tenascin-C and also increased deposition of collagen III and V in submucosa (Altraja, A. et al., 1996) (Chakir, J., et al., 2003) (Chetta, A. et al., 1997). Proteoglycan deposition in the ECM and bronchial fibroblast production of proteoglycans is also correlated with AHR in asthmatic patients (Huang, J. et al., 1999). Additionally, there are also changes in ECM deposition in airway cartilage, which are associated with perichondrial fibrosis observed both in severe asthma and COPD (Haraguchi, M. et al.,1999).

1.1.1.3 Increased ASM mass

Increased ASM layer thickness and myofibroblast numbers in the proximal airways have been a feature of remodelling that, by far, appears to differentiate severe asthma from mild/moderate forms of the disease (Pepe, C. et al., 2005). This is also evidenced by analyses of post-mortem tissue from subjects with severe and poorly controlled asthma demonstrating an apparent increase in ASM mass (Bai, T.R. et al., 2012).

The definite cause of increased ASM layer thickness in asthma is unknown, but it may be an aftermath of increased ASM proliferation or survival, either because of intrinsic abnormalities in the ASM or because of their interactions with the local inflammatory environment. Each may contribute to a varying degree and the predominant pathway may differ from airway generation to generation. A number of other mechanisms have been suggested as an explanation for an augmented ASM content in asthma. For example, enhanced collagen deposition in and around

ASM bundles may contribute to the overall increase in the ASM content (Thomson, R.J. & Schellenberg, R.R., 1998). Another unique mechanism proposed that may explain the increase in ASM content in asthma involves the participation of actin–containing fibromyocytes, which are distinct from myofibroblasts on the basis of their ultrastructural characteristics and represent differentiated myocytes that are able to migrate toward the surface epithelium to form new ASM bundles (Jeffery, P.K., 2001) (Parameswaran, K. et al., 2002). Some reports have even described increased proliferation in ex vivo asthmatic ASM cells, which has been attributed to alteration in mitochondrial biogenesis (Trian, T. et al., 2007).

As well as adapting to a proliferative phenotype, ASM cells also take on the secretory and immunomodulatory characteristics and play an active part in inflammatory and remodelling processes by releasing a variety of pro-inflammatory cytokines, chemokines and ECM proteins (Xia, Y.C. et al., 2013). It has been shown that chemokines and inflammatory mediators relevant to the environment within the airways of patients with asthma have the ability to induce smooth muscle cell migration, proliferation and to enhance their contractility in vitro, implying this as a mechanism significantly contributing to overall air flow obstruction in asthmatic patients (Joubert, P. et al., 2005). In addition to mitogenic stimuli, cell-matrix interactions are also important regulators of ASM proliferation (Johnson, P.R.A. et al., 2004)

1.1.1.4 Goblet cells and mucous gland hyperplasia

Goblet cells and sub mucosal gland hyperplasia are common features of severe or fatal asthma both in adults and children (Aikawa, T. et al., 1992). In an asthmatic airway goblet cells (which are glandular simple columnar epithelial cells) can

become hyperplastic, hypertrophic and metaplastic and have been shown to extend into peripheral airways where they usually do not exist (Xiao, C. et al., 2011). A functional consequence of goblet cells and glandular hyperplasia is increased mucous production and resultant airway narrowing due to sputum secretion and airway wall thickening (Carroll, N. et al., 1993).

1.1.1.5 Increased vascularity and angiogenesis

Angiogenesis is a pre-requisite for airway remodelling in bronchial asthma. Several growth factors are thought to play important roles in inflammation and angiogenesis through effects on inflammatory cell infiltration (Amishima, M. et al., 1998). There are a number of vascular alterations occurring in the airways of all asthma types and severities including increased size of airway wall blood vessels, increased mucosal blood flow and angiogenesis. These changes in airway wall microvasculature result in airway wall oedema and reduced airway calibre (Li, X. & Wilson, J.W., 1997). Angiogenesis is also known to increase the delivery of inflammatory and remodelling mediators to the airway wall and subsequently has an effect on structural and inflammatory processes of airway remodelling. Increased airway vascularity in asthma is associated with enhanced expression of vascular endothelial growth factor (VEGF) (Hoshino, M. et al., 2001).

1.1.1.6 Loss of cartilage integrity

Cartilage is a key determinant of airway wall integrity and stiffness. There has been evidence suggesting decreased cartilage mass and increased cartilage proteoglycan degradation in asthmatic airways (Haraguchi, M. et al.,1999). Cartilage degradation and loss of its integrity can result in a more powerful bronchoconstriction as a result of load reduction on ASM bundles for a given

degree of ASM cell contraction and contribute to chronic airway obstruction in severe cases of asthma (Noble, P.B. et al., 2002).

1.1.2 Role of inflammation in airway remodelling

The airways of asthmatic patients are characterised by T-helper (Th) cell-2 inflammatory responses which consist of airway infiltration by large numbers of eosinophils, mast cells and Th2 lymphocytes. These inflammatory cells produce inflammatory mediators that induce bronchoconstriction, mucous production and perhaps airway remodelling. The inflammatory mediators involved in this process are Th2 cytokine interleukin (IL)-4, IL-5, IL-13 and IL-9, histamine, lipid mediators, granulocyte-macrophage colony stimulating factor (GM-CSF) and transforming growth factor (TGF-β), which is a potent fibrogenic and immunomodulatory factor involved in the structural changes of asthmatic airways, especially sub-epithelial fibrosis and the presence of inflammatory infiltrates (Duvernelle, C. et al., 2003). TGF-β and some of the other mediators such as IL-11 and 17, having had strong airway remodelling properties by altering ECM, are therefore associated with severe airway disease (Chakir, J. et al., 2003). Histamine is a key mediator of allergic inflammatory reactions, and has been shown to play a role in the induction of airway remodelling by inducing lung fibroblast proliferation and connective tissue growth factor (CTGF) expression (Kunzmann, S. et al., 2007).

1.1.3 Impact of airway remodelling on lung function

According to previous studies, structural modifications of airway remodelling such as fibroblast accumulation and ASM hypertrophy in proximal airways, combined with airway inflammatory processes, seem to be directly related to the magnitude of functional consequences of asthma, AHR and airflow obstruction (Boulet, L.-P.

et al., 1997), and are also believed to be responsible for the chronicity of disease (Benayoun, L., et al., 2003).

Airway remodelling often starts in childhood, with airflow limitation associated with persistence of asthma and abnormal lung function in adulthood (Larsen, G.L., 1999). Among the children with mild-to-moderate persistent asthma, the majority develop abnormal patterns of lung growth and decline in lung function in early adulthood. Risk factors identified for reduced growth of lung function include maternal smoking during pregnancy, reduced lung function at enrolment, and increased AHR. Among these factors, reduced lung function at baseline was the strongest predictor of longitudinal lung-function impairment (McGeachie, M.J. et al., 2016). In another demographic study, in an unselected birth cohort, more than one in four children had wheezing that persisted from childhood to adulthood or that relapsed after remission. The factors predicting persistence or relapse were: AHR, sensitisation to house dust mites, female sex, smoking and early age of onset. These findings, together with persistently low lung function, suggest that outcomes due to airway remodelling in adult asthma may be determined in early childhood (Sears, M.R. et al., 2003).

The childhood asthma management program or 'CAMP' study (Larsen, G.L., 1999) showed a clear association between duration of asthma and reduced lung function, increased methacholine airway responsiveness, more asthma symptomatology, and greater use of as-required inhaled β2 agonists, which are all measures of asthma severity. As such, the study suggested early diagnosis and intervention to ameliorate these adverse effects of persistent asthma (Zeiger, R.S., et al., 1999). In another study, within a sample of the general population, people who identified themselves as having asthma had substantially greater declines in

lung function over time than those who did not have asthma. This even included people with asthma who did not smoke showing on average, a significantly greater decline in FEV_1 than other non-smokers in the study, suggesting that treatment should aim to keep the lung function of people with asthma at the highest achievable level (Lange, P. et al., 1998). In conclusion, these studies and the identification of airway remodelling in children confirms that structural changes of airway remodelling occur early in asthma and may be responsible for the reduced lung function associated with debilitating nature of this chronic airway disease. However, long term effects of airway remodelling are still largely unknown and more work is required to establish its role in permanently altering lung function in asthma.

1.1.4 Effects of current asthma treatments on airway remodelling

1.1.4.1 Bronchodilators (β2 agonists and Theophylline)

A number of studies have indicated that addition of an inhaled LABA gives better clinical control than increasing ICS and may also decrease exacerbation rates in patients with asthma who remain symptomatic despite low to moderate doses of ICS (Greening, A.P. et al., 1994) (Taylor, D.R. et al., 1998). However, there is rather little pathological data on the relative effects of these therapeutic regimens on the airway inflammation and their implications for the long-term "remodelling" process occurring in the airways of asthma patients. There is also no evidence that short acting β2 agonists (SABA) affect airway remodelling.

β2 agonists reduce muscular tone in the airways and therefore improve expiratory airflow. In one study, addition of LABA (salmeterol) for three months in symptomatic asthmatic patients, who already were on ICS, was shown to decrease

the density of blood vessels in their lamina propria as an index of remodelling when compared with patients taking ICS alone. However, there was no significant change in percentage area of lamina propria occupied by vessels within any of the patient groups and there was no relationship demonstrated between physiological changes and changes in vascularity indices over the three months' period of the study. It therefore concluded that the improvement in airway responsiveness observed with salmeterol was unlikely to be related to any change in vascularity, and more likely was due to the pharmacological effect of salmeterol on relaxing vascular smooth muscle (Orsida, B.E. et al., 2001). BAL studies have also not shown any significant influence of salmeterol on airway inflammatory cells mediated by the eosinophilic pathway and deposition of tenascin-C in patients with newly diagnosed and stable asthma (Lindqvist, A. et al., 2003). Another study found that, adding salmeterol for 12 weeks to ICS causes a significant reduction in the airway eosinophilic infiltrate as assessed by immunostaining, when compared with increasing ICS dose alone (Li, X. et al., 1999), therefore suggesting that salmeterol has an anti-inflammatory effect in asthma, which may be independent of ICS. Despite this, the evidence for the effect of LABA on airway remodelling is rather sparse and is only based on a few studies observing their effect in combination with ICS. These studies showed that in addition to their anti-inflammatory effects, ICS and LABA have anti-proliferative properties as they both inhibit proliferation of ASM cells in vitro. In combination, LABA and ICS, can simultaneously activate glucocorticoid receptors and CCAAT/enhancer binding protein-α (CEBPα), which stimulates the promoters $p27^{Kip}$ and $p21^{Waf1/Cip1}$(cell cycle inhibitor proteins). As a consequence, the proliferation of ASM is suppressed (Roth, M. et al., 2002) (Roth, M. et al 2012). Another in vitro

study found that combination treatment with LABA and ICS was superior in regulating excessive matrix production in human fibroblast cultures, when compared with monotherapy with either ICS or LABA (Todorova, L. et al., 2009). LABA/ICS treatment is also shown to decreases MMP-9, TIMP-1, and TGF-β levels in sputum samples and decreased airway wall thickness, as assessed by means of high-resolution CT (Wang, K. et al., 2010). These findings suggest a possible advantage with combination therapy of LABA/ICS to affect remodelling. Theophylline is a widely used anti-asthma drug and is classed as a bronchodilator. There is increasing evidence that it may have immunomodulatory effects on the asthmatic airway, due to the fact that its withdrawal is shown to be associated with a significant increase in asthma symptoms and fall in spirometry especially at night, and this was associated with an increase in CD4+ and CD8+ lymphocytes and activated eosinophils in bronchial biopsies (Kidney, J. et al., 1995). Despite the evidence for an anti-inflammatory effect, there are no long term studies suggesting its influence on airway remodelling.

1.1.4.2 Corticosteroids

Anti-inflammatory treatment with ICS provides the mainstay of current asthma treatment together with bronchodilation achieved by short- and long-acting β2 agonists. The effects of ICS treatment on airway remodelling in asthma have been widely studied using BAL fluid and bronchial biopsies and have produced inconsistent results describing both positive and neutral effectiveness. Lower airway fibroblasts are thought to play a critical role in airway inflammation and remodelling, suggesting that they might represent an important target for the major anti-asthmatic drugs. Studies show that ICS such as beclomethasone dipropionate (BDP) have significant anti-proliferative effects on lung fibroblasts

treated with basic fibroblast growth factor (bFGF) and the combination of BDP with long or short acting β2 agonists such as formoterol or salbutamol, respectively, strengthen these effects and inflammatory mediator release by fibroblasts (Descalzi, D. et al., 2008). ICS have also been reported to modulate inflammation and possibly induce epithelial shedding through cell apoptosis (Dorscheid, D.R., et al., 2001).

Some studies have also reported that corticosteroids as monotherapy can decrease ASM proliferation induced by a number of mitogens including thrombin, foetal calf serum (FCS), bFGF and epidermal growth factor(EGF) but are less effective in altering ECM protein synthesis and cytokine release by ASM cells (Stewart, A.G., et al., 2012). Others have shown that ICS decrease mucous production (Laitinen, L.A. and A. Laitinen., 1995) and tenascin deposition in the airway basement membrane of asthmatics as compared with placebo (Laitinen, A. et al., 1997). Another study has demonstrated a modest decrease in the thickness of reticular basement membrane with long term use of high-dose ICS (Sont, J.K., et al., 1999). The 'inhaled steroids as regular treatment' trial showed that the decrease in lung function associated with exacerbations might be attenuated by regular treatment with low-dose ICS (O'Byrne, P.M. et al., 2009). These clinical findings indicate that corticosteroids might affect remodelling, especially if used during critical periods of asthma development and airway injury such as exacerbations.

Despite above evidence suggesting that corticosteroids may influence airway remodelling through their anti-inflammatory properties, they seem to have only a small functional effect, if any, on airway remodelling. This was further supported by a recent study, which showed failure of ICS to decrease collagen deposition in

the lungs of asthmatic subjects and suggested this might be due to persistently elevated TGF-β expression in asthmatic airway tissue (Chakir, J. et al., 2003). CAMP study has also reported no significant or long term prevention of declining lung function in children with mild to moderate asthma treated with ICS alone (Larsen, G.L., 1999).

1.1.4.3 Anti-leukotrienes

Montelukast, a cysteinyl leukotriene-1(CysLT-1) receptor antagonist is commonly used in the treatment of asthma as an add-on therapy. Studies have shown that Montelukast can significantly reduce the airway eosinophilic infiltration, mucus plugging, ASM hyperplasia, and sub-epithelial fibrosis, but not ASM hypertrophy in an animal model of asthma (Muz, M.H. et al., 2006). CysLT1 receptor blockade also inhibited the presence of Charcot-Leyden–like crystals in airway macrophages and the increased IL-4 and IL-13 messenger ribonucleic acid (mRNA) expression in lung tissue and protein in BAL fluid seen in OVA-treated mice, suggested an important role for cysteinyl leukotrienes in the pathogenesis of chronic allergic airway inflammation associated with fibrosis and airway remodelling (Henderson, W.R. et al., 2002). Another study has reported a significant decrease in airway wall myofibroblasts and also reduction in lymphomononuclear cells after only 8 weeks of treatment with Montelukast (Kelly, M.M. et al., 2006). This evidence therefore suggests that Montelukast may have an inhibitory effect on airway structural cells that play a key role in airway remodelling in persistent allergic airway inflammation, and it may therefore be a useful therapy to attenuate airway remodelling in atopic asthma. However, long term studies are needed to fully understand the clinical significance of these findings.

1.1.4.4 Anti Immunoglobulin- E (IgE)

IgE plays an important role in allergic asthma. In fact, allergic asthma is specifically characterised by the presence of IgE antibodies against one or more common environmental allergens, such as pollen, house dust mite, animal dander and moulds. The risk of developing asthma increases with increasing IgE levels, which contribute to the pathophysiology of allergic processes by attaching to mast cells and regulating their mediator release. These mediators can in turn initiate a cascade of inflammation involving B and T lymphocytes, eosinophils, ASM, fibroblasts, and epithelial cells, resulting in the release of a multitude of cytokines and chemokines to intensify airway inflammation, asthma symptoms, and possibly remodelling (Zietkowski, Z. et al., 2010).

Omalizumab, a humanised recombinant murine monoclonal anti-IgE antibody, has been designed to treat patients with allergic asthma by binding to the Fc portion of free IgE to prevent its attachment to the high-affinity IgE receptor Fc epsilon RI (FceRI) of mast cells, basophils, and dendritic cells. This action decreases circulating IgE levels, which in turn down-regulate IgE receptor expression (Hoshino, M. & Ohtawa, J., 2012). In a 28-week, randomised, placebo-controlled trial in the European Union (INNOVATE study), Omalizumab significantly reduced frequency of exacerbations, oral corticosteroid use and total emergency visit rates, and also improved overall lung function, asthma symptoms and quality of life (Humbert, M. et al., 2005). An earlier study however showed Omalizumab reduces sputum and tissue eosinophilia and also IgE-positive T and B lymphocytes in the airways, but seems to have no effect on AHR in mild to moderate asthma (Djukanović, R. et al., 2004). Huang et al also demonstrated that Omalizumab inhibits pro-inflammatory cytokines and growth

factor generation, most notably tumour necrosis factor (TNF)-α and TGF-β, in human bronchial epithelial cell cultures (Huang, Y.-C. et al., 2005).
Other anti IgE treatments such as a recombinant humanised monoclonal antibody (rhuMAb-E25), which was developed by immunising mice with human IgE and selecting a monoclonal antibody that recognises IgE at the same site as the FceRI receptor for IgE can also reduce asthma symptoms, corticosteroid use and blood IgE levels, but has negligible effect on lung function in moderate to severe allergic asthma after 20 weeks of therapy (Milgrom, H. et al., 1999). Although Omalizumab reduces airway inflammation, there is little evidence to suggest an effect on airway remodelling. There are few clinical studies with Omalizumab which show some improvement in lung function. Indeed, if changes in lung function occur, they are usually modest (Rodrigo, G.J. et al., 2011) (Fahy, J., 2006). Nonetheless, these studies suggest that prolonged use of anti-IgE in a selected patient population such as with significant eosinophilic inflammation may limit airway remodelling to some extent.

1.2 ASM cells

Smooth muscle cells which surround the airways such as bronchi and bronchioles develop from mesenchymal cells during the developmental stage and are referred to as ASM. They are packed together in the form of bundles interconnected by ECM. It is well known that the ASM derived from asthmatic airway has an altered expression profile which may be important for asthma disease pathogenesis. Additionally, the ECM can affect ASM cell behaviour as demonstrated by a number of in vitro studies.

1.2.1 ASM morphology and physiological functions

Ultrastructural examination of almost confluent cultured tracheo-bronchial smooth muscle cells of either human or animal origin shows that these cells form multiple layers showing characteristic elongated spindle-like shape. The cells appear flattened with several cytoplasmic processes and an elongated oval nucleus containing little or no heterochromatin, which is characteristic of cells during transcription and division. Whilst in the cytoplasm, organelles associated with the synthetic phenotype predominate. The cytoplasm contains mitochondria, highly developed Golgi cisternae and various profiles of rough endoplasmic reticulum. Myofilaments are rarely seen in synthetic smooth muscle cells during proliferation, but are present when the contractile phenotype is expressed (Hirst, S.J., 1996). The relative quantitative expression of many of these cytoplasmic structures in human and animal airway smooth muscle, is therefore critically dependent upon cell phenotype and culture conditions (Warley, A. et al., 1994).

The primary physiological function of ASM contractility is yet to be explained fully. However pathogenically, ASM contribute both from hyper-contractility and ability to secrete a variety of chemicals in response to different stimuli such as in asthma. ASM cells also display a degree of heterogeneity in their behaviour, which is quite obvious between ASM derived from different sections of the airway and is also observed at the tissue level, especially with regards to their contractile properties, as seen in a number of in vitro studies of ASM of animal origin (Gunst, S.J., 2014) (Ma, X. et al., 1997). Heterogeneity of ASM cell behaviour additionally has an impact on ASM related research, although there is evidence that ASM cells in culture retain the physiologic responsiveness to a wide range of stimuli including inflammatory mediators, growth factors, contractile agonists and

immune receptor cross-linking and also express smooth muscle cell-specific markers over several population doublings. In addition, the synthetic functions of these cells appear to be comparable to those studied in vivo (Lazaar, A.L. & Panettieri, R.A., 2001).

1.2.2 Altered phenotype of ASM cells in asthma

A number of studies have shown that ASM cells derived from asthmatic airways exhibit differences in their synthetic functions, expression and behaviour as compared to ASM cells derived from healthy airways free of asthma or any other respiratory disease. What is known of an 'asthmatic' ASM cell is its ability to contribute to the most characteristic features of asthma such as bronchoconstriction (contractile phenotype), inflammation (synthetic phenotype) and ASM hyperplasia (proliferative phenotype). The phenotype of healthy or diseased ASM cells is mainly determined by expression of key phenotypic markers. There is now increasing evidence that the synthetic functions of ASM cells in culture derived from asthmatic and non-asthmatic donors differ. Some of these differences include an altered profile and increased production of ECM proteins, pro-inflammatory mediators and adhesion receptors, collectively indicating that ASM cells from asthmatics have the capacity to alter their micro-environment, actively participate in repair processes and functionally respond to changes in their environment (Wright, D.B. et al., 2013).

AHR is characterised by an exaggerated contraction and therefore narrowing of the airways in response to a wide variety of chemical, physical and toxic stimuli and is a consistent and defining feature of the clinical syndrome of asthma. Two types of AHR have been observed in asthmatic airways: variable and persistent AHR. Variable AHR is considered to be due to increased sensitivity of the ASM

to contractile stimuli and is related to inflammatory responses, associated with current exposures, asthma activity and disease severity. Whereas persistent or baseline AHR is found mainly in chronic asthmatics and by contrast, likely relates to structural (and/or physiologic) airway changes referred to as airway remodelling. As such, the baseline persistent component of AHR probably reflects chronicity of disease and is less likely to be a measure of current disease severity/activity (Cockcroft, D.W. & Davis, B.E., 2006). Persistent AHR in asthma may be explained by both increased ASM mass and by increased contractile abilities of the ASM. When cultured in collagen gels, ASM obtained from asthmatics enhanced gel size reduction compared to non-asthmatic derived ASM cells after histamine exposure, suggesting increased ASM cell contraction (Matsumoto, H. et al., 2007). In other studies, AHR, FEV_1% predicted, and airway responses to deep inspiration are shown to be associated with increased selective expression of the contractile proteins in ASM cells such as total smooth muscle myosin heavy chain (sm-MHC) isoforms, alpha smooth muscle actin (α-SM-actin), desmin, myosin light chain kinase (MLCK) and components of the ECM (Leguillette, R. et al., 2009) (Slats, A.M. et al., 2008). Increased expression of sm-MHC has been observed in airways after repeated allergen challenge in animal models of AHR, and this was associated with increased contractile force generated by the ASM cells (Wright, D.B. et al., 2013).

In addition, recent studies have shown that allergic animal models of AHR with house dust mite or OVA challenge, have increased ASM mass with enhanced α-SM-actin expression, which correlates with AHR and resistance (Wright, D.B. et al., 2013) (Plant, P.J. et al., 2012). Increased ASM mass in asthma could lead to an increase in force development and airway narrowing as well as advanced

biosynthetic capabilities (Lambert, R.K., et al.,1993). Increased ASM mass in asthmatic airways may result from an increase in ASM cell number (hyperplasia) and an increase in ASM cell size (hypertrophy) mediated by inflammatory cytokines such as TGF-β. This is shown to increase cell size and total protein synthesis, expression of α-SM-actin and sm-MHC, formation of actomyosin filaments, and cell shortening to acetylcholine, as well as α-actin synthesis (Goldsmith, A.M. et al., 2006) and migration of sub-epithelial myofibroblasts to the ASM layer (Johnson, P.R.A. et al., 2012) (Wright, D.B. et al., 2013). Alterations in calcium homeostasis may also lead to excessive ASM proliferation in severe asthma via increased mitochondrial biogenesis which, in turn, enhances cell proliferation, leading to airway remodelling (Wright, D.B. et al., 2013) (Trian, T. et al., 2007). Moreover, alterations in the ECM surrounding the ASM bundles likely attribute to increased ASM bulk (Araujo, B.B. et al., 2008). The increased ASM bulk seen in asthmatic subjects correlates with the disease severity and is a feature of airway remodelling largely insensitive to all currently available asthma therapies such as corticosteroids, due to cell-type-specific absence of signalling proteins controlling the inhibitory effect of steroids on cell proliferation in asthmatics (Roth, M. et al., 2004). Furthermore, ASM cells derived from asthmatic donor biopsies proliferate at a higher rate, when treated with serum, as compared to those derived from non-asthmatic subjects, implying an intrinsic difference between asthmatic and non-asthmatic ASM cells (Wright, D.B. et al., 2013) (Johnson, P.R.A. et al., 2012). This functional difference is even retained in ASM cultures for several weeks (around five passages) without the presence of endogenous asthmatic environment (Roth, M. et al., 2004) (Trian, T. et al., 2007) (Araujo, B.B. et al., 2008) (Wright, D.B. et al., 2013).

It has also been reported that asthma-derived ASM cells in culture, compared to non-asthmatic derived ASM cells, express increased amounts of collagen type-I, fibronectin, perlecan, nuclear respiratory factor-1 (NRF-1), peroxisome proliferator-activated receptor co-activator-1α (PGC-1α), mitochondrial transcription factor A (mtTFA), eotaxin-1 (CCL11), VEGF and CTGF. These ECM proteins from asthmatic cells also enhance ASM cell proliferation when compared with non-asthmatic ECM proteins. Asthma derived ASM cells however, express less sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA)2B, prostaglandin (PG)E2, laminin α1, collagen type-IV, chondroitin sulphate and hyaluronan (Johnson, P.R.A. et al., 2004) (Wright, D.B. et al., 2013) (Mahn, K. et al., 2009) (Chan, V. et al., 2006). It is important to note that the pathological micro-environment surrounding the ASM cells in vivo is also involved in this modification of asthmatic ASM phenotype. Inflammatory characteristics of asthmatic environment affect ASM cells and can induce cell growth, migration or secretion of chemokines, cytokines, and a number of other growth factors (Wright, D.B. et al., 2013).

1.2.3 ECM of the lung

ECM is a multi-component dynamic network of macromolecules, which basically provides support to the surrounding ASM bundles. The amount and the composition of the matrix may have functional consequences by altering the physiological properties of the airway wall and can particularly influence the contraction and proliferation of ASM cells (Meiss, R.A., 1999). In the airways, the ECM is primarily composed of structural proteins such as fibronectin, glycosaminoglycans, laminin, type IV collagen, tenascin, entactin, integrins, and metalloproteinases. Bronchial epithelial cells secrete fibronectin, MMP-2 and

MMP-9, and probably some other matrix components, whereas fibroblasts in the pulmonary interstitium secrete most components of the ECM (Johnson, S. & Knox, A., 1999) (Campagnone, R. et al., 1987). Several proteases contribute to the turnover of ECM, including MMPs (such as collagenases, stromelysins, and matrilysin), plasmin and the plasminogen activators (Okada, Y., 2017).

The different components of the ECM bi-directionally communicate with cells through a family of transmembrane glycoprotein cell surface receptors called integrins. These exist as heterodimers of α and β transmembrane subunits in a variety of combinations. The extracellular domains recognise short peptide sequences (e.g., Arg-Gly-Asp [RGD] found on some ECM proteins such as fibronectin and vitronectin), while the intracellular domains are involved in the formation of focal adhesion complexes and in signalling events with implications for cell adhesion, migration, differentiation and cell survival (Freyer, A.M. et al., 2001). Integrins are therefore important initiators and modulators of signal-transduction processes and play a key role in regulating both mitogenic signalling and cell migration. Additionally, they also have an important architectural role in ASM cell structure by spanning the membrane to link the ECM to the actin cytoskeleton. Integrins modulate the efficiency of the extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, acting at several distinct levels in controlling both mitogenesis and cell migration. (Juliano, R.L. et al., 2004).

ECM proteins can also bind to non-integrin cell receptors such as discoidin domain receptors (DDRs), glycoprotein-VI (GPVI) and leukocyte-associated immunoglobulin-like receptors (LILR-1). DDRs are receptor tyrosine kinases (RTKs), which are activated by native triple-helical structure of collagen. RTKs

control a wide array of cellular responses, including the regulation of cell growth, differentiation, migration, metabolism and survival (Lemmon, M.A. & Schlessinger, J., 2010). Collagen activation of DDR1/2 induces phosphorylation of a docking site for the Shc phosphotyrosine binding domain, whose presence is controlled by alternative splicing. Activation of DDR1/2 by collagen results in the up-regulation of MMP-1 expression, which acts as a collagenase and enhances ECM turnover. Thus, these findings suggest that the discoidin-related DDR tyrosine kinases are novel collagen receptors with the potential to control cellular responses to the ECM modulation (Vogel, W. et al., 1997).

1.2.4 ASM interactions with ECM in asthma

The composition of ECM surrounding the ASM bundles of healthy and asthmatic subjects has been extensively explored, with some studies suggesting no histological differences and others showing some variations (Fernandes, D. et al., 2006) (Yick, C.Y. et al., 2012). The altered ECM is likely to be deposited by the mesenchymal cells, including myofibroblasts and ASM, which are increased in number in asthmatic airways. In turn, the altered asthmatic ECM is likely to influence the function of the resident airway cells, and may be directly responsible for increasing proliferation, migration, ECM synthesis, inflammatory mediator release, and survival of resident mesenchymal cells. Therefore, the deposited ECM may perpetuate the disease phenotype (Wright, D.B. et al., 2013) (Fernandes, D. et al., 2006).

The deposition of collagen I, III, V, fibronectin, tenascin, hyaluronan, versican and laminin α2/β2 is increased, whereas the deposition of collagen IV and elastin is decreased within an asthmatic airway. This imbalance in the ECM profile could be due to increased de novo synthesis of ECM proteins, decreased activity of its

degrading enzymes (namely MMPs) or up-regulation of the TIMPs in asthma. One of the characteristic features of asthma is an increase in the amount of ASM within the airways. The ECM proteins/ MMPs/ TIMPs in and around the ASM may play a contributory role in this increased cell growth (Johnson, P.R., 2001) (Bonacci, J.V. et al., 2003). Cell contact with altered ECM substrate also enhances the migration of ASM cells (Parameswaran, K. et al., 2004), survival signals (Freyer, A.M. et al., 2001) and the release of cytokines via integrins as identified by the use of functional blocking antibodies (Peng, Q. et al., 2005). Interestingly, the altered ECM deposited by ASM cells from asthmatic individuals can differentially modulate cellular function when compared to ASM derived ECM from non-asthmatics. For example, when non-asthmatic ASM cells are grown on ECM produced by asthmatic cells they have increased proliferation. This suggests that the altered profile of ECM in asthma can drive an otherwise functionally healthy ASM cell into behaving more like a highly proliferative and secretory asthmatic phenotype and vice versa (Johnson, P.R.A. et al., 2004) (Figure 1).

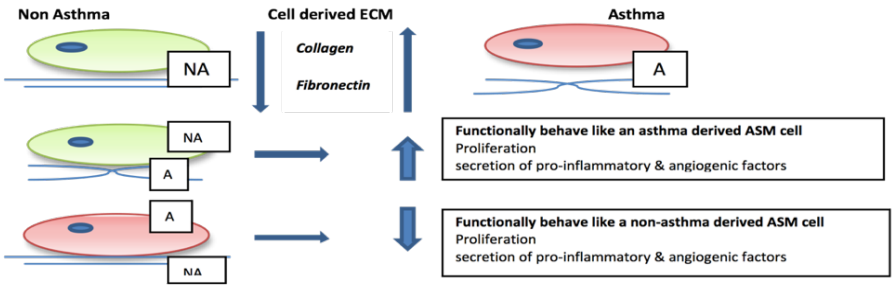


Figure 1. Effect of ASM derived ECM on ASM phenotype and function. Alterations in the ECM microenvironment dictate ASM function so that non-asthmatic derived ECM reverses the enhanced proliferative and secretory capacity of ASM derived from asthmatic subjects and vice versa (Johnson, P.R.A. et al., 2004). A = Asthmatics; NA = Non asthmatics.

The ECM formed by ASM cells from asthmatics in vitro is highly enriched in fibronectin and enhances IL-13 stimulated eotaxin release in ASM cells from patients with and without asthma (Chan, V. et al., 2006). A complete profile of ECM components surrounding ASM bundles in asthma of varying severity is still required and transcriptome-based studies may be useful in 2D and 3D culture environments to explore and identify signalling pathways and pharmacologically relevant targets implicated in airway remodelling (Manabe, R.I. et al., 2008).

1.3 Mast cells

Mast cells are present in all healthy human tissues, both within connective tissue and at mucosal surfaces. They form a heterogeneous population of cells with obvious differences in their development, structure, mediator content and functionally in their ability to interact with their local environment. They store and secrete a vast number of biological substances including the classical autacoid mediators such as histamine, PGD2, leukotriene (LT)C4, proteases, pro-inflammatory cytokines including IL-4, IL-5, and IL-13 (which regulate IgE production and eosinophilic inflammation) and pro-fibrogenic cytokines, including TGF-β and bFGF- 2.

Mast cells play a central role in tissue homoeostasis, sensing the local environment through various innate cell surface receptors, enabling them to respond rapidly to perceived tissue injury with a view to initiating a co-ordinated programme of inflammation and tissue repair (Bradding, P. & Holgate, S.T., 1999). However, when the tissue insult is chronic, the ongoing release of multiple pro-inflammatory mediators, proteases, cytokines and chemokines leads to tissue damage and remodelling (Bradding, P. & Arthur, G., 2016). Mast cells are commonly found in

the bronchi of healthy individuals and asthmatic patients. Degranulated mast cells are a common feature of both extrinsic and intrinsic asthma, as suggested by the presence of tryptase, histamine and PGD2 in the BAL fluid (Gordon, J.R., Burd, P.R. & Galli, S.J., 1990). Additionally, mast cells are often found adjacent to blood vessels and appear to be closely related to ASM bundles in asthmatic bronchi (Bousquet, J. et al., 2007). Mast cells release preformed, stored mediators and synthesise other factors de novo on activation. The classical type 1 hypersensitivity reaction in acute asthma or the early response to allergen challenge results from IgE cross-linking by allergen and, thus, FceRI signalling. This results in release of histamine, tryptase and chymase, and heparin, and synthesis of lipid mediators including LTC4 and PGD2, which have roles in bronchoconstriction, oedema, and recruitment of inflammatory cells (Robinson, D.S., 2004).

Mast cells, particularly activated, degranulated forms are more common within ASM bundles of those with asthma and this observation has potential implications for the pathogenesis of asthma and the pathophysiological role that mast cells have in this disease. It is possible that mast-cell infiltration of the ASM is a general feature of obstructive lung disease and is not specific to asthma but there is increasing evidence that the infiltration of ASM by mast cells is associated with the disordered airway function found in asthma (Brightling, C.E. et al., 2002) (Beasley, R. et al., 1989). Human mast cells have traditionally been divided into two major subtypes based on the protease granule content (tryptase alone vs. tryptase and chymase). Collectively, in asthma, mast cells play a key role in all three characteristics of asthma (inflammation, AHR, and airway remodelling) by releasing mediators that cause increased bronchoconstriction, induce ASM cell

proliferation, as well as recruit and activate other inflammatory cells (Fajt, M.L. & Wenzel, S.E., 2013).

1.3.1 ASM and mast cell interactions in asthma

Mast cell infiltration of the asthmatic airway is T-cell-dependent. Th2 cytokines from T cells and other sources act in mast cell migration and expansion from circulating and other tissue precursors (Robinson, D.S., 2004). As mentioned before, mast cells are shown to be increasingly localised within the ASM bundles in patients with asthma but not in normal individuals or those with eosinophilic bronchitis (Brightling, C.E. et al., 2002). Mast cells produce a variety of lipid mediators, chemokines, cytokines and enzymes, that may interact with ASM cells to cause hyper-reactivity to constrictive stimuli and proliferation, and activated ASM can in turn produce stem cell factor (SCF) and other chemokines, cytokines, and growth factors that may act in recruitment, differentiation, and retention of mast cells (Robinson, D.S., 2004).

It is obvious from various studies that the human ASM cells have the ability to produce a wide variety of pro-inflammatory cytokines and chemokines such as IL-1b or TNF-α. Activated ASM produces RANTES (regulated on activation, normal T cell expressed and secreted), b-chemokine family members monocyte chemoattractant proteins (such as chemokine ligand (CCL)11, CCL5 and CCL2) as well as neutrophil and eosinophil chemoattractant proteins (such as GM-CSF, IL-6, chemokine CXC ligand (CXCL)8, IL-8 and IL-11) (Hirst, S.J., 2003). IL-4 and IL-13 can also activate ASM production of CCL11 (Hirst, S.J. et al., 2002). In addition, activated ASM cells produce both soluble and membrane bound SCF, which is a chemotactic and survival factor for mast cells and plays an important role in recruitment, survival and differentiation of mast cell precursors and mature

mast cells. This may contribute to localisation of mast cells in ASM bundles reported in both normal and asthmatic airways; however, those present on the ASM layer in cases of fatal asthma were mostly degranulated (Kassel, O. et al., 1999) (Carroll, N.G. et al., 2002). A couple of Leicester, UK group studies have demonstrated that human lung mast cells abundantly express chemokine receptor (CXCR)3 and ASM cells secrete three CXCR3 ligands (CXCL9, CXCL10, and CXCL11). An increased expression of CXCL10 by the ASM bundles in bronchial biopsies from asthmatic individuals as compared with normal subjects further demonstrated the importance of CXCR3/CXCL10 axis in mast cell recruitment by the asthmatic ASM in vivo (Brightling, C.E. et al., 2012) (Brightling, C.E. et al., 2005). Recent data also suggest that ASM may attract mast cells through release of TGF-β in addition to SCF after exposure to tryptase, providing another mechanism to stimulate further accumulation of mast cells, which may in turn stimulate the secretion of chemotactic factors release by ASM cells and so on, leading to a state of chronic ongoing mast cell activation. Both factors have been localised to ASM in asthmatic airways (Berger, P. et al., 2003). Thus, in the inflammatory setting of asthma, ASM chemokine release acts in recruitment and retention of mast cells. This may then be further amplified by mast cell products (such as IL-4 or TNF-α).

ASM cells also express a variety of adhesion molecules that could act in recruitment and retention of mast cells and their precursors, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 (Page, S. et al., 2001). Mast cells also interact with ASM cells through the effects of chemokines, cytokines, proteases, and growth factors. For example, mast cell infiltration of the ASM in asthma could lead to direct activation of airway

narrowing via release of histamine, leukotrienes, and other mediators. In addition, leukotrienes may play a role in ASM proliferation and other changes of remodelling (Holgate, S.T. et al., 2003). Similarly, tryptase and TNF-α can both increase ASM responsiveness to histamine and other constrictors. Tryptase may do this partially through activation of protease-activated receptors (PAR)-2 receptors (Anticevich, S.Z. et al., 1995) (Johnson, P.R. et al., 1997).

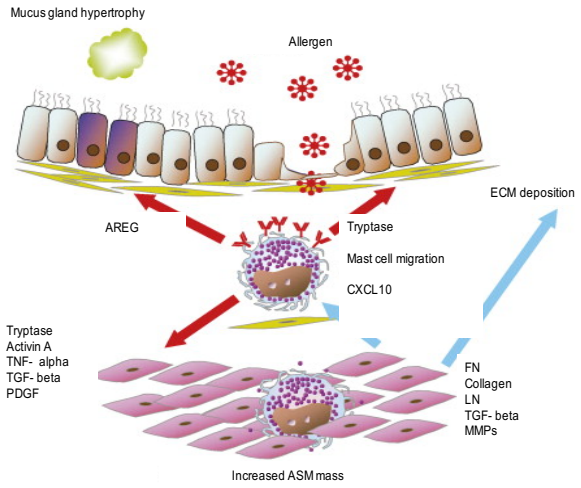


Figure 2. Mast cells interactions in asthma and their role in airway remodelling. Copyright license no. 3364740619878. AREG = Amphiregulin; ASM = Airway smooth muscle; ECM = Extracellular matrix; FN = Fibronectin; LN = Laminin; MMPs = Matrix metalloproteinases; PDGF = Platelet-derived growth factor; TGF-β = Transforming growth factor-β; TNF-α = Tumour necrosis factor-α.

Mast cells may also play a role in ASM proliferation through tryptase and growth factors such as platelet-derived growth factor (PDGF) (Hirst, S. J. et al., 1996). In contrast, other mast cell derived factors such as chymase (Lazaar, A.L. et al., 2002), heparin (Okona Mensah, K.B. et al., 1998), and IL-4 (Hawker, K. M. et al., 1998) inhibit mitogen-induced ASM proliferation. Chymase inhibits ASM proliferation by modifying cell-matrix interactions (Lazaar, A.L. et al., 2002). Whereas mast cell derived TGF- β and IL-13 enhance responses to LTD4 and

induce ASM proliferation and production of ECM proteins via up-regulation of the CysLT1 receptor.

TGF- β1 is a potent pro-fibrotic Th3 type cytokine, which may induce structural changes within the airways, such as sub-epithelial fibrosis, thickening of the reticular lamina, and ASM proliferation by modulating the expression of insulin-like growth factor binding protein (IGFBP)-3 (Espinosa, K. et al., 2003) (Cohen, P. et al., 2000). Some studies however have shown that selective treatment of ASM cells with TGF- β1 inhibits EGF and thrombin-induced deoxyribonucleic acid (DNA) synthesis and therefore can modulate ASM proliferation in both a dose and time dependent manner (Cohen, M.D. & R A Panettieri, J., 2012). Activin A, which is a homodiameric protein and part of the TGF- β superfamily is also released from mast cells and may play an important role in the process of airway remodelling by promoting the proliferation of ASM (Cho, S.H. et al., 2003).

The interaction of mast cells and ASM may also influence a wide variety of other inflammatory and structural cells in the asthmatic airway. For example, IL-6 induced from ASM can potentiate IgE synthesis and Th2 cell development, whereas GM-SCF and CCL11 will enhance eosinophilic inflammation (Robinson, D.S., 2004). ASM and mast cell density correlate significantly with indices of AHR such as methacholine dose in PC_{20} airway responsiveness and are thought to be important factors determining the asthmatic phenotype (Bradding, P., 2003).

1.4 MMPs

MMPs are a large family of zinc-dependent proteolytic enzymes capable of degrading ECM components. MMPs play a central role in many biological processes, such as embryogenesis, normal tissue remodelling, wound healing and

angiogenesis, and in diseases such as asthma, atheroma, arthritis, cancer and tissue ulceration (Visse, R. & Nagase, H., 2003).

Enzyme	MMP	Human chromosome location
Collagenases		
Interstitial collagenase: collagenase 1	MMP-1	11q22-q23
Neutrophil collagenase; collagenase 2	MMP-8	11q21-q22
Collagenase 3	MMP-13	11q22.3
Collagenase 4	MMP-18	NA
Gelatinases		
Gelatinase A	MMP-2	16q13
Gelatinase B	MMP-9	20q11.2-q13.1
Stromelysins		
Stromelysin 1	MMP-3	11q23
Stromelysin 2	MMP-10	11q22.3-q23
Stromelysin 3	MMP-11	22q11.2
Matrilysins		
Matrilysin 1; Pump-1	MMP-7	11q21-q22
Matrilysin 2	MMP-26	11p15
Membrane-type MMPs (MT-MMPs)		
Transmembrane		
MT1-MMP	MMP-14	14q11-q12
MT2-MMP	MMP-15	15q13-q21
MT3-MMP	MMP-16	8q21
MT5-MMP	MMP-24	20q11.2
GPI anchor		
MT4-MMP	MMP-17	12q24.3
MT6-MMP	MMP-25	16p13.3
Others		
Macrophage elastase	MMP-12	11q22.2-q22.3
No trivial name	MMP-19	12q14
Enamelysin	MMP-20	11q22.3
XMMP (Xenopus)	MMP-21	ND
CA-MMP	MMP-23	1p36.3
CMMP (Gallus)	MMP-27	11q24
Epilysin	MMP-28	17q21.1

Table 1. Matrix Metalloproteinases. Different groups of MMPs are listed with their trivial names and chromosomal location (Visse, R., 2003).

So far 24 MMPs have been identified in humans and most of these MMPs are homologous proteins. MMPs can be divided into 6 groups according to their common features and primary functions. These are: collagenases, stromelysins,

matrilysins, gelatinases, membrane-type metalloproteinases (MT-MMPs) and other MMPs (zinc- and calcium-dependent endopeptidases) (Table 1). ECM proteins are examples of typical MMPs substrates, although there is also increasing evidence emerging of non-ECM and intracellular targets, therefore a lot of MMPs have wide substrate specificity (Kukacka, J. et al., 2005).

1.4.1 Structure and functions of MMPs

MMPs are synthesised as inactive zymogens or pro-enzymes and require activation through the cleavage of their pro-peptide domain. All MMPs have a conserved pro-domain and a catalytic domain to which the Zn^{2+} ion of the active site (acting to inhibit enzyme function) is held.

MMP pro-domains contain the protein sequence PRCXXPD, and their catalytic domains consist of a 3-histidine motif (HEXXHXXGXXH) to which the active site Zn^{2+} is held. Ligation of the catalytic domain zinc ion with the free thiol of the pro-domain cysteine is what retains the latent state of proMMPs, and this bond must be broken for an MMP to gain activity (Bellayr, I.H. et al., 2009). With the exception of a few MMPs (*7*, 23 and 26), a variable C- terminal hemopexin-like domain, linked by a short hinge, makes up the catalytic domain of MMPs. The extracellular activities of the MMPs are multiple, cleaving not only the components of the ECM, but also many of the bioactive molecules at or around the cell surface (Tocchi, A. & Parks, W.C., 2013). Unlike most of the soluble MMPs, membrane bound MT-MMPs are already active at the cell surface because they are cleaved intracellularly by furin like pro-protein convertases at their specific recognition sequence, located at the C-terminal of the pro-peptide (Murphy, G. & Nagase, H., 2011). It is important to note that hemopexin domains probably serve an important role in substrate specificity among MMPs (Ra, H.J.

& Parks, W.C., 2007). MMPs are involved in the regulation of cell–cell and cell–ECM interactions in both physiological and pathological states. A major function of MMPs is thought to be the removal of ECM in tissue resorption. However, the ECM is not just a simple extracellular scaffold; it also acts as a reservoir of biologically active molecules such as growth factors. Degradation of ECM components by MMPs can alter cellular behaviour and phenotypes. Therefore, the functions of MMPs are much more complex in both health and disease and more subtle than straightforward demolition of ECM. What adds to this complexity is also the expanding number of non-ECM proteins that are MMP substrates and their biological activities (McCawley, L.J. & Matrisian, L.M., 2001).

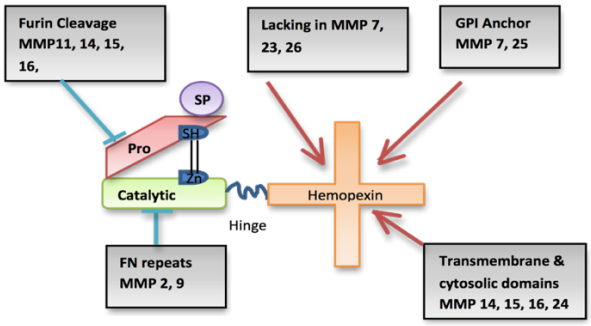


Figure 3. Domain structure of MMPs. The pro-domain of a typical MMP is ~ 80 amino acids and contains the consensus sequence PRCXXPD. The catalytic domain contains three conserved histidines in the sequence HEXXHXXGXXH, which ligate the active site Zn^{2+}. Several MMPs have a furin-recognition within the C-terminal half of the pro-domain. MMPs have a flexible proline-rich hinge region and a hemopexin-like C-terminal domain with the exceptions of MMP-7, -23 and -26. Membrane-type MMPs have transmembrane and cytosolic domains, and MMP-7 and -25 are held to the cell surface by a glycosylphosphatidylinositol (GPI) anchoring signal. MMP-2 and -9 have gelatin-binding domains with motifs similar to fibronectin (FN). Reconstructed with permission (Tocchi, A. & Parks, W.C., 2013).

Specific MMPs do have greater affinity for specific substrates and the concentrations of the active enzyme and the preferred substrate relative to other

substrates may determine their overall efficacy. The hemopexin domains of the collagenase MMPs (MMP-1, MMP-8, MMP-13 and MT1-MMP) are essential for cleaving native triple helical collagen monomers. Collagenases locally unwind the triple helix to allow access of the individual α- chains to the active site centre. Therefore, the collagen-binding site may be composed of elements from both the catalytic and the hemopexin domains (Murphy, G. & Nagase, H., 2011). It is also interesting to note that inter-domain flexibility is key for the specificity of the collagenases and possibly other MMPs as well (Sela-Passwell, N. et al., 2010). MMP proteolysis is usually confined to cellular compartments, such as secretion vesicles and cell membranes, where the concentrations of both the enzyme and substrate can be maintained at catalytically favourable levels (Murphy, G. & Nagase, H., 2011).

1.4.2 Activation of Pro MMPs

Biochemical studies of the activation process have shown that the proMMPs undergo a conformational change, leading to self-cleavage of more than 80 N-terminus amino acids of the pro-peptide region, generating a short-lived intermediate form (Birkedal-Hansen, H. et al., 1976). Pro MMPs can also be activated by proteinases or in vitro by chemical agents, such as thiol-modifying agents (acetate, $HgCl_2$, and N-ethylmaleimide), oxidised glutathione, chaotropic agents, sodium dodecyl sulphate (SDS) and reactive oxygen. Low pH and heat treatment can also lead to activation of proMMPs. The activation mechanism known as the 'cysteine switch' involves proteolytic cleavage of the pro-peptide causing eventual destabilisation of the cysteine–zinc interaction. The initial proteolytic attack occurs at an exposed loop region between the first and the second helices of the pro-peptide. The cleavage specificity of the target region is

dependent on the sequence found in each MMP. This reaction is intramolecular rather than intermolecular. Once a part of the pro-peptide is removed, this probably destabilises the rest of the pro-peptide, including the cysteine switch–zinc interaction, which then allows the intermolecular autocatalytic reaction by partially activated MMP intermediates generated in this process or by other active MMPs leading to release of active MMPs, starting with Tyr or Phe at the N-terminus (Nagase, H., 1997) (Nagase, H. et al., 1990) (Suzuki, K. et al., 1990). Proteolytic activation of the proMMPs is therefore a stepwise process, which is likely to occur in vivo. Studies have shown that a large number of proteases such as trypsin, chymotrypsin, plasma kallikrein, plasmin and thermolysin can activate proMMPs -1, -3, -7 and -10. MMP-3 and -10 can also directly activate proMMP-7, proMMP-8, proMMP- 9 and proMMP-13 into fully active forms (Nagase, H. et al., 1990) (Knäuper, V. et al., 1996) (Okada, Y., 2017). It is important to mention that the bait region sequences in the pro-peptide generally specify which proteases can become an activator of a particular MMP (Nagase, H., 1997). In common with other MMPs, proteolytic removal of the pro-domain is required for MMP-1 activation.

1.4.3 Endogenous inhibitors of MMPs

TIMPs are a family of homologous proteins referred to as the tissue inhibitors of metalloproteinases. TIMPs have an important function in the regulation of connective-tissue turnover in vivo. They are specific inhibitors that bind MMPs in a 1:1 stoichiometry and play a role in regulation of the activity of MMPs. TIMPs (21 to 29 kDa) have two domains of relatively small size, N- and C-terminals made of 125 and 65 amino acids respectively, with each containing three conserved disulphide bonds. The two domains have been found to exhibit several

biochemical and physiological functions, including inhibition of active MMPs, proMMP activation, cell proliferation, matrix binding, inhibition of angiogenesis and the induction of apoptosis. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates and their expression is usually regulated during development and tissue remodelling (Brew, K. et al., 2000). Under pathological conditions associated with unbalanced MMP activities, changes of TIMPs levels are considered to be important as they directly affect the level of MMP activity in these circumstances (Williamson, R.A. et al., 1990). The N-terminal domain folds as a separate unit and is capable of inhibiting MMPs (Murphy, G. et al., 1991). The overall shape of the TIMP molecule is like a wedge, which slots into the active-site cleft of an MMP in the same manner as the substrate.

TIMPs inhibit all MMPs which have been tested so far, except that TIMP-1 fails to inhibit MT1-MMP (Will, H. et al., 1996). There is a clear potential for the application of TIMPs as endogenous inhibitors, especially because the results of clinical trials with small molecule MMP inhibitors have been rather disappointing, especially in cancer treatment (Zucker, S. et al., 2000). In addition to metalloproteinase-inhibiting activities, TIMPs have other biological functions. TIMP-1 has cell growth–promoting activity for a variety of human cells and TIMP-2 has erythroid-potentiating activity (Stetler-Stevenson, W.G. et al., 2002) (Hayakawa, T. et al., 2002). TIMP-2, but not TIMP-1, inhibits endothelial cell growth induced by bFGF independent of protease inhibitory activity, suggesting that TIMP-2 may have additional actions which may limit neo-vascularization associated with solid tumour growth and metastasis in vivo. However, the mechanism of this activity mostly remains to be discovered (Murphy, A.N. et al.,

1993). TIMP-1 and TIMP-2 also have anti-apoptotic activity, but TIMP-3 on the other hand has pro-apoptotic activity, possibly through the stabilisation of death receptors such as tumour necrosis factor receptor-1 (TNF-R1), Fas receptor (FasR), and TNF-related apoptosis inducing ligand receptor-1 (TRAIL-RI) in cancer cells (Ahonen, M. et al., 2003).

Other proteins such as plasma macroglobulins are general endopeptidase inhibitors that inhibit most proteinases including MMPs, by trapping them within the macroglobulin after proteolysis of the bait region of the inhibitor. For example, MMP-1 binds preferentially with α 2-macroglobulin more readily than with TIMP-1 in vitro (Cawston, T.E. & Mercer, E., 2001). Several other proteins have been reported to inhibit MMPs, such as tissue factor pathway inhibitor (TFPI)-2, which is a serine protease inhibitor. TFPI-2 reduced the ability of the interstitial collagenases MMP-1 and MMP-13 to degrade triple-helical collagen, a main weight bearing molecule of ECM within human atheroma in vitro. TFPI-2 expression in situ correlated inversely with MMP levels in human atheroma, as opposed to the levels of the "classical" tissue inhibitors of MMPs or TIMPs. In addition, TFPI-2 also reduced the activity of the gelatinases MMP-2 and MMP-9 (Herman, M.P. et al., 2001). A C-terminal fraction of the pro-collagen C-terminal proteinase enhancer protein has also been found to inhibit MMP-2 (Mott, J.D. et al., 2000).

1.4.4 Signalling pathways regulating transcription of MMP genes

Due to the significant effect of MMPs on tissue architecture, their expression is regulated by a number of mechanisms including transcription, translation, enzymatic activation, inhibition and autolysis. MMPs expression is primarily

regulated by gene transcription, typically involving intracellular signalling cascades. MAPKs are serine/threonine/tyrosine-specific protein kinases belonging to the CDK/MAPK/GSK3/CLK (CMGC) kinase group. MAPKs are involved in directing cellular responses to a wide array of stimuli, such as mitogens, heat shock, osmotic stress and pro-inflammatory cytokines. They regulate cell proliferation and differentiation, gene expression, mitosis, cell survival and apoptosis (Pearson, G., 2001). Activation of MAPK pathways by IL-1 activates the MAPK kinase kinases (MAPKKKs), transforming-growth-factor-β-activated kinase-1 (TAK 1) and Raf kinase, which then phosphorylate and activate several MAPK kinases such as MKK6, MKK4, MKK7, and MEK. These MAPKKs in turn phosphorylate and activate the MAPKs, p38, c-Jun N-terminal kinase (JNK) and ERK, which phosphorylate by translocating to the nucleus and activating various transcription factors (Cargnello, M. & Roux, P.P., 2011) (Leppa, S. et al., 1998). With relevance to MMP transcription, JNKs and ERKs phosphorylate and activate the activating protein-1 (AP-1) family member c-Jun, which dimerises with c-Fos to lead to transcription of multiple MMP genes (Karin, M., 1995). The interaction of AP-1 with other transcription factors may also regulate tissue-specific expression of MMPs. In addition, these MAPKs phosphorylate and activate other transcription factors such as transcription factor 2 (ATF2) and ETS domain-containing protein (Elk-1), also contributing to collagenase (MMP-1, MMP-13) gene transcription (Davis, R.J., 2000) (Vincenti, M.P. & Brinckerhoff, C.E., 2001). Similar findings were also shown in a recent study by my group, where ECM protein tenascin-C induced MMP-1 expression, that was dependent on ERK1/2, JNK and p38 MAPK activation and was inhibited by function blocking antibodies against the β1 and β3 integrin subunits,

therefore suggesting that MMP-1 expression by ASM is partly regulated by integrin mediated MAPK signalling (Rogers, N.K. et al., 2014).

Another important cytokine-induced signalling pathway involves translocation of nuclear factor-κB (NF-κB) from the cytoplasm to the nucleus. On binding of IL-1 to its receptor (IL-1R1), TAK-1 becomes active, eventually leading to the activation of the NF-κB-inducing kinase (NIK). In turn, NIK activates the inhibitor of κB kinase (IKK) complex, which is required for phosphorylation of inhibitor of κB (IκB) and NF-κB (NF-kBI). After phosphorylation, IκB and NF-κBI become ubiquitinated and present themselves as targets for degradation by the proteosomes. Degradation of these inhibitors allows translocation of NF-κB subunits to the nucleus and in this process activate MMP promoters, including those for MMPs of the collagenase family (Vincenti, M.P. et al., 1998) (Vincenti, M.P. & Brinckerhoff, C.E., 2002).

1.5 MMP-1

MMP-1, also known as fibroblast interstitial collagenase is the most commonly expressed protease in the interstitial collagenase subfamily of MMPs. This group includes MMP-1, MMP-8, MMP-13, and MMP-18. The key property of these enzymes is their ability to cleave or break down interstitial collagens I, II, and III at a particular site, three-quarters of the way from the N-terminal.

These enzymes can also digest a number of other ECM and non-ECM molecules (Visse, R. & Nagase, H., 2003). The MMP-1 gene is located on chromosome 11q22 and is expressed in a variety of cell types in human lung including bronchial epithelial cells, alveolar epithelial cells and macrophages, endothelial cells and lung fibroblasts, as well as ASM cells and a number of cancer cells (Brinckerhoff, C.E. et al., 2000). Under normal physiological conditions, MMP-1 is always expressed

at low levels. However, during a variety of pathological processes, MMP-1 expression is significantly increased due to up-regulation of MMP-1 gene expression (Rutter, J.L. et al., 1997).

1.5.1 Activation of latent MMP-1

Like other MMPs, the activation of MMP-1 is also a multi-step process, including initial partial cleavage of the pro-domain, followed closely by certain conformational re-arrangements to allow activation of the inherent autocatalytic action of MMP-1, removing further remaining pro-peptide sequences and generating a transient partially active isoform. Further proteolysis then generates a mature form of the enzyme (He, C. et al., 1989).

1.5.2 Substrates of MMP-1

Interstitial collagens are natural substrates for most collagenases including MMP-1. In vertebrates, collagens are the major and most important structural proteins of connective tissue such as skin, bone, tendons, blood vessels and basement membranes. Collagens I, II and III are the most commonly occurring and they not only provide the scaffolding of the tissue in the form of ECM, but also guide cells to migrate, proliferate and differentiate (Yang, C. et al., 2004). Therefore, the degradation of these macromolecules is an integral part of many biological processes such as organogenesis, tissue remodelling, angiogenesis and wound repair. The interstitial collagens are long triple-helical structures comprised of three left-handed poly-Pro II-like helices stabilised by hydrogen bonds between the backbones of three α chains. In order for a collagenase to cleave collagen, it first binds and then unwinds the rigid triple-helical structure, before it hydrolyses the peptide bonds of collagens (Chung, L. et al., 2004). MMP-1 is active against a number of collagens including collagen I, II, III, VII, VIII, X and gelatin I. It is

also active against a number of other matrix substrates such as aggrecan, perlecan, proteoglycan and versican (Murdoch, A.D., 1996) and can activate or inactivate other bioactive non-matrix proteins such as IGFBPs 2, 3, and 5, PAR-1, CXCL8 and CXCL12 (Boire, A. et al., 2005). Other non matrix substrate proteins of MMP-1 are serpines such as α1-antichymotrypsin and α1-proteinase inhibitor for proMMP-1 & 2 and proTNF-α (McCawley, L.J. & Matrisian, L.M., 2001).

1.5.3 MMP-1 in the lung

MMP-1 is increasingly expressed during lung development and is localised to the pseudo stratified epithelial layer of airways. However its role in normal lung development has not received much attention due to the lack of MMP-1 expression in mice experimental models (Masumoto, K. et al., 2005). MMP-1 expression in healthy adult lung is low. In healthy non-smokers, BAL fluid contains no detectable MMP-1 protein or collagenase activity (Ward, K. et al., 1990). However, both levels of MMP-1 protein and collagenase activity are found to be elevated in BAL fluid of patients with acute irritant asthma (Takeda, N. et al., 2009), emphysema (Finlay, G.A. et al., 1997), idiopathic pulmonary fibrosis (IPF) and stage 3 sarcoidosis (Henry, M.T. et al., 2002). A study using murine lung explants to investigate the effects of enzymatic digestion on the rate and magnitude of airway narrowing induced by acetylcholine (ACh), found that treatment of explanted airways with two different proteases (collagenase and elastase) results in differential effects on the dynamics of airway narrowing. By simulating computational airway contraction models, the effects of collagenase on airway dynamics were shown to be due to reduction in airway wall stiffness as opposed to elastase, which mostly exerts its functional effect through reduction in parenchymal tethering (Khan, M.A. et al., 2010). Some recent studies have used

an approach of examining ASM containing 3D collagen gel contractions induced by exogenous application of MMP-1 or activated mast cell supernatants. These show direct contraction of ASM-containing gels in response to MMP-1, -2, -9, and -12 and indicate that mast cells may enhance, but are not required for, ASM contractile responses to catalytically active MMPs (Margulis, A. et al., 2009) (Ohta, Y. et al., 2008). The exact mechanism of gel contraction by MMP-1 is unknown. However, it does act to remodel the ultrastructure of collagen fibres (Ohta, Y. et al., 2008). In a guinea pig model, inhalation of collagenase and elastase caused bronchial hyperreactivity and increased pulmonary permeability (Nagai, H., et al., 1991).

In a couple of studies by the Rajah, R. et al group, it has been shown that airway tissue extracted from asthma patients has less intact IGBP 2 & 3, specifically showing that this is due to MMP-1 activity (Rajah, R. et al., 1996) (Rajah, R. et al., 1999). Destruction of IGBP-3 by MMP-1 and the resultant increased availability of IGF protein is shown to have further impact on airway biology other than increased ASM proliferation, by increasing antigen-induced airway inflammation and hyperresponsiveness through the modulation of vascular leakage and VEGF expression in murine models of asthma (Kim, S.R. et al., 2012). Furthermore, IGF through αVβ3 integrin receptors has been shown to stimulate ASM migration and replication in vascular murine models, which may also be relevant in asthmatic airways (Clemmons, D.R. et al., 1999).

In a recent sepsis study, systemic infusion of exogenous MMP-1 increased lung vascular permeability in normal wild-type mice, showing this to be dependent on MMP-1 mediated PAR-1 activation (Tressel, S.L. et al., 2011). MMP-1 also promotes vascular endothelial growth factor receptor-2 (VEGFR2) expression

and proliferation of endothelial cells through stimulation of PAR-1 receptor and NF-κB mediated active transcription of the VEGFR2 gene, leading to angiogenesis and vascular remodelling (Mazor, R. et al., 2013). A few studies have also been carried out to evaluate effects of MMP-1 on airway epithelial cells. One such study concluded that healing of an alveolar epithelial cell monolayer was enhanced in the presence of exogenous collagenase, suggesting that collagenases can modulate the wound repair process by decreasing cell adhesion and cell stiffness, and by increasing cell migration in response to type I collagen (Planus, E., et al., 1999). In another animal study, treatment of hamster tracheal surface epithelial cells with collagenase caused increased mucin release from them, suggesting MMP-1 might have a role in the regulation of airway goblet cell mucin secretion under pathological conditions of mechanical strain on epithelial cells by the underlying ASM layer (Kim, K.C. et al., 1993). Last but not least, increased activity of MMP-1 and serine proteases in asthmatic airways have been linked with proteolytic activation of a multifunctional cytokine TGF-β, which may have further pro-remodelling effects including enhanced production of pro-collagen I by ASM cells in asthma (Burgess, J.K. et al., 2009).

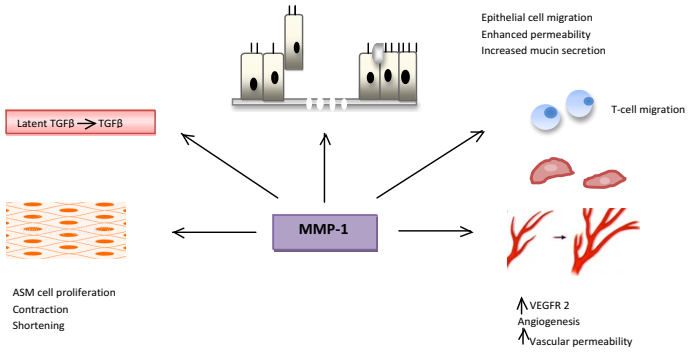


Figure 4. Schematic representation of different MMP-1 functions within the lung. ASM = airway smooth muscle; MMP-1 = Matrix metalloproteinase-1; TGF-β = Transforming growth factor-β; VEGFR2= Vascular endothelial growth factor receptor 2.

1.5.4 Role of MMPs and TIMPs in Asthma

Lately, it has been demonstrated in various studies that MMPs may make a significant contribution to the pathogenesis of asthma. Many MMPs, including MMP-1, -2, -3, -8 and -9, have been detected by various methods in the airways of asthma patients (Cataldo, D. et al., 2000) (Dahlen, B., Shute, J. & Howarth, P., 1999). Additionally, it has been found that there is usually an imbalance between MMPs and TIMPs levels in the airways of asthma patients. TIMP-1 levels have been shown to be significantly higher in untreated asthmatic patients than in ICS treated patients or healthy controls and are also far greater than those of MMP-1, MMP-2, MMP-3, and MMP-9 combined in asthmatics. One study also showed that TIMP-1 levels were directly correlated with cytokine IL-6 levels and the number of alveolar macrophages in BAL fluid of asthma patients. This observation has important implications because increased TIMP-1 expression can lead to airway fibrosis, a hallmark of airway remodelling in patients with chronic asthma (Mautino, G. et al., 1999). A number of studies have shown increased sputum cell expression of MMP-1 mRNA in asthmatic individuals as compared to their healthy counterparts (Cataldo, D., 2004). Since MMP-1 is a major collagenase capable of degrading fibrillar collagen, increased levels of MMP-1 gene expression could be associated with the airway remodelling in asthmatics that mainly consists of cycles of tissue degradation and impaired repair (Bousquet, J. et al., 2007). Increased expression of MMP-1 as well as MMP-2, -9 and the ECM protein fibronectin, has been localised to the outer area of the small airways and in the peri-bronchiolar parenchyma in patients with fatal asthma, whilst TIMP expression is only increased in the large airways of asthmatic patients, thus concluding that the outer area of the small airways is probably a major site of

MMP mediated ECM remodelling and its functional consequences in fatal asthma (Dolhnikoff, M. et al., 2009).

1.6 Study Hypothesis

The overarching hypothesis here is that the airway environment in asthma perpetuates airway remodelling (Henderson, N. et al., 2006) (Rogers, N.K. et al., 2014). Mast cells, particularly activated forms are more common within ASM bundles of those with asthma (Brightling, C.E. et al., 2002) (Bradding, P. & Arthur, G., 2016) and generate pro-inflammatory cytokines including IL-4, IL-5, and IL-13, which regulate IgE production, eosinophilic inflammation and pro-fibrogenic cytokines, including TGF-β and bFGF-2. Pre-formed mast cell serine proteases including tryptase, chymase, and carboxy-peptidase are secreted from granules and interact with various cell types via PAR receptors or other signalling pathways. The abundance of cytokines, mediators and proteases expressed and released by mast cells and their proximity to ASM cells, means that mast cell activation within the ASM bundles is likely to have serious implications on airway pathophysiology in asthma (Bradding, P. & Arthur, G., 2016). MMP-1 is present in remodelled airways and plays an important role in the breakdown of proteins comprising the ECM of ASM tissue (Rogers, N.K. et al., 2014). Proteolytic activation of secreted pro-enzyme forms of MMPs has been achieved in vitro by many mechanisms including organomercurials, chaotropic agents, and proteases (Nagase, H. et al., 1990). It is also already well known that components of the ECM in asthmatic airways can promote airway remodelling by affecting: cell proliferation (Johnson, S. & Knox, A., 1999), migration (Henderson, N. et al., 2006), apoptosis (Freyer, A. M. et al, 2001) and β-agonist signalling of ASM cells (Freyer, A.M. et al., 2004). Therefore, I further hypothesised that mast cell

proteases are responsible for ASM derived MMP-1 activation in asthma, leading to ongoing structural changes responsible for airway remodelling. To prove these hypotheses, a combination of in vitro and human studies have been used in this project to examine the interactions between mast cells, MMP-1 expression/activity, ECM remodelling, asthma symptoms and severity. Some of these results have previously been presented and published as abstracts (Naveed, S. et al., 2015) (Naveed, S. et al 2013) (Naveed, S. et al., 2016). Recently a paper based on the main findings of this study has also been published in the American Journal of Respiratory and Critical Care Medicine (Naveed, S.-U.-N. et al., 2016).

Chapter 2: Methods

2.1 Outline of clinical studies and procedures

2.1.1 Stable asthma study

2.1.1.1 Patient recruitment

Eleven healthy (non-smoking, age matched) and 16 asthmatic volunteers meeting the inclusion criteria (outlined in section 2.1.1.2) underwent baseline clinical sampling including lung function, airway challenge test and bronchoscopy at City Hospital, Nottingham. The subjects had no history of: asthma exacerbation, change in therapy or use of oral steroids for at least six weeks prior to recruitment. The asthma patients were asked to stop their inhaled medication (SABA and combination inhalers) 48 hours prior to their first visit. These patients were then systematically phenotyped during their first screening visit. The overall study design and timing of assessments is summarised below in Table 2. The study was approved by the Nottingham Research Ethics Committee (12/EM/0199).

Days	0 visit 1	1	2	3	4	5	6	7	10	14	15 visit 2
ACQ'5 diary											
PEFR diary											
Spirometry	x										x
PC_{20} airway challenge	x										
FeNO measurements	x										
Bronchoscopy											x

Table 2. Main study visits and assessments (stable asthma cohort). ACQ'5 = Juniper 5 asthma control questionnaire; PC_{20} = Provocation concentration of methacholine (mg/ml) causing a 20% fall in forced expiratory volume in one second; FeNo= Fractional exhaled nitric oxide levels.

2.1.1.2 Study subjects

Adults aged 18-65 years old, with no significant smoking or other respiratory history were eligible if they met diagnostic criteria for asthma (physician diagnosis of asthma). Listed below are additional recruitment criteria.

Inclusion criteria for patients with asthma

1. 18-65 years of age
2. Physician diagnosed asthma
3. Post bronchodilator FEV_1>80% predicted
4. Able to perform acceptable and reproducible spirometry required for methacholine challenge test
5. Able to give informed consent

Exclusion criteria for patients with asthma

1. Participation in a clinical drug trial within 3 months
2. Pregnant or breast feeding
3. Any other chronic respiratory condition
4. Smoker within the last year or previous smoking history of >10 pack years
5. History of life threatening exacerbation of asthma
6. >1000mcg inhaled corticosteroid / day
7. Exacerbation of asthma within the last 6 weeks
8. Use of oral steroids, theophylline or leukotriene antagonists within the last 6 weeks
9. Any severe systemic illness (malignancy, diabetes, tuberculosis)
10. Inability to give informed consent
11. Known cerebral or aortic aneurysm
12. Recent myocardial infarction

13. Recent eye surgery

Inclusion criteria for healthy subjects

1. 18-65 years of age
2. Non-smoker or less than 10 pack year smoking history
3. Post bronchodilator FEV_1>80% predicted normal value
4. No known respiratory illness
5. Able to give informed consent

Exclusion criteria for healthy subjects

1. Diagnosis of asthma or any other respiratory disease
2. Smoker within the last year or previous smoking history of >10 pack years
3. Respiratory infection within the last 6 weeks (e.g. pneumonia)
4. Use of oral steroids, theophylline or leukotriene antagonists within the last 6 weeks
5. Any severe systemic illness (e.g. malignancy, diabetes, tuberculosis)
6. Inability to give informed consent
7. Pregnant or breast feeding
8. Known cerebral or aortic aneurysm
9. Recent myocardial infarction

2.1.1.3 Defining asthma severity status

Asthmatic volunteers were defined as mild, moderate or severe based on the global initiative for asthma (GINA) 2004 criteria (Table 3). Despite the shift in asthma terminology in recent years away from a system based on disease severity towards one based on asthma control, these definitions were included to allow direct comparison with the asthma exacerbation study. However, given the shift in

emphasis towards asthma control, subjects have also been defined according to their asthma control status (see section 2.1.1.4).

	Symptoms/Day	Symptoms/Night	PEF or FEV_1	PEF variability
Step 1 Intermittent	< 1 time a week Asymptomatic and normal PEF between attacks	</= 2 times a month	>/= 80%	< 20%
Step 2 Mild Persistent	> 1 time a week but < 1 time a day Attacks may affect activity	> 2 times a month	>/= 80%	20-30%
Step 3 Moderate Persistent	Daily Attacks affect activity	> 1 time a week	60%-80%	> 30%
Step 4 Severe Persistent	Continuous Limited physical activity	Frequent	</= 60%	> 30%

Table 3. GINA Classification of asthma severity. The presence of one of the features of severity is sufficient to place a patient in that category. PEF = Peak expiratory flow; FEV_1 = Forced expiratory volume in one second.

2.1.1.4 Defining asthma control status

In this study, the modified asthma control questionnaire (ACQ'5) was selected to be used to assess asthma control. ACQ'5 is an instrument developed by Juniper and colleagues for assessing asthma control in both research trials and clinical practise (Juniper, E.F. et al., 2005). Questions rely on recall of the previous 7 days assessing symptoms (5 items-self-administered) comprising breathlessness, nocturnal waking, symptoms on waking, wheeze and activity limitation, (1 item-self-administered) frequency of SABA use, and (1 item completed by clinic staff) pre-bronchodilator FEV_1% predicted. All seven items are scored on a 7-point scale without weighting (0= good control, 6 = poor control) and the overall score is the mean of the responses.

2.1.1.5 Subject recruitment

About half of subjects in this study were recruited through adverts placed on the Nottingham Respiratory Research Unit (NRRU) website. Additional paper adverts were placed throughout noticeboards at Nottingham University Medical School. The other half of asthmatic and healthy volunteers for this study were selected from the Nottingham asthma research database, incorporating around 400 asthma patients and 200 healthy volunteers. Initial contact with volunteers was made by an invitation letter and a telephone call. Approximately 45 subjects were screened by telephone. Of these 32 attended NRRU for the first of two potential screening visits. The remaining 13 subjects who initially replied to the advert either declined to enter the study (mostly due to unwillingness to undergo a bronchoscopy) or were not suitable (due to smoking history and/or significant other chronic medical conditions). The flow of participants through each stage of recruitment is illustrated in figure 5.

2.1.1.6 First visit (screening)

At the first screening visit a more in depth medical history was obtained followed by a full examination, pre and post bronchodilator spirometry (for all study subjects), single breath FeNO measurements and methacholine PC_{20} test (for asthma patients only). This visit also included review of allergic status for asthma patients. However, specific allergen testing for atopy was not performed. To determine overall asthma control and asthma related morbidity, asthma patients were asked to complete twice daily PEFR and the ACQ'5 for 2 weeks (Table 2).

2.1.1.7 Second visit

On the second visit (2 weeks after the first visit) PEFR and ACQ'5 diaries were collected from asthma patients and bronchoscopy performed on all study

volunteers. Asthma patients also underwent a spirometry prior to their bronchoscopy. During bronchoscopy, bronchial washings and biopsies were taken from the right bronchus intermedius (Table 2).

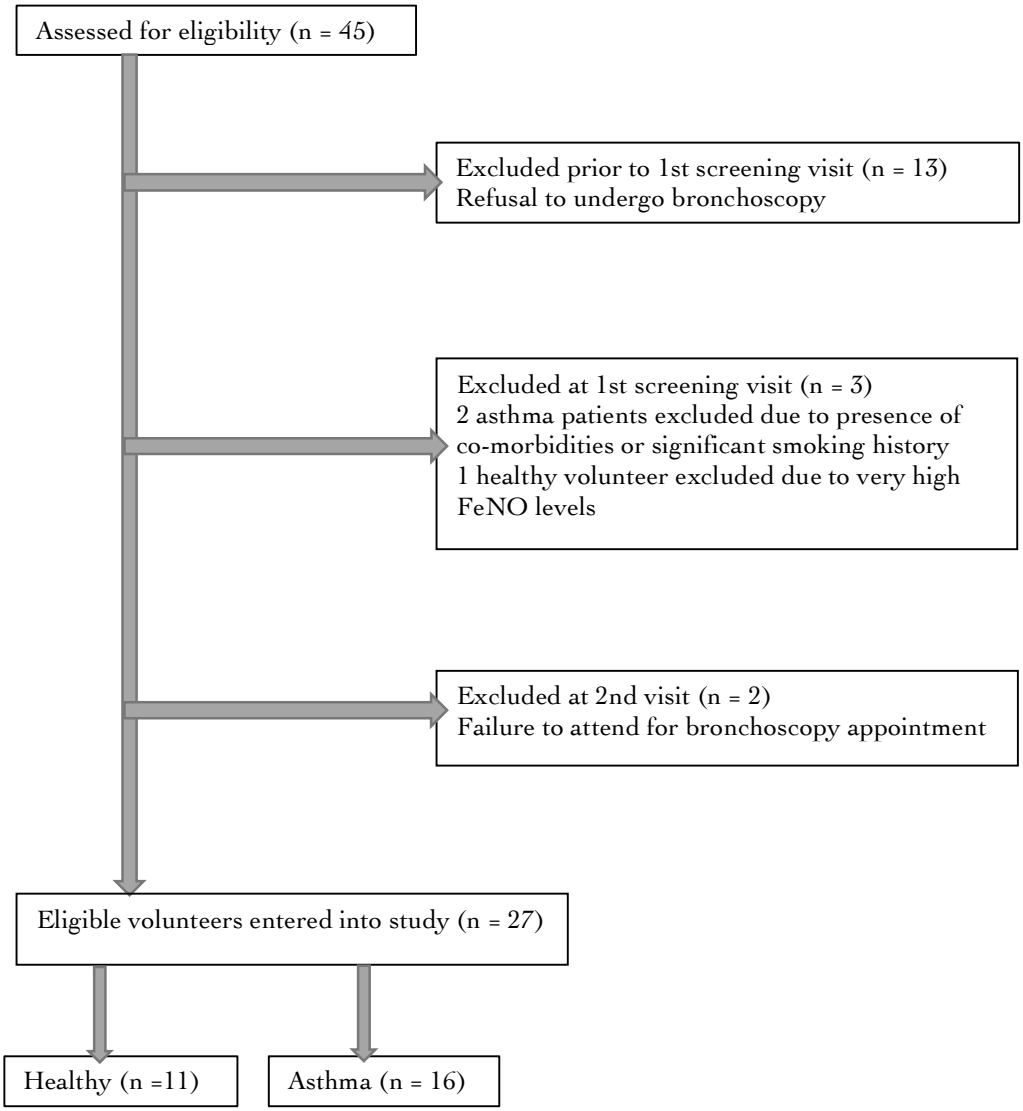


Figure 5. Stable asthma study recruitment – consort diagram.

2.1.1.8 Pulmonary function tests

Clinical spirometry was performed according to British Thoracic Society (BTS) and European Respiratory Society (ERS) guidelines (Bush, A. & Cramer, D., 1994) using a MicroLab spirometer MK8 (MicroMedical, Rochester, UK).

Asthma study participants were also instructed to perform home peak flow measurements on waking up each morning and before going to bed for two weeks, recording the best of three attempts of PEF using a Wright's mini peak flow meter (Medical Supplies, UK).

Spirometry is one of the basic measures of asthma control and FEV_1 provides a highly reproducible and objective measure of airway obstruction. PEF is also useful to confirm asthma control but is considered inferior to FEV_1 as it lacks accurate reference values for many populations, may underestimate airflow limitation in individuals with airway remodelling and offers no advantage in reproducibility. In this study FEV_1 was recorded pre and post-bronchodilator for all study participants to assess severity of disease in asthma subjects and to confirm healthy status of non-asthmatics.

2.1.1.9 Methacholine airway challenge test

AHR is an objective measure of variable airway obstruction and reflects the increased sensitivity of the airways to inhaled stimuli. Methacholine (acetyl-p-methylcholine chloride) was used as the direct ASM stimulus and a non-specific bronchoconstrictor. Ten doubling concentrations of methacholine stored in sterile vials and pre-warmed at room temperature were used according to guidelines (Crapo, R.O. et al., 2000) (Popa, V., 2001). These concentrations are: 0.03, 0.06, 0.125, 0.25, 0.50, 1, 2, 4, 8, 16 mg/ml.

During the test, participants were first asked to inhale a vapour of 0.9% saline, the mist being generated by a jet nebuliser (Medisafe, UK). This was followed by FEV_1 measurements using a conventional spirometer and the best of 3 results was taken as a reference (or baseline) reading. This was followed by 3 ml of the first (lowest) concentration of methacholine delivered through a nebuliser, using the 2-

min tidal breathing method as described (Popa, V., 2001) (Sterk, P.J. et al., 1993). Next, FEV_1 was measured at 30 and 90 seconds after the nebulisation was completed. An acceptable-quality FEV_1 at each time point was obtained. Additionally, any associated symptoms were noted. Depending upon whether a patient reported symptoms in the long questionnaire based on the Borg scale or not, doubling (for those with symptoms) or quadrupling (for those without symptoms) doses of methacholine were inhaled by the patients for 2 minutes on set time intervals until a 20% fall in post-saline (baseline) FEV_1 was observed, or the maximum cumulative dose of 16mg was reached. To keep the cumulative effect of methacholine relatively constant, the time interval between the commencement of two subsequent concentrations was kept to a maximum of 5 minutes. If the FEV_1 fell less than 20%, 3 ml of the next highest concentration of methacholine was given, and steps of FEV_1 and clinical assessment were repeated as above. If the FEV_1 fell more than 20% from baseline (or the highest concentration was given), no further methacholine was given, signs and symptoms noted and inhaled salbutamol administered. Spirometry was then repeated after 10 minutes to ensure reversal of methacholine induced bronchoconstriction.

2.1.1.10 Exhaled nitric oxide test

Nitric oxide is released by epithelial cells in the bronchial wall of the lungs. When the airways are inflamed, the level of nitric oxide is increased. The proportion of nitric oxide present in patients' breath provides a direct measure of the degree of underlying airway inflammation (Munakata, M., 2012). FeNO is a robust, non-invasive biomarker of allergic or non-allergic airway inflammation. The FeNO concentration in expired breath was measured by NIOX MINO® device (Circassia.com). This technology provides measurements, which are repeatable

and according to recommendations for NO measurement established jointly by the ATS/ERS (Dweik, R.A. et al., 2011).

After ensuring that the device is in functional mode, asthma patients were asked to empty their lungs first and then inhale deeply through the filter on the device to total lung capacity (TLC). This is followed by a slow exhalation manoeuvre through the filter until the sound and top light on the device turns off. The device exhibited steady light and a continuous sound during exhalation phase to ensure correct exhalation pressures were delivered. The FeNO value was displayed on the device screen as parts per billion (ppb) along with a unique patient ID after a wait of about 1 minute. The cut-off point of 25 ppb was used in asthma patients as significant.

2.1.1.11 Bronchoscopy

All bronchoscopies were performed in the endoscopy unit at City Hospital, Nottingham using an Olympus PF260 bronchoscope. Subjects with asthma were instructed to continue using their usual inhaler treatment and were also administered nebulised salbutamol (2.5mg) prior to the procedure. The sedation was achieved with intravenous Midazolam. The bronchoscope was passed via the nose and in some instances via mouth if nasal passage was inaccessible. Bronchial washing was performed by instillation of 15 ml sterile normal saline into the right upper lobe bronchus. On average about 10 ml fluid was recovered back, filtered, centrifuged and the supernatant immediately stored at -80°C. Six bronchial biopsies were taken from the secondary division of the right intermediate bronchus using 1.8mm radial jaw (1523, Boston Scientific, USA) serrated needle free biopsy forceps. Two biopsies were placed immediately in 4% formaldehyde and the other four placed in 500µl of Dulbecco's Modified Eagles' Medium

(DMEM) for future ASM tissue dissection and primary cell culture.

2.1.2 Asthma exacerbation study

Fourteen healthy (non-smoking, non-atopic) and 32 (non-smoking) asthmatic volunteers meeting the inclusion criteria outlined in section 2.1.2.2 were recruited for this study by Dr David Jackson of the Imperial College Respiratory Research Unit (ICRRU) at St Mary's Hospital, London. The study was approved by the St Mary's Hospital ethics committee (09/H0712/59). All patient procedures were also performed at ICRRU by Dr Jackson and his team. A few of these procedures relevant particularly to my project are explained here briefly to provide clarity. The study subjects underwent baseline clinical sampling including lung function and bronchoscopy on day 0, about 2 weeks prior to rhinovirus (RV16) inoculation. Subjects were then seen on days 2, 3, 4, 5, 7, 10 and 42 post-inoculation. Infection was induced with RV16 via nasal spray into both nostrils. A second bronchoscopy was performed during the rhinovirus infection on day 4 post-inoculation to allow measurement of a wide range of inflammatory markers of infection and permit comparison between healthy and asthmatic subjects.

2.1.2.2 Study Subjects

Inclusion criteria for asthmatics

Adults aged 18-55 years old, with no significant smoking or other respiratory history were eligible if they met diagnostic criteria for atopic asthma (physician diagnosis of asthma, bronchodilator reversibility >12%, bronchial hyper-reactivity as measured by a provocative concentration of histamine causing a 20% reduction in FEV_1 (PC_{20}) <8 mg/mL, and ≥1 positive skin prick test to a panel of aeroallergens). Listed below are the full criteria.

- Age 18-55 years
- Doctor diagnosis of asthma
- Histamine PC_{20} < 8 mg/mL
- Mild-to-moderate disease based on 2004 GINA criteria
- Treatment with either SABA alone or with maintenance ICS, daily dose 200 - 1000 µg BDP equivalent
- Worsening asthma symptoms with infection since last change in asthma therapy
- Atopic on skin testing (≥ 1 positive skin prick test on a panel of 10 aeroallergens)

Exclusion criteria for asthmatics

- History of severe asthma defined by GINA
- Smoking history over past 6 months or > 5 pack year history
- Current symptoms of allergic rhinitis
- Current or previous history of significant respiratory disease (other than asthma)
- Any clinically relevant abnormality on screening or detected significant systemic disease
- Asthma exacerbation or viral illness within the previous 6 weeks
- Treatment with oral corticosteroids in the previous 3 months
- Current use of any nasal medication, anti-histamine, anti-leukotrienes, LABA, or anti-IgE therapy
- Presence of serum neutralising antibodies to rhinovirus-16 at screening
- Pregnant or breast-feeding women
- Contact with infants or elderly at home or at work

Inclusion criteria for healthy subjects

- Age 18-55 years
- No history or clinical diagnosis of asthma or any other significant respiratory disease
- No history of allergic rhinitis or eczema
- Negative responses on skin prick testing
- PC_{20} > 8 µg/mL
- Absence of significant systemic disease

Exclusion criteria for healthy subjects

- History of atopy, asthma or any significant respiratory disease
- Smoking history over past 6 months or > 5 pack year history
- Current symptoms of rhinitis
- Any clinically relevant abnormality on screening or detected significant systemic disease
- Viral illness within the previous 6 weeks
- Current use of any nasal medication or anti-histamine
- Presence of serum neutralising antibodies to RV16 at screening
- Pregnant or breastfeeding women
- Contact with infants or elderly at home or at work

2.1.2.3 Defining asthma severity and control

Asthmatic volunteers in the asthma exacerbation study were defined as mild or moderate based on the GINA 2004 criteria as before. Asthma control groups were defined by Juniper modified ACQ cut-off points of ≤0.75 for well-controlled and ≥1.50 for poorly-controlled asthma, respectively. Asthmatics that fell between

these 2 categories have been termed as partially-controlled (Juniper, E.F. et al., 2006).

2.1.2.4 Pulmonary function tests

Spirometry was performed for the asthma exacerbation cohort at each visit using a MicroLab spirometer V002ML3500 (MicroMedical, UK). Study participants were also instructed to perform home spirometry on waking each morning, recording the best of three attempts of PEF and FEV_1 using a Piko-1 device (nSpire, UK). The % change in morning PEF and FEV_1 from baseline during infection was calculated for each subject as the % fall from the mean of the 7 days period prior to inoculation. Maximal fall from baseline (%) for each subject represented the greatest fall from baseline over the 2-week period following inoculation (Jackson, D.J. et al., 2014).

2.1.2.5 Lower respiratory tract (LRT) symptom scores

Daily diary cards of upper and lower respiratory symptoms were commenced 2 weeks before baseline bronchoscopy and continued until 6 weeks post-inoculation. LRT symptoms were recorded in a similar fashion to cold symptoms and included cough on waking, wheeze on waking, daytime cough, daytime wheeze, daytime chest tightness, daytime shortness of breath, nocturnal cough, wheeze or shortness of breath. A grading system of 0–3 was applied to these symptoms. LRT symptom scores were analysed in 2-week blocks, as the baseline and acute infection stages both contained a bronchoscopy on the fourth day of each 2- week block. Daily symptom scores during infection, corrected for baseline symptoms and the symptoms related to bronchoscopy, were calculated by subtracting scores obtained during the baseline 2- week block from the corresponding days of the acute infection 2-weeks block (Message, S.D. et al., 2008) (Beale, J. et al., 2014)

(Jackson, D.J. et al., 2014).

2.1.2.6 Bronchoalveolar lavage

During bronchoscopy procedure, BAL was performed by instillation of 150ml sterile normal saline in 5 x 30 ml aliquots with a 10 second dwell time into the right upper lobe bronchus (Jackson, D.J. et al., 2014). Recovered BAL fluid was then filtered, centrifuged and the supernatant stored at -80°C. Bronchial fluid samples obtained from this cohort were 10x more diluted than bronchial fluid samples from the stable asthma cohort. This was because of the difference in volumes of normal saline being instilled into airways as a part of lavage vs. wash technique.

2.1.2.7 Nasal lavage

Nasal lavage for viral load was performed at each visit, with sterile 0.9% sodium chloride using a 10ml syringe attached to a hollow nasal adapter. This is used to obstruct the nostril and prevent leak of lavage fluid (Nicholson, G.C. et al., 2011). A total of 5 ml of sterile normal saline was instilled into the left nostril and the fluid withdrawn into the 10ml syringe and flushed back into the nasal cavity 20 times over a one-minute period. Fluid was collected, aliquoted and stored at -80°C (Jackson, D.J. et al., 2014).

2.2 Cells and tissues

2.2.1 Primary ASM culture

Bronchial biopsy tissue was either processed for immunohistochemistry or used for culture of ASM cells as described below, with cells used at passage five or less. I followed the standard operating procedure (SOP)-THE107 for isolation of ASM bundles from bronchial biopsies. After pH and temperature equilibration of biopsies, ASM bundles were dissected from surrounding tissue under the

microscope and seeded in clear plastic 6-well culture plates in DMEM, supplemented with 10% foetal bovine serum (FBS), penicillin (50U/ml) and streptomycin (50ug/ml) solution. Cultures were maintained at 37°C in a humidified incubator under 95% air/5% CO_2 for several days. Following dissection of bundles, small pieces of smooth muscle were allowed to adhere to the tissue plastic of individual wells of a 6 well plate and then dried in such a manner that when media was added to the plates, they did not detach and float. Once a single well was confluent (this usually took around 1-3 weeks from the first bronchial biopsy explants) or if the monolayer showed signs of slowed growth, the cells were trypsinised and re-seeded in a T25 culture flask. At this stage they were deemed passage 1. Once confluent in these small flasks, the cells were passaged into bigger T75 flasks as explained below at a ratio 1:4. The resulting ASM cells displayed typical a 'hill and valley' appearance and exhibited positive staining for fluorescently labelled α- smooth muscle actin (α-SMA) antibody (Cy3-conjugated;1/250).

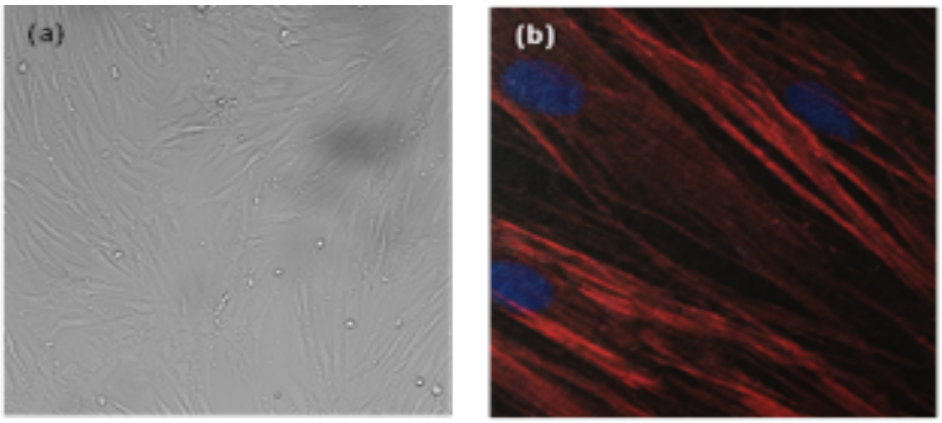


Figure 6. Characterisation of ASM cells obtained from bronchial biopsies. (A) Cultured primary ASM cells viewed by light microscopy showing typical 'hill and valley' appearance. **(B)** Cultured primary ASM cells stained with fluorescently labelled α-SMA antibody. α-SMA = α smooth muscle actin.

Bronchial biopsies of all 16 patients with asthma and 11 healthy controls from the

stable asthma cohort were processed for ASM culture. Primary ASM cells were isolated from 11 asthmatics and 7 healthy bronchial biopsies. No ASM cells were obtained from the remaining samples. All our experiments were conducted on ASM cells from ≥3 donors from each group between passages 3-5.

2.2.2 Passaging of ASM cells

Further passaging of primary ASM cells was carried out in accordance with SOP-THE048. Once there were several outgrowths of cells occupying approximately 70% of the culture T25 vessel, depending on distribution, cells were trypsinised. To trypsinise, the vessel surface was first washed gently with 5 ml warmed phosphate-buffered saline (PBS) followed by 1ml trypsin, which was aspirated immediately and 2ml trypsin added to the vessel to lift ASM cells from surface. About 5ml DMEM+ 10%FBS+ 1% Penicillin/Streptomycin solution was then added to stop trypsin activity. The resultant cell suspension was collected in a sterilised universal plastic container and centrifuged the at 500rpm for 5 minutes. The supernatant was carefully aspirated without disturbing the cell pellet, which was re-suspended in a total of 1ml DMEM+ 10%FBS+ 1%Penicillin/Streptomycin solution. Cell numbers were counted by taking 10µl of this solution through a pipette and transferring it on to a haemocytometer. Cells were then plated into culture grade plastic flasks with pre-warmed media inside, at a density of 2,500 - 3,500 cells per cm^2 in a T75 containing 10ml of DMEM media mixed with serum and antibiotic solution. Cells were fed as required i.e. every 2 days until flasks were nearly 90% confluent and ready to passage further or freeze.

2.2.3 Cryopreservation of ASM cells

ASM cell preservation in liquid nitrogen and subsequent thawing was carried out in line with SOP-THE048. Primary ASM cells in culture were cryopreserved at

250,000 cells per ml of freeze medium. The method used for lifting the ASM cells from the surface of culture vessel was essentially the same as for passaging cells. The cell pellet obtained after centrifugation of the cell suspension was re-suspended in 1ml of freeze medium (10%DMSO+ 90%FBS) and transferred immediately to cryovials, followed by a quick transfer to -80°C freezer for 24-48 hours. Subsequently, the cryovials were moved to a liquid nitrogen cell storage facility.

2.2.4 Thawing ASM cells and subcultures

After removing a cryovial from liquid nitrogen, it was transferred immediately into a microbiological safety cabinet and the cap twisted slightly to release any pressure build-up. The cryovial was then thawed via partial immersion in a water bath at 37°C. The ASM cells were then transferred to a universal container, containing 20ml of warmed media mixed with 10% FBS and 1% antibiotic solution, followed by centrifugation at 500rpm for 5 minutes at 25°C. The resultant supernatant was aspirated and discarded, and cell pellet re-suspended in 1ml of DMEM media containing FBS and antibiotics as above. The re-suspended pellet was subsequently seeded in a vented T75 tissue culture flask with approximately 10ml of DMEM containing FBS and antibiotics. Once ASM cells approached 90% confluence, they were then subjected to subculture as before. For ASM proliferation experiments, cells were first counted using a haemocytometer and plated into 6 or 12 well experiment plates (cell culture grade) at the cell density of 10,000 - 20,000 cells per well in DMEM containing serum but no antibiotics. Plates were then returned to the incubator at 37°C and 5%CO_2 for at least 24 hours to allow cell adhering, before replacing culture media. Primary ASM were first serum starved for at least 24 hours for all cell proliferation experiments.

2.2.5 Immunofluorescence of cultured ASM cells

Immunofluorescence was performed in accordance with SOP-THE120, on cultured primary cells to confirm α-SMA expressing ASM phenotype. After aspiration of cell culture supernatant, cells were fixed with 3.7% formaldehyde solution overnight. Next morning, this solution was aspirated and the cells were washed three times with PBS+Tween (PBST) 0.05% for 5 minutes for each wash. Subsequently, cells were blocked with 1% bovine serum albumin (BSA) in PBST for 1 hour at room temperature with slight agitation on a rocking plate. After removing the blocking solution, cells were then incubated with Cy3 conjugated α-SMA antibody (1:200) at 4°C overnight. The following day, the antibody solution was aspirated and cells washed three times as before. The second time wash solution however contained 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) in the concentration of 1:1000 as a DNA probe to help clarify cell visualisation. After the last wash, the cell plates were stored at 4°C until they were ready to be analysed under light microscopy with fluorescence (Figure 6B).

2.2.6 Mast cell culture and activation

Human mast cells were from the HMC-1 cell line and a generous gift from Professor Peter Bradding, University of Leicester. I contributed to the SOP-THE048 for mast cell culture by optimising culture conditions for the HMC-1 cell line. The HMC-1 cell line has been established from a patient with mast cell leukaemia. These are small delicate cells, which are widely used in research for studies of human mast cell function because they exhibit many important features of tissue mast cells, such as histamine, tryptase, heparin expression and similar antigen-profile on cell surface including receptor tyrosine kinase/KIT (Sundstrom, M. et al., 2003).

Mast cells from HMC-1 cell line are suspension cells. However, I found out that they do not grow well in roller flasks and cell growth was much better under static culture conditions. During subcultures, I kept pipetting to a minimum because HMC-1 were quite fragile cells, often exhibiting lower viability/cell counts when handled aggressively. I also optimised cell plating density of 250,000 cells /ml and passaging cell density of 0.5-1.0 million cells/ml of culture medium, respectively to achieve maximum cell viability. HMC-1 mast cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with glutaMAX™ and supplemented by 10% FBS in T75 cell culture flasks. The culture vessels were incubated at 37°C and 95%air/5% CO_2 for 3-5 days or until they reached the optimised cell density required for further passaging. During this time, HMC-1 suspension cells were left alone with only media replenishment every 1-2 days. Cell counts and viability were determined using a haemocytometer with tryptan blue exclusion assay (section 2.4.1).

For activation, HMC-1 cells were first serum starved for 24 hours, followed by addition of phorbol 12-myristate 13-acetate (PMA) and calcium ionophore (A23187) at working concentrations of 50ng/ml and 25ng/ml to the culture medium, respectively. The cells were incubated with PMA and calcium ionophore for various periods of time (4, 8, 16, 20, 24 hours) and cell viability was measured at each of these time points. HMC-1 cell viability was not affected at 4, 8 and 16 hours of incubation after addition of PMA and calcium ionophore. It however started to decline sharply after that. I therefore, decided to use an incubation time of 16 hours for mast cell activation (in order to achieve maximum mediator release, while preserving cell viability) for all future experiments. After incubating HMC-1 cells for 16 hours with PMA and calcium ionophore, cells were pelleted

by centrifuging at 1200 rpm in an IEC Centra - 4B Centrifuge for 5 minutes. The culture supernatant was then harvested and sterile-filtered before being aliquoted in batches and frozen at -80°C. For experiments, frozen mast cell supernatant aliquots were thawed at room temperature under sterile conditions prior to their use in ASM cell cultures. For control experiments, mast cell supernatants were prepared in a similar manner as above, except that the activation stimuli (PMA and calcium ionophore) were added directly to the supernatants after removal of the cell pellet, providing an identical solution other than the contents of mast cell granules. Inhibition of proteases was performed by pre-incubation of supernatants for 30 minutes at room temperature with protease inhibitor solutions, used at the manufacturer's recommended concentrations (section 2.3.1).

2.3 In vitro preparation of ASM derived ECM

ASM cell derived ECM was prepared as previously described (Rogers, N.K. et al., 2014) and by adapting the SOP-THE146. Primary ASM cells were grown to 100% confluence in 12 well culture plates (treated or not treated with activated mast cell supernatants for 24 hours). The culture medium was aspirated and the cell monolayer washed three times with PBS. A solution of ammonium hydroxide (50mM) and Triton X-100 (0.05%) was added to the culture wells and plates incubated at 37°C until cells detached. The resultant solution containing detached cells was then aspirated and culture plates washed again with ammonium hydroxide (50mM) and PBS, respectively to remove any left-over debris. Following this, DNase-1 (20U/ml) was added to each well and culture plates incubated once more at 37°C for one hour. This solution was also aspirated and plates once again washed with PBS three times, resulting in a coating of ECM left on the culture plate surfaces (Figure 7). The plates were then wrapped in cling

film and refrigerated prior to use. ECM coated plates were warmed to room temperature prior to seeding of new cells onto them for ASM cell proliferation experiments.

2.3.1 ASM proliferation experimental protocol

Primary ASM cells from 3 asthma and 3 healthy donors at passage 3 were plated in 12 well plastic culture plates (n=3) under normal culture conditions (in DMEM medium supplemented with 10% FBS and incubated at 37°C). The cells were allowed to grow to 100% confluence over 3-5 days to allow them to deposit ECM on the culture plate surfaces. Where relevant, activated or control mast cell supernatants were added to the culture medium in a ratio of 3 parts culture media to 1 part activated or control mast cell supernatant. Culture plates were incubated for a further 24 hours after mast cell supernatant treatment. After this, ECM preparation was carried out in each well according to the procedure described above. Primary ASM cells from an asthma or healthy donor at passage 3 were re-seeded onto the ECM preparations in each well at the plating cell density of 10,000 – 20, 000 cells per well under reduced serum conditions (DMEM containing only 1%FBS). The culture plates were incubated for another 48 hours at 37°C, before MTT assay and/or cell counts using a haemocytometer were carried out (Figure. 7).

Where needed, activated or control mast cell supernatants were pre-mixed with protease inhibitors prior to their use in ASM cell proliferation experiments. The protease inhibitor concentrations used were according to manufacturer's instructions and as follows:

1. cOmplete™ Mini, EDTA-free protease inhibitor cocktail; 1 tablet dissolved in 10mL water and used at 5µl/ml of mast cell supernatant. This contains a mixture

of serine and cysteine protease inhibitors.

2. Serine protease inhibitor cocktail; this was purchased as a powder, which was reconstituted in 1 ml of distilled water to obtain a 100 x stock solution. This was serially diluted 2 fold and a minimum of 1.56 x protease inhibitor cocktail solution was used in inhibitory experiments. A 1 x concentration of the serine protease cocktail consists of 500µM AEBSF, 420nm aprotinin, 20µM elastatinal and 1µM GGACK.

3. Ilomastat, an MMP inhibitor used at a concentration of 25µM, reconstituted in dimethyl sulphoxide (DMSO) 20mg/ml.

4. Specific tryptase inhibitor (APC366) used at a concentration of 10µM, reconstituted in 20% ethanol/water.

5. Specific chymase inhibitor (Chymostatin) used at a concentration of 10 µM, reconstituted in DMSO 20mg/ml.

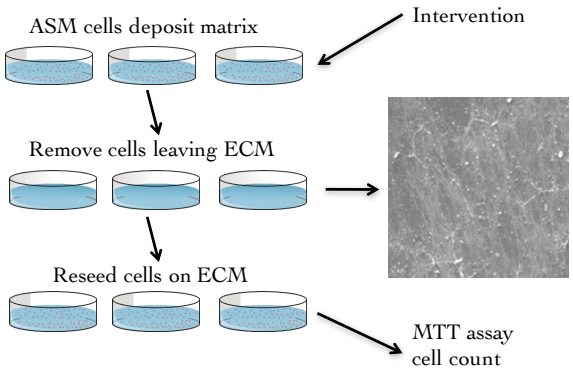


Figure 7. Schematic representation of ASM proliferation experimental protocol. Electron microscopic appearance of ECM matrix deposited by ASM on culture surface following cell removal (black and white). ASM= Airway smooth muscle; ECM= Extracellular matrix.

2.4 In vitro ASM proliferation assays

2.4.1 Cell counting

Cell proliferation was first measured by direct cell counting using a Neubauer haemocytometer as described in SOP-THE048. After harvesting and re-

suspending cells in 1ml of media, a 20µl aliquot of cells was placed in the chambers of a haemocytometer. Cells in one quadrant of the grid (coloured in blue as seen in Figure. 8) were counted and multiplied by 10^4 to give the number of cells per ml of culture media. These results were also confirmed by imaging culture plates at 4x magnification on a Nikon Diaphot 300 inverted Microscope using SPOT Advanced Imaging™ software. Images were taken across the centre of the well from a reference mark on the side of each well. The usual culture media was then aspirated and replaced with DMEM containing 1% FBS (reduced serum). This imaging process was repeated at 24 and 48 hours, imaging the same fields of view each time. Cell counts were then conducted using ImageJ with the cell counter plug-in (Schneider, C.A. et al., 2012).

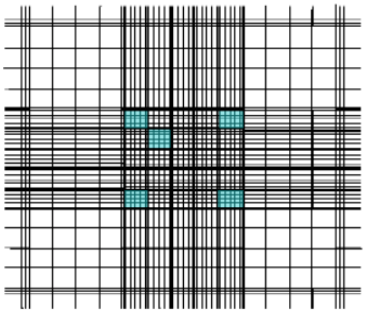


Figure 8. Haemocytometer grid layout.

2.4.2 Cell viability

Cell viability was calculated according to the protocol in SOP-THE049 using 0.4% solution of trypan blue (Thermofischer Sceintific) in PBS (pH 7.2 to 7.3). After determining total cell counts by using a haemocytometer as described above, 0.1 ml of trypan blue stock solution was added to 0.1 ml of cells. After loading a haemocytometer, cells were examined immediately under a microscope at low magnification. If cells took up trypan blue, they were considered non-viable. The number of blue staining cells and the number of total cells were counted. Cell

viability of at least 95% was used for healthy log-phase cultures, such as for HMC-1 suspension cells. This was calculated using the formula below.

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100

To calculate the number of viable cells per ml of culture for ASM proliferation experiments, another formula as below was used and the final number corrected for the dilution factor.

Number of viable cells × 10^4 × 1.1 = cells/ml of culture media

2.4.3 MTT reduction assay

MTT assay was first described in 1983 as a semi-automated colorimetric assay, based on the assumption that the mitochondria of living cells reduce the water soluble tetrazolium salt (3-(4,5-dimethylthiaaol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan crystals by mitochondrial dehydrogenases, but the dead cells lose this ability. The formazan crystals are then solubilised in isopropanol or DMSO and the concentration determined by reading the absorbance spectrum or optical density at 570 nm on a multi-well scanning spectrophotometer. The colour formation serves as a useful and convenient marker of only the viable cells and usually shows a high degree of precision (Mosmann, T., 1983). This assay can therefore be used to measure cytotoxicity, cell proliferation and activation. There are no unnecessary washing steps in the assay, eliminating the risk of cell damage and loss. The main advantages of a colorimetric assay like this are its efficiency and precision, as well as the lack of any radioisotope toxicity exposure to the cells. As most of the culture medium is removed before the addition of MTT, there is no risk of any interference from protein precipitation when using isopropanol or DMSO. MTT assay has been shown to correlate well with other cell proliferation assays and can be used to

complement other more established experimental systems, such as cell counts and carboxyfluorescein succinimidyl ester (CFSE) assay (Kaur, D. et al., 2010) (Marshall, N.J. et al., 1995). Although the MTT assay is a reliable way of determining cell proliferation and viability, care is required when using it for this purpose and it is important to standardise the seeding cell density and incubation times, especially in assessing effects of drugs or other chemical treatments on cells in vitro (Marks, D.C. et al., 1992).

MTT reduction was used in this study as a surrogate marker for ASM cell number and has been commonly used for similar purpose and in other proliferation studies in the past by researchers across the world (Johnson, S. & Knox, A., 1999) (Saunders, R. et al., 2009) (Henderson, N. et al., 2006). I followed the protocols in SOP-THE036 for the MTT reduction assay to measure cell proliferation. ASM cell culture media was first aspirated from culture plates and cell monolayer washed with PBS, followed by replacing it with a solution of DMEM: Nutrient mixture F12 (phenol red free) containing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT powder) at a concentration of 0.5mg/ml in a light protected environment. After 4 hours of incubation at 37°C, this solution was aspirated and isopropyl alcohol added to solubilise the resultant formazan crystals. The violet coloured solution was re-suspended and then transferred to a 96 well plate in triplicates. The light absorbance of the plates was measured using a Flexstation®3 benchtop multi-mode microplate reader at wavelengths of 570nm with background subtraction of 690nm. Comparison of direct cell counting and MTT assay confirmed excellent agreement between the two assays. In addition to assessing ASM proliferation in response to different stimuli, MTT data was also used to normalise MMP-1 levels in ASM cell culture supernatants established by

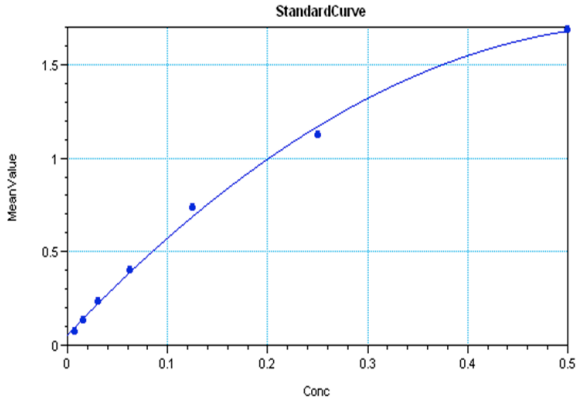
ELISA.

2.5 Protein determination assays

2.5.1 Human total MMP-1 DuoSet ELISA

For the quantitative determination of total MMP-1 levels in ASM proliferation experiments and bronchial fluid samples, a commercially available sandwich ELISA kit (Duoset) was used. This piece of kit has been validated and used in a number of studies before (Symeonidis, C. et al., 2011) (Truchetet, M.-E. et al., 2013). All solutions required for the assay were prepared approximately 15 minutes prior to use at room temperature from previously reconstituted stocks according to SOP-THE051. The Human MMP-1 Duoset ELISA kit was used following the manufacturer's protocol in a 96-well microplate. All other reagents and equipment provided with the kit were also prepared according to the manufacturer's protocol. Any light sensitive solutions were protected from direct light exposure. After reagents, samples and standards were prepared, 100µl of capture antibody was added to each well on a 96-well plate and incubated overnight at room temperature. The next day, the plate was washed three times with 400µl/well of wash buffer (0.05% Tween®20 in PBS, pH 7.2-7.4). Next, the plate was blocked with 300µl of Reagent Diluent (1% BSA in PBS, pH 7.2-7.4, 0.2mm filtered) for 1 hour. The plate was washed again three times with wash buffer as before. Standard controls or samples (neat or diluted with Reagent Diluent to a total volume of 100µl) were then added to the wells on the microplate in duplicates and incubated at room temperature for 2 hours. The MMP-1 standard was diluted with Reagent Diluent, to create a maximum top concentration of 10ng/ml, which was subjected to 2 fold serial dilutions to achieve a 7- point standard curve. The plate was washed three times with wash buffer and

the desired conjugate was added to the plate followed by its incubation for 1 hour at room temperature. The wash process was repeated and substrate solution was added and plate incubated for 20-30 minutes at room temperature, protecting it from the light. Finally, stop solution (2N sulphuric acid) was added to each well on the plate and the absorbance was determined spectophotometrically on a Flexstation® 3 microplate reader set to wavelength 450nm within 30 minutes. Background wavelength correction was done to 570 nm in order to avoid optical imperfections, which might occur if the readings were made at only 450nm. Data analysis was carried out using ELISA plate reader Softmax Pro software. The standard curve was analysed using 4 parametric logistic curve-fit algorithms and this was used to determine the concentration (ng/ml) of MMP-1 in the samples (Figure 9).



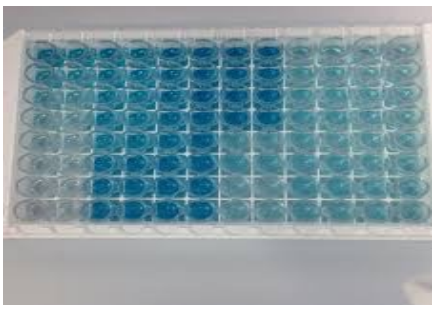


Figure 9. A representative standard curve for MMP-1 ELISA. Representative curve was created from serial dilution of the standards using a 4-parametric logistic curve fitting. Human anti-IgG sandwich ELISA microplate showing colour development after addition of substrate to the wells. ELISA= Enzyme-linked immunosorbent assay.

2.5.2 Human active MMP-1 Fluorokine E activity assay

MMP-1 activity was measured in ASM cell culture supernatants and bronchial fluid samples using a commercially available specific fluorescent activity assay kit as described previously (Rogers, N.K. et al., 2014) (Geraghty, P. et al., 2011) and in SOP-THE128. Adequate care was taken to protect light sensitive solutions from direct light exposure and, where required, steps of the assay were carried out in subdued light. The microplate was prepared by adding 100µl of assay diluent (RD1-64) to each well. The MMP-1 standard was reconstituted with calibrator diluent (RD5-25) to create a stock solution of working concentration 25ng/ml. This was further subjected to 2-fold serial dilutions and a 10-point standard curve was prepared with concentrations ranging from 12.5ng/ml - 0.012ng/ml. Samples (150µl/well) were added to the plate in quadruplicates. The plate was then incubated at room temperature for 3 hours. Subsequently, samples and standards were discarded and the plate washed with wash buffer four times. Next, 200µl of 4-Aminophenylmercuric acetate (APMA) was added to all standard wells on the plate (APMA was diluted 15 minutes prior to its use to 1:168 concentration using Reagent Diluent 2). Reagent Diluent 2 (200µl) was added to the sample wells. The plate was incubated at 37°C in a dark humidified chamber for 2 hours. This was followed by the usual wash process repeated four times. Next, 200µl of substrate was added to each well and the plate was incubated in a humidified, light protected chamber at 37°C for 17 hours. Consequently, the relative fluorescence units (RFU) of each well were determined using a fluorescence plate reader set with the following parameters.

1. Excitation wavelength to 320 nm
2. Emission wavelength to 405 nm

3. Endpoint mode; 1 x 20 mS integration time

4. Plate speed = 6.

The active MMP-1 concentration of each sample was determined by first finding the RFU value on the y-axis and extending a horizontal line to the standard curve. At the point of intersection, a vertical line was extended to the x-axis and the corresponding active MMP-1 concentration was read for each sample and corrected for the dilution factor.

2.5.3 Sample concentration

Measurement of MMP-1 levels and activity in BAL samples from the asthma exacerbation cohort required these samples to be concentrated in order to fall within assay detection limits. Samples were concentrated two-fold using Vivaspin®500 ultrafiltration spin columns with 10,000 molecular weight cut off membranes. 2ml of lavage sample was added to concentrators and centrifuged at 15,000 x g in a fixed angle rotor at 40 degrees. Once the desired concentration was achieved, assembly was removed and the sample recovered from the bottom of the concentrate pocket with a pipette.

2.5.4 Western blotting

Western blotting was carried out to identify pro and active MMP-1 in ASM cell culture supernatants and bronchial fluid samples according to the protocol (SOP-THE048). Commercially bought polyacrylamide Mini-PROTEAN® TGX™ gels (4–15%, 1.5mm thickness) were used for protein electrophoresis. Equal volumes of sample (ASM cell culture supernatants or bronchial fluid) or control and 2x gel loading buffer (1M Tris, 0.1% SDS (w/v), 0.08 DTT (w/v), 0.01% bromphenol (w/v) blue, 55% glycerol (w/v)) were mixed, heated to 95°C for 5 minutes and centrifuged at 13,000 rpm for 3 minutes. Subsequently, 10-30µl of each of

prepared sample or control was loaded onto pre-cast gels alongside kaleidoscope precision plus® protein standards and the gels were run at 100V in a protein electrophoresis system for 90 minutes, or until the blue dye front reached the bottom of the gel. The polyvinylidene fluoride (PVDF) membrane was soaked in methanol for 5 minutes and washed with water before being soaked in western transfer buffer solution along with fibre pads, filter papers and gels. The gels were blotted using the Bio-Rad® wet blot transfer cell onto methanol- activated PVDF membrane in an ice-cooled electrophoretic system for 1 hour at 100V. The membrane was then blocked in blocking buffer (5% Marvel skimmed milk in 0.1% PBS-T) for 1 hour at room temperature. Following this, the membrane was incubated overnight at room temperature with the primary human MMP-1 monoclonal antibody (clone 36665, R&D Systems) used at a concentration of 2µl/ml, mixed in blocking buffer. Next, to remove excess antibody, the membrane was washed with PBS-T for 15 minutes and this process was repeated three times. The secondary horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse antibody (R&D Systems) was incubated with the membrane for 60 minutes in blocking buffer, followed by a further three washes with PBS-T. The membrane was then drained and placed in a plastic dish with 2.5ml of 1:1 solution of enhanced chemiluminescence (ECL) reagents 1 and 2 for 1 minute. After draining excess fluid, the membrane was placed in a plastic sheet protector and the covered membrane was subsequently placed in an autoradiography film cassette with X-ray film for various lengths of time in a dark room for optimal protein band detection. After each exposure, the X-ray film was submerged in developing solution, fixed with fixture solution and finally washed with lukewarm water and air dried under dark conditions before being photographed using ImageJ software

(Schneider, C.A. et al., 2012).

2.5.5 Gelatin zymography

Zymography is commonly used to detect and characterise proteases such as MMPs (Henderson, N. et al., 2007). The advantages of gel zymography are: the ability to distinguish the presence of both latent and active gelatinases simultaneously, acceptable quantification and relative ease of performance. Because the technique makes use of the enzymatic cleavage of the gelatin substrate, this provides a much more sensitive assay than western blots (Frankowski, H. et al., 2011). In order to investigate the presence of a specific protease activity related to MMP-2 and -9 in the bronchial fluid samples obtained from asthmatic and control subjects, gelatin zymography was used according to the protocols established in SOP-THE042. First, 10µl of undiluted or concentrated bronchial fluid samples was mixed with 10µl of sample buffer (4% SDS, 0.15M Tris (pH 6.8), 20% (v/v) glycerol, 0.5 % (w/v) bromophenol blue and allowed to stand for 10 minutes at room temperature. Next, 15µl of the sample buffer mix was loaded onto a pre-cast 15 well Novex™ polyacrylamide gel supplemented with 0.1% gelatine and run with 1x tris-gylcine SDS zymogram running buffer at 125V for 100 minutes, in an electrophoresis mini-cell chamber. The gels were removed from the mini-cell chamber and incubated with 1x zymogram renaturing buffer at room temperature, with gentle agitation for 60 minutes. The gels were then equilibrated for another 30 minutes with 1x zymogram developing buffer at room temperature and incubated overnight at 37°C with 100ml of fresh developing buffer in a humidified chamber, to allow enzymatic digestion of the substrate. Finally, the gels were stained for 15-30 minutes with 0.5% coomassie blue stain in acetic acid, methanol and water (1:4:5)

and de-stained in acetic acid, methanol and water (1:4:5) to reveal areas of protease activity as clear bands. The gels were scanned with a resolution of 300 dot per inch or higher and images saved in the tag image file format (TIFF). For objective quantification of bands, densitometry with the ImageJ software was used (Schneider, C.A. et al., 2012). Since polyacrylamide gel contains SDS, the speed of migration in electrophoresis is determined by the molecular weight of the separated proteins, resulting in shifted local condensation of full length pro-enzyme and truncated forms of MMPs. Subsequently, when compared to respective molecular weight standards, the positions of the unstained areas enable identification of target MMPs; the size of the areas correlates with the amount of the target MMPs present (Waas, E.T. et al., 2002).

2.5.6 Silver staining

Silver staining is another protein detection method which involves electrophoretic separation of proteins on a polyacrylamide gel. It combines excellent sensitivity, whilst using very simple and cheap equipment and chemicals (Wells, K. & Cordingley, J.S., 1991). The simple rationale of silver staining is that the proteins bind silver ions, which are reduced under appropriate conditions to build up a visible image made of finely divided silver metal (Chevallet, M. et al., 2006). Proteolytic cleavage of MMP-1 by tryptase was examined by using the same initial protocol of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as in western blotting (SOP-THE048) followed by silver staining. Recombinant pro-MMP-1 (10ng) and recombinant human mast cell tryptase (in varying concentrations of 0-0.2 IU) were co-incubated for 30 minutes at 37°C. The resultant products were diluted in PBS and resolved on commercially bought SDS-PAGE gel by electrophoresis. The gels were then fixed in a solution of 30%

ethanol/10% acetic acid for at least 30 minutes. Following this, the gels were rinsed twice in 20% ethanol, for 10 minutes each wash, and then twice in water, for 10 minutes each wash. Subsequently the gels were sensitised by soaking each gel (one at a time) in 0.8mM sodium thiosulfate for 1 minute. The gels were washed again twice in water for 1 minute each wash and finally, transferred to 12mM silver nitrate solution in individual containers. The gels turned yellow at this stage, but this did not affect the final quality of the results. Silver nitrate impregnation of gels lasted from 20 minutes to 2 hours depending on the quality of the results. Using gloved hands rinsed with deionized water, the gels were pulled out one by one from the silver nitrate solution and dipped for 10 seconds in the water bath and finally transferred to the developer solution. A brown to grey precipitate developed within a few seconds of gels being dipped in the developer solution. This precipitate was re-dissolved by shaking of the developer-gel-containing box to avoid particulate surface background appearance on the gel surface. With this protocol, the most intense bands only took a few minutes to appear (depending on the temperature). When the adequate degree of staining had been achieved, the gels were transferred to the stop solution for at least 30 minutes. The stop solution was made on the day of use and contained 40g of Tris and 20ml of acetic acid per litre. Finally, the gels were washed in water (at least twice, for 30 minutes each wash) and imaged immediately afterwards to avoid dehydration of gels. The images were analysed by Syngene-GeneSnap 6 software.

2.6 MMP-1 gene knockdown

MMP-1 siRNA was used for MMP-1 gene knockdown according to the protocol previously described by our colleagues (Rogers, N.K. et al., 2014) and in SOP-THE021. This resulted in more than 75% reduction in MMP-1 protein expression

by ASM cells in cultures. Primary ASM cells were seeded at a cell density of approximately 20,000 cells/well on 6-well culture plates in 2mls of DMEM culture medium containing 10% FBS and incubated until approximately 50% confluent. ASM cells were then serum starved for 24 hours, followed by their transfection using (1µl/ml) INTERFERin® and (1nM) MMP-1 siRNA/scrambled negative siRNA duplexes, diluted to the final concentration of 0.2M in fortified cell culture medium Opti-MEM®. The plates were then incubated at 37°C in a CO_2 incubator for 24 hours until they were ready to be assayed for MMP-1 by ELISA. Approximately 78% reduction in total MMP-1 expression by primary ASM cells was achieved by MMP-1 siRNA gene knockdown (Figure 10).

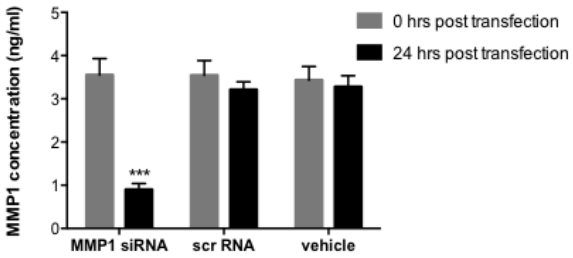


Figure 10. Suppression of MMP-1 protein by siRNA. ASM cells were transfected with MMP-1 targeting siRNA or scrRNA**.** The MMP-1 targeting siRNA decreased MMP-1 protein by 78%. Control siRNA and transfection reagent vehicle control had no effect on MMP-1 protein expression of ASM cells. **p<0.01, two-way analysis of variance. siRNA = small interfering RNA; scrRNA = scrambled control RNA.

2.7 Immunohistochemistry

2.7.1 Endobronchial tissue preservation

Endobronchial biopsies obtained from stable asthma study subjects were preserved in 4% formaldehyde solution and kept in the fridge at 4°C until they were ready to be embedded in paraffin blocks. Paraffin tissue embedding and serial tissue sectioning was kindly performed by our colleagues in clinical pathology, Nottingham University Hospitals NHS Trust, Nottingham, UK.

2.7.2 Quantification of mast cells and proliferating ASM cells

Endobronchial biopsies were immunostained as described (Markwick, L.J. et al., 2012), with images captured using a Digital Nanozoomer (Hamamatsu Photonics). Immunohistochemistry was performed on sections cut from formalin fixed tissue embedded in paraffin blocks. Slides were dewaxed first in Histoclear and rehydrated in a series of graded alcohol solutions (100%, 95%, 70%). The slides were then washed with PBS and water. Following this, slides were treated with 3% hydrogen peroxide solution for 15 minutes. To block non-specific binding sites, slides were first blocked in 2.5% horse serum for 30 minutes. Following this, slides were incubated with primary antibody overnight at 4°C in an airtight humidified chamber (see materials sections for antibody concentrations). The slides were then washed twice with PBS and subjected to the standard Vector VIP detection procedure according to the manufacturer's specifications (vectorlabs.com). The slides were then briefly washed in PBS and counterstained with haematoxylin for 30 seconds. After this, the slides were immersed in water at room temperature for 10 minutes, followed by a brief wash. Next, the slides were subjected to inverted alcohol gradient and washed with Histoclear®. The slides were then air dried, mounted and cover-slipped. Slides were allowed to set overnight prior to imaging and stored at 4°C in between imaging. ASM bundles were identified by immunostaining with anti α-SMA and ASM cell nuclei were counted using 4',6-diamidino-2-phenylindole (DAPI) staining. In adjacent serial sections, proliferating ASM and tryptase positive mast cells were identified by anti-Ki67 and anti-mast cell tryptase antibodies, respectively. Proliferating ASM and mast cells within ASM bundles were quantified per 100 ASM cells by an observer blinded to the clinical details. All slides were also assessed by a

respiratory pathologist.

2.8 Statistical analyses

Data are presented as mean (±SD/SEM) values for normally distributed data or as median (interquartile range) values for non-parametric data. Differences between groups were analysed by unpaired t-tests or Mann-Whitney tests. Multiple comparisons were made using 2-way analysis of variance (ANOVA). Tukey's post hoc test was used to confirm statistically significant differences between group means. Differences were considered significant for all statistical tests at p values less than 0.05. Correlations between data sets were examined using Pearson's correlation for normally distributed data and Spearman's rank correlation coefficient for nonparametric data. Some data was log-transformed and mean values calculated for presentation in data tables, however, where the original dataset was non-parametric in distribution, the Spearman's rank correlation is shown. Microsoft office Excel was used to record all study results. Statistical analyses were performed using GraphPad® Prism *7* for windows and mac (version *7*.01 and *7*.0a respectively, 1992-2016 GraphPad® Software, Inc. USA).

Chapter 3: Results
MMP-1 expression and activation in asthma

3.1 Introduction

Mast cells are usually only found adjacent to blood vessels in the lamina propria in healthy human airways. However, in asthma, they migrate into three key structures: the airway epithelium, the airway mucous glands and the ASM bundles. This anatomical relocation places mast cells deep within these functionally important airway elements, suggesting that the targeted delivery of mast cell mediators is likely to be central to the abnormal airway pathophysiology (Bradding, P., 2008).

ASM cells routinely express low levels of MMP-1 mRNA and protein (Rajah, R. et al., 1999). MMP-1 expression is induced following stimulation of ASM cells with collagen-1 (Schuliga, M. et al., 2010), cyclical strain (Hasaneen, N.A., 2005) and PDGF (Ito, I. et al., 2009). Despite functional proximity of ASM cells with ECM, previously only one study (Schuliga, M. et al., 2010) has investigated the regulatory effects of ECM on MMP-1 expression and induction by ASM cells. This showed that the ASM cultures maintained on fibrillar collagen had higher levels of MMP-1 and -14 mRNA than the cultures maintained on non- fibrillar collagen (gelatin) or plastic, without any apparent increase in cytotoxicity or apoptosis of ASM cells under all culture conditions. More recently my group has shown differential induction of MMP-1 in ASM cells by the ECM protein tenascin-C (also co-localised in the ASM bundles of patients with asthma) and this occurred partly through integrin mediated MAPK signalling. Additionally,

contraction of 3D collagen gel embedded ASM cells was reduced by the MMP inhibitor ilomastat and by MMP-1 gene knockdown, suggesting that ECM changes in airway remodelling via MMP-1 could contribute to an environment promoting greater airway narrowing in response to bronchoconstrictor stimuli and worsening asthma symptoms (Rogers, N.K. et al., 2014). It is important to mention here that most previous in vitro studies have only analysed a single measure of MMP-1 expression (the change in secreted protein levels) and also do not differentiate between latent and active forms of MMP-1, which unfortunately does not provide a full picture of MMP-1 regulation and activity in the diseased vs. healthy airway environment.

3.2 Hypotheses and aims

3.2.1 Hypotheses

1. Asthmatic airways in stable asthma patients would have increased MMP-1 expression in bronchial fluid when compared to healthy airways.

2. MMP-1 would be present in an inactive form in clinically stable asthma and may then be activated by asthma-related inflammatory products such as mast cell proteases.

3.2.2 Aims

To test these hypotheses, this chapter aims to:

1. Compare the basal total and active MMP-1 protein expression in bronchial fluid of asthma patients with their healthy counterparts.

2. Understand the activation of MMP-1 by asthma-relevant stimuli, in particular, most prominent mast cell mediators tryptase and chymase.

3.3 Results

3.3.1 MMP-1 is expressed in the airways of patients with asthma

Demographic details of the 16 asthma patients and 11 controls recruited in the stable asthma study have been shown in table 4 (chapter 5) and the patient recruitment protocol and inclusion/ exclusion criteria are explained in chapter 2. Briefly, individuals with stable asthma, defined by the GINA criteria and healthy controls were enrolled onto the study. Asthma patients underwent: spirometry (pre and post bronchodilator treatment), methacholine airway challenge test (as a measure of AHR and indirectly airway remodelling), FeNO (as a measure of airway inflammation) and Juniper ACQ'5 (as a measure of asthma severity and general disease control). All study subjects underwent bronchoscopy and had bronchial washings and a bronchial biopsy collected from the right bronchus intermedius. Bronchial biopsies were then used to grow primary ASM cells under culture conditions.

The total MMP-1 ELISA DuoSet (R&D Systems) assay was used to measure total basal MMP-1 protein levels in bronchial fluid samples from asthma patients and their healthy counterparts. MMP-1 ELISA measured total MMP-1 protein levels including active, inactive and TIMP- inhibited forms of MMP-1. The expression of total MMP-1 protein was 5.4 fold greater in bronchial washings from patients with asthma than those from controls (n=27, control mean 0.14 ng/ml, 95% C.I. 0.095-0.197, asthma 0.79, 0.35-1.21. p=0.002, Figure 11).

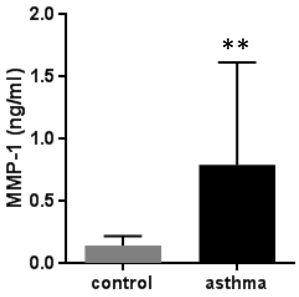


Figure 11. MMP -1 expression in stable asthma. Total MMP-1 protein measured by ELISA in bronchial washings from 16 patients with asthma and 11 healthy control subjects. Those with asthma have significantly higher MMP-1 levels. **p = 0.002, Analysis was conducted using the Mann-Whitney U test. Error bars indicate standard error mean (SEM). ELISA= Enzyme-linked immunosorbent assay.

Western blotting of these bronchial fluid samples identified a single 55 kDa band consistent with pro-MMP-1 in those with asthma but not in healthy controls. The 43 kDa active MMP-1 species was not detected in either group (Figure 12). This confirmed increased expression of mainly the pro-form of MMP-1 in bronchial wash samples from stable asthma patients, compared to healthy subjects, as the bronchial fluid samples from these showed virtually no MMP-1 expression at all.

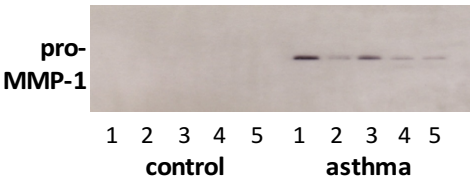


Figure 12. MMP -1 expression in stable asthma. Western blot of bronchial washings from five representative patients with asthma and five healthy control subjects. A single band of 55 kDa consistent with pro-MMP-1 is present in those with asthma but not detectable in healthy control subjects.

In keeping with the western blot analysis, MMP-1 activity was barely present in those with stable asthma and not detected in control subjects, when measured by MMP-1 Fluorokine activity assay (R&D Systems). Additionally, this assay also replicated ELISA findings as before, showing that the total MMP-1 protein levels were significantly elevated in those with asthma but low in healthy control

subjects (n=27, p= 0.01, Table 4, Figure 13).

Disease	n=number of patients	Severity (GINA)	Mean Total MMP- 1 (ng/ml)	Mean Active MMP-1 (ng/ml)
Healthy	11	Healthy	0.177 ± 0.024	0.000*
Asthma	9	Mild	0.528 ± 0.203	0.009 ± 0.005
Asthma	5	Moderate	1.481 ± 0.75	0.030 ± 0.016
Asthma	2	Severe	1.351 ± 0.08	0.027 ± 0.006

Table 4. MMP -1 expression and activity in stable asthma. Mean (±SEM) MMP-1 levels (total and active) in bronchial wash fluid samples measured with a Fluorokine MMP-1 activity assay. 0.000* value represents below detection threshold.

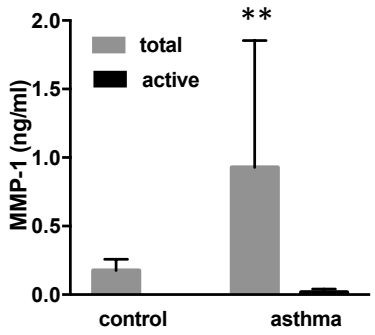


Figure 13. MMP -1 expression and activity in stable asthma. Total and active MMP-1 measured by Fluorokine activity assay in patients described in Figure 12. Total MMP-1 protein is low in healthy control subjects and significantly elevated in those with asthma **p = 0.01. MMP-1 activity is undetectable in control subjects and low in those with asthma. Two-way analysis of variance with Tukey's correction for multiple comparisons.

Primary ASM cells were cultured from endobronchial biopsies of six of the patients with asthma and six healthy controls. ASM cells were seeded on tissue culture plates at a cell density of 0.2x 10^5 per well (which was kept constant throughout the study experiments) and allowed to grow to 70% confluence. Total MMP-1 protein was 3.3 fold higher in ASM culture supernatants from those with asthma than from healthy controls (N=3, control mean 0.48 ng/ml, 95% C.I. 0.23-0.74, asthma 1.57, 0.30-1.23, p=0.002, Figure 14).

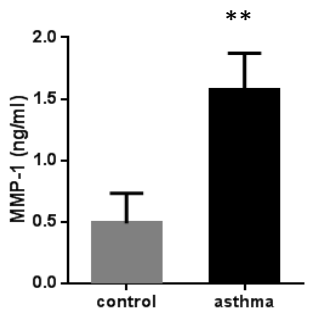


Figure 14. MMP -1 expression in asthma derived ASM cultures. Total MMP-1 protein measured by ELISA in cell culture supernatants from ASM cells grown from bronchial biopsies from patients with stable asthma and healthy control subjects. **p = 0.002, Analysis conducted using Mann-Whitney U test. Error bars indicate SEM.

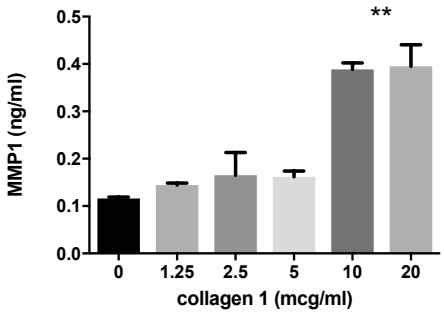
3.3.2 Effect of cell density on MMP-1 expression

It is important to note here that the basal MMP-1 levels secreted by ASM cells appear to be related to seeding cell densities, with a higher seeding density producing higher concentrations of total MMP-1 in culture supernatants, greater than would be expected from the increase in cell number alone. The effect of cell density and cell contact on MMP secretion by the cells has been noted in other studies as well, especially those investigating the role of MMPs in cancer cells (Bachmeier, B. et al., 2005). Cell density can influence binding of MMPs to the cell surface because MMP-binding sites, which are expressed on sparsely plated cells, are progressively lost as the cells approach confluence (Menashi, S. et al., 1998). In low cell density cultures, MMP-1 is retained at the cell surface by α2β1 integrin, but at higher densities, it is released into the culture supernatant (Dumin, J.A. et al., 2001).

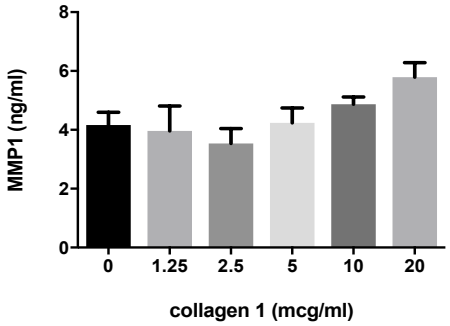
As mentioned before, MMP-1 expression is induced following stimulation of ASM cells with the ECM protein collagen-I (Schuliga, M. et al., 2010). I designed experiments to evaluate the effect of cell density on MMP-1 expression in a dose response manner, by using exogenous collagen-1 of human placental origin in increasing concentrations to treat primary ASM cell cultures for 24 hours under

serum free conditions in four different seeding cell densities (2,500 - 30,000 cells per cm^2). The cell culture supernatant was then harvested and total MMP-1 protein was assayed using MMP-1 DuoSet ELISA. Collagen - I treatment of ASM cells produced dose dependent increases in MMP-1 expression in ASM culture supernatants, when plated at low cell densities (2,500 - 5000 cells per cm^2). Almost maximum effect of collagen induced MMP-1 expression by ASM cells at lower cell densities seems to be produced by 10µg/ml of collagen-1, followed by a plateau effect with further increases, in the dose of collagen-1. At higher cell densities, basal MMP-1 levels in the ASM cell culture supernatant are generally quite high (10-40 ng/ml) and therefore the dose dependent effect of collagen is lost (Figure 15). As I was interested particularly in the effects of ECM proteins and other inflammatory mediators on MMP-1 expression, it was decided to use low seeding densities (2,500 - 5,000 per cm^2) for all ASM experiments, in order to minimise the effect of cell density or contact on MMP-1 induction and subsequent expression by ASM cells in culture under different conditions.

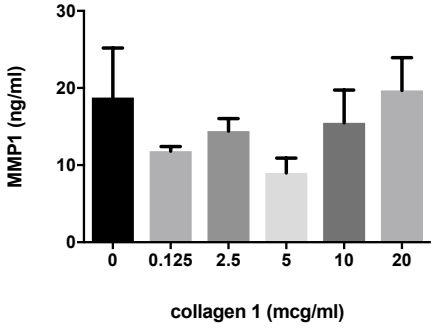
A) ASM cell density 2,500 cells per cm^2



B) ASM cell density 5,000 cells per cm^2



C) ASM cell density 15,000 cells per cm^2



D) ASM cell density 30,000 cells per cm^2

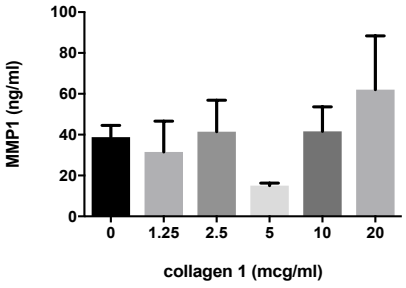


Figure 15. Dose-dependent effect of collagen on MMP-1 protein expression by ASM cells at different cell densities. Dose response effect of collagen-1 is only maintained at lower ASM cell densities of 2,500 – 5,000 per cm^2 or 10,000 - 20,000 cells per well (**$p<0.01$).

3.3.3 ASM derived MMP-1 is activated by mast cell derived tryptase

The supernatants of unstimulated primary ASM cell cultures from patients with stable asthma were examined by western blotting. This revealed a single band

consistent with pro-MMP-1 at 54kDa. Primary ASM cells in culture were then treated with the supernatants of activated or control mast cells from a HMC-1 cell line for 24 hours, before the culture media was harvested and analysed by western blotting. The full mast cell activation protocol has been explained in the methods chapter. Activated, but not control, mast cell supernatants resulted in the activation of MMP-1, identified by the appearance of the active 43 kDa MMP-1 species, which could be inhibited using a pan protease inhibitor (Figure 16).

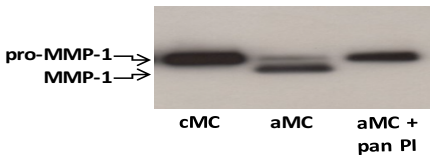


Figure 16. Mast cell products activate MMP-1. Western blot for MMP-1 in airway smooth muscle supernatants treated with control or activated mast cell supernatants. Activated mast cell supernatants cause MMP-1 activation shown by the appearance of the smaller MMP-1 band, which is blocked by a broad-spectrum protease inhibitor. aMC = activated mast cell supernatant; cMC = control mast cell supernatants; pan PI = broad-spectrum protease inhibitor.

To determine which protease was responsible for MMP-1 activation, more specific protease inhibitors were used. Generation of active MMP-1, detected by western blotting, was blocked by pan-serine and cysteine protease inhibitors, serine protease inhibitor, broad spectrum MMP inhibitor (ilomastat) and tryptase inhibitor (APC 366) specifically, but not by chymase inhibitor (chymostatin) (Figure 17A & 17B). It is important to mention here that activated or non-activated mast cell supernatants were mixed with protease inhibitors for 30 minutes prior to their use in ASM cell culture experiments. The concentrations of different protease inhibitors used were according to manufacturer's instructions and are explained in the methods.

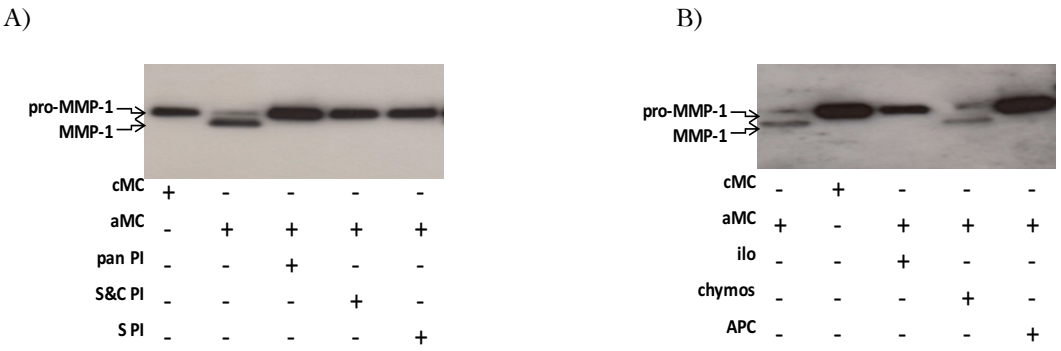


Figure 17. Mast cell tryptase activates MMP-1 (western blotting). (A) MMP-1 activation by activated mast cell supernatants is inhibited by inhibitors of pan proteases, serine and cysteine proteases and serine proteases. **(B)** MMP-1 activation by activated mast cell supernatants is inhibited by inhibitors of metalloproteinases and tryptase, but not chymase. aMC = activated mast cell supernatants; APC366 = Inhibitor of tryptase; chymos = Inhibitor of chymase; cMC = control mast cell supernatants; ilo = Inhibitor of metalloproteinases; pan PI = broad-spectrum protease inhibitor; S&C PI = Inhibitor of serine and cysteine proteases; SPI = Inhibitor of serine protease.

Next, to quantify MMP-1 activity in vitro, MMP activation by activated mast cell supernatants was also analysed with the Fluorokine MMP-1 activity assay. ASM cell cultures were treated with activated and control mast cell supernatants as before and incubated for 24 hours, after which the culture media was harvested and MMP-1 activity was examined by Fluorokine fluorescent activity assay. These experiments correspond to the experiments shown in Figures 17A & 17B. It should be noted that the Fluorokine assay is dependent upon MMP-1 mediated cleavage of its substrate. The presence of the pan protease inhibitor or the MMP inhibitor ilomastat may directly reduce the assay measurement independent of the activity of MMP-1 in the samples. The addition of activated mast cell supernatant to primary ASM cultures resulted in generation of the active MMP-1 species and was associated with an increase in MMP-1 activity. The enhancement was inhibited by the broad spectrum protease inhibitor and MMP inhibitor, which may also directly inhibit the assay reading and are therefore represented by two partially shaded (hatched) areas on the graph (Figure 18). Most importantly, the tryptase inhibitor APC 366 blocked MMP-1 activation, but the chymase inhibitor

chymostatin did not. These inhibitors do not interfere with the assay and confirm the earlier findings of the western blots.

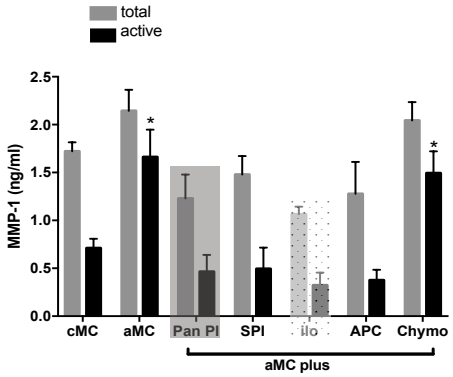


Figure 18. Mast cell tryptase activates MMP-1 (Flourokine activity assay). MMP-1 activity assay, showing total and active MMP-1 in ASM supernatants treated with control (cMC) or activated mast cell supernatants (aMC). Activated mast cell supernatants cause MMP-1 activation. MMP-1 activation is blocked by a broad spectrum protease inhibitor (pan PI), inhibitors of serine proteases (SPI), MMPs (ilo), tryptase (APC), but not chymase (chymo). *p = 0.05 vs cMC. Hatched bars represent values which are potentially affected by the presence of metalloproteinase inhibitors.

3.3.4 MMP-1 is directly cleaved by tryptase

To determine if mast cell tryptase activated proMMP-1 by direct or indirect cleavage, recombinant mast cell tryptase (0-0.2 IU) and pro-MMP-1 (10ng) were co-incubated at room temperature for 30 minutes. The resultant products were then analysed by SDS-PAGE gel electrophoresis and silver staining. Increasing concentrations of mast cell tryptase were associated with production of a cleaved MMP-1 protein at 43 kDa consistent with active MMP-1 (Figure 19).

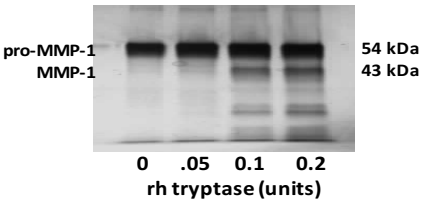


Figure 19. Mast cell tryptase directly activates MMP-1. Silver stain of SDS- PAGE gel electrophoresis showing tryptase cleaves pro-MMP-1 by direct proteolysis in vitro shown by the dose-dependent appearance of a 43-kDa band of active MMP-1. rh = recombinant human.

3.4 Chapter discussion

In summary, this chapter has demonstrated increased expression of pro MMP-1 in stable asthma and also enhanced expression of MMP-1 by asthma derived ASM cells in vitro. Furthermore, supernatants from activated (degranulated) mast cells activate ASM derived proMMP-1 in cultures. ProMMP-1 is activated by the mast cell protease tryptase, but not by chymase. Additionally, by direct interaction of the two proteins, mast cell tryptase directly cleaves the pro-domain of MMP-1 in vitro.

MMP-1 is a 54 kDa enzyme also known as interstitial collagenase, which is released by a wide variety of stromal cells such as fibroblasts, ASM and epithelial cells and is capable of digesting all major components of the ECM. Its main function is during tissue homoeostasis and wound repair (Martins, V.L. et al., 2012).

The observation of increased expression of total MMP-1 protein in bronchial fluid derived from asthma airways in this study is in keeping with a couple of earlier studies demonstrating an overexpression of MMP-1 mRNA in sputum samples and increased MMP-1 content in airways of patients with asthma, emphysema and current smokers when compared to non-smoker healthy controls (Cataldo, D.D. et al., 2004) (Dolhnikoff, M. et al., 2009) (Betsuyaku, T. et al., 2003). Asthma derived ASM in vitro also had higher basal expression of MMP-1 as compared to healthy ASM cells, and ASM derived MMP-1 facilitated the agonist induced ASM contraction in vitro (Rogers, N.K. et al., 2014).

The reason for MMP-1 mRNA over expression in bronchial secretions from asthma patients could be linked to the action of some pro-inflammatory cytokines

commonly encountered in the asthmatic airway environment, that are known to be able to up-regulate the MMP-1 gene expression in different cells. For instance, an increased mRNA expression for monocyte chemoattractant protein-1 (MCP-1) in sputum cells from patients with asthma was found to have a direct correlation with the ratio between MMP-1 and TIMP-1 mRNA. Furthermore, intensity of MMP-1 mRNA expression was inversely correlated with the FEV_1 in patients with asthma, indicating that MCP-1 may be partly responsible for an increased MMP-1 proteolytic activity and therefore participate in the inflammation and airway remodelling in asthma (Cataldo, D.D. et al., 2004). Additionally, it has been reported that MMP-1 mRNA expression of mononuclear phagocytes at various stages of differentiation is induced by Th2 cell membrane factors and IL-4 (Chizzolini, C. et al., 2000). In 3D collagen gels, human ASM cells increase MMP-1 expression and activity in response to their stimulation with cytokines such as TNF-α, IL-1β and IL-13 (Margulis, A. et al., 2009). Therefore, these mechanisms may be relevant to pathological conditions characterised by Th2 inflammatory responses such as asthma, resulting in MMP-1 mediated tissue destruction and remodelling.

MMP-1 protein levels were also higher in fibroblasts from asthmatic patients than those from healthy volunteers in a study by a Swedish research group, but this difference was not of statistical significance (Todorova, L. et al., 2010). The authors however acknowledged that only the level of MMP-1 protein, but not activity, was measured in the study, which may have pathological significance for asthma. Interstitial collagenase MMP-1 has a unique ability to cleave the native helix of fibrillar collagen, by hydrolysing a single bond located one quarter of the way from the collagen C terminus, followed by further degradation of these into

hydroxyproline molecules by other members of the MMP family. This suggests that MMP-1 may contribute to the dissolution of the main structural framework of the lung matrix, resulting in disorganisation of the lung architecture in asthma and other chronic lung diseases leading to increased disease related morbidity (Sweet, D.G., 2001). In another related study, patients with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) who were found to have elevated MMP-1 and/or MMP-3, had higher mortality and disease progression rates as compared to the group without detectable MMP-1 or MMP-3 (Fligiel, S.E.G. et al., 2006). The results in this chapter have shown that MMP-1 protein is not only increasingly expressed in bronchial fluid samples from asthma patients, but there is also enhanced MMP-1 activity in asthmatics as compared to healthy controls. Moreover, asthma derived primary ASM cells also show increased MMP-1 protein expression in culture supernatants, where it is present in an inactive form. From the extensive literature on MMPs, it is clear that in order to be able to degrade connective tissue elements, MMP-1 needs to be activated. Since the mechanisms of MMP activation in vivo are poorly understood and both circulatory MMP-1 protein levels and mast cell numbers seem to be elevated in the asthmatic airway environment, it made sense to examine the potential of mast cell proteases to activate the precursor forms of MMP-1. Activation of pro MMP-1 by mast cell proteases was first reported about 40 years ago (Birkedal-Hansen, H. et al., 1976). More recently, both the active and the precursor forms of MMP-1 were reported to bind to mast cell granules (Krejci, N. C. et al., 1992). In the airways, mast cells have been mostly shown to express the serine proteases tryptase and chymase. Both of these can degrade pericellular matrix proteins and components of the basement membrane, including collagen, laminin, fibronectin,

and elastin (Lohi, J. et al., 1992). These enzymes also serve as specific markers to differentiate mast cells from other inflammatory cells (Schwartz, L.B., 1994). Additionally, tryptase and chymase are thought to directly or indirectly activate pro-MMPs (more specifically tryptase activates pro–MMP-3 and chymase is known to activate both pro-MMP-1 and pro-MMP-3). MMP-3 in turn can activate other MMPs, including MMP-1, MMP-2, and MMP-9, leading to amplification of the cascade of matrix degradation and remodelling processes (Gruber, B.L. et al., 1989) (Lees, M. et al., 1994). In the past it has also been reported that human mast cell tryptase, present in rheumatoid synovium, can activate pro-MMP-1 in crude culture medium in vitro. However, the full activation of latent MMP-1 in this particular experimental model was dependent on proMMP-3 or stromelysin activation by tryptase, in a time and dose-dependent manner. MMP-1 in turn, activated other latent collagenases specifically MMP- 7 and -13. Once activated, these MMPs seemed to initiate a step-wise process of matrix degradation (Gruber, B.L. et al., 1989). A vascular study has also shown that the mast cell serine proteases such as trypsin, neutrophil elastase, tryptase and chymase, can indirectly cause MMP-1 activation and collagen type I proteolysis via matrix derived MMP-10, leading eventually to capillary tubular network collapse and regression in 3D collagen matrix models (Saunders, W.B. et al., 2005). Thus, it would appear that mast cell mediator release in vivo may contribute to localised matrix degradation, both by the stimulation of MMPs production by resident cells and by the subsequent activation of their precursors by mast cell serine proteases. The results presented in this chapter showed that the mast cell proteases released by activated mast cells in vitro were able to process and activate the precursor form of MMP-1. Furthermore, inhibition of MMP-1

activation by specific mast cell protease inhibitor APC366 (for tryptase), but not by chymostatin (for chymase), demonstrated that proMMP-1 was effectively activated to its active form of 43kDa by the tryptase but not the chymase component of mast cell derived proteases. Further investigation of the mechanism of proMMP-1 activation in vitro by tryptase was carried out by incubating recombinant human proMMP-1 (10ng) with various concentrations of recombinant human lung mast cell tryptase at room temperature for 30 minutes and by monitoring its digestion by SDS-PAGE electrophoresis and silver staining. This experimental design clearly demonstrated that recombinant tryptase, at concentrations of 0.1 IU and above, is able to process proMMP-1 into a form corresponding to the size of the active form of MMP-1, by direct proteolysis in the absence of any other activation factor. The pathophysiological relevance of this crucial finding of MMP-1 activation by serine protease tryptase through direct proteolysis emphasises the importance of this pathway and its subsequent participation in ECM homeostasis in asthma airways making it an attractive target for future development of selective MMP inhibitors as therapeutic compounds for chronic asthma.

In conclusion, the data presented here are consistent with the observations of others showing enhanced expression of MMP-1 in an inflammatory environment of asthma airways (Cataldo, D.D. et al., 2004) (Chizzolini, C. et al., 2000), and also in response to a number of mediators associated with asthma and airway remodelling including: collagen, tenascin C, cyclical strain, pro-inflammatory cytokines and serine proteases (Rajah, R. et al., 1996) (Ito, I. et al., 2009) (Schuliga, M. et al., 2010) (Margulis, A. et al., 2009) (Hasaneen, N.A. et al., 2005). Just like other MMPs, MMP-1 activation also requires proteolytic removal

of the pro-domain. Whilst MMP-1 activation by serine proteases has been illustrated before (Saunders, W.B. et al., 2005), the The current study is the first to have reported ability of serine protease tryptase to directly initiate MMP activation possibly leading to collagen and ECM proteolysis. It would appear that these pathways may overlap and cooperate functionally to regulate overall ECM turnover and therefore may be relevant to a wide variety of physiological and pathological conditions characterised by tissue remodelling, including asthma.

Chapter 4: Results
Effects of MMP-1 activation on ASM derived ECM

4.1 Introduction

The ECM is one of the main components of the airway cellular microenvironment. It is a highly dynamic and a remarkably versatile structure, continuously undergoing a remodelling process whereby ECM components are being deposited, degraded or modified. It performs many other functions in addition to its all important structural role. ECM dynamics are also imperative during restructuring of tissue architecture (Hynes, R.O., 2009)

The major process involved in ECM remodelling is cleavage of ECM components and this is important for regulating ECM turn over as well as for releasing biologically active molecules such as growth factors (Bonnans, C. et al., 2014). ECM remodelling is the result of multiple circumstantial processes that vary according to the initiating stimulus. Regardless of this, the key mechanistic features of matrix remodelling are common to most aetiologies and at the very minimum require two events: synthesis and deposition of ECM components and their proteolytic breakdown (Lu, P. et al., 2011). Numerous proteases have been implicated in the proteolytic degradation of the ECM and the most prominent among those are members of the MMP family (Stamenkovic, I., 2003). MMPs target a vast range of ECM and other extracellular proteins. For example, MMP-3 and -10 target proteoglycans, fibronectin and laminin. While MMP-1 prefers collagen III, MMP-8 and -13 selectively choose and target collagen I and II, respectively. Additionally, both MMP-2 and -9 degrade denatured collagen or

gelatin (Cawston, T.E. & Young, D.A., 2009). The components of the ECM in asthmatic airways can promote airway remodelling by directly affecting: cell growth and synthetic function (Johnson, S. & Knox, A., 1999) (Schuliga. M, et al., 2011), cell migration (Henderson, N. et al., 2007), adhesion, differentiation (Hynes, R.O., 2009), apoptosis (Freyer, A.M. et al., 2001), MMP activation (Rogers, N.K. et al., 2014) (Henderson, N. et al., 2007) and β-agonist signalling of ASM cells (Freyer, A.M. et al., 2004) and asthma phenotype (Ward, J.E. & Hirst, S.J., 2008).

4.2 Hypotheses and aims

4.2.1 Hypotheses

1. Mast cell induced MMP-1 activation is capable of processing ASM derived ECM, which may effect ASM proliferation.

2. MMP-1, a collagenase over-expressed in asthma, remodels airway ECM to support airway remodelling.

4.2.2 Aims

To test these hypotheses, this chapter aims to:

1. Determine the effect of MMP-1 activation on ASM-derived ECM and subsequent ASM cell behaviour.

2. To investigate if mast cell and MMP-1 interactions contribute to ASM cell proliferation, resulting in structural changes that constitute airway remodelling.

4.3 Results

4.3.1 Activated mast cell treatment of ASM cells generates a pro-proliferative ECM

Most MMPs can be activated by a variety of serine proteases and other MMPs in vitro; my work so far has clearly established that the mast cell protease tryptase

can specifically cleave and activate collagenase MMP-1 (results chapter 3). To determine if MMP-1 activation would lead to proteolytic modification of ASM-derived ECM and promote ASM growth, ASM-derived ECM preparations from three patients with stable asthma and controls were generated. The ECM preparation protocol is already explained in detail in the methods chapter. Briefly, primary ASM cells were grown on 6 well culture plates to 100% confluence to allow them to deposit ECM. The cultures were treated or untreated with activated or control mast cell supernatants for 24 hours, followed by removal of the top cell layer using ammonium hydroxide and DNase-1 solutions, which resulted in ECM substrates left on the culture plates . ASM cells were then seeded onto these substrates at a constant seeding cell density of $0.2x\ 10^5$ per well and cell proliferation measured 48 hours later with MTT reduction assay (as an arbitrary measure of ASM proliferation) and cell counts using a haemocytometer and trypan blue dye.

Results obtained using MTT reduction assay showed increased proliferation of both control and asthma-derived ASM cells on activated mast cell (aMC) treated ASM-derived matrices, while neither control mast cell supernatants (cMC) nor the activation vehicle alone (PMA and calcium ionophore directly added to mast cell supernatant without cells) had any effect on ASM proliferation ($p= 0.0001$, Figure 20).

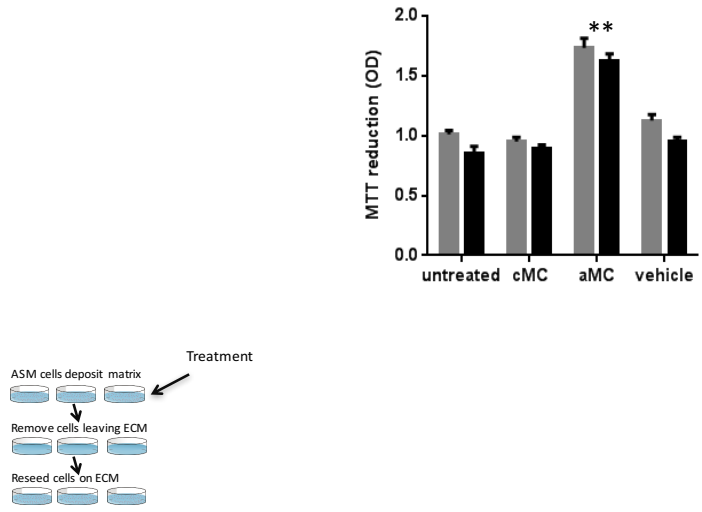


Figure 20. Activated mast cell treatment of ASM generates pro-proliferative ECM. ASM from patients with asthma or control subjects was treated with control or activated mast cell supernatants or mast cell activation vehicle during ECM deposition. ASM cells were removed from ECM preparations and a new generation of ASM cells seeded onto these and incubated for 48 hours in the presence of 1% serum. Cell proliferation was measured by MTT assay. ASM proliferation was enhanced by activated, but not control, mast cell treatment. **p= 0.0001, two-way analysis of variance. aMC = activated mast cell supernatants; cMC = control mast cell supernatants; vehicle = mast cell activation vehicle (IMDM with glutaMAX™ + 10% FBS and containing PMA and calcium ionophore, without mast cells); MTT = 3-(4,5-dimethylthiaaol-2-yl)-2,5-diphenyltetrazolium bromide; OD = Optical density.

Next, to confirm the pro-proliferative quality of activated mast cell remodelled ASM matrix, ASM cells were seeded onto ASM-derived ECM preparations from three patients with asthma and three controls (after ECM treatment with activated and control mast cell supernatants as before); ASM proliferation was then quantified by cell counting after 48 hours of incubation in the presence of 1% serum containing medium. Results obtained using cell counts were similar to those using MTT reduction assay. Activated mast cell-treated ASM cells produced a matrix that enhanced proliferation of control and asthma-derived ASM over 48 hours (mean difference for control cells 0.73, 95% CI of difference 0.52–0.93, $p < 0.001$; mean difference for asthma cells 0.78, 95% CI of difference 0.58–0.99, $p < 0.001$), as assessed by cell counting when compared with control mast cell supernatants (Figure 21).

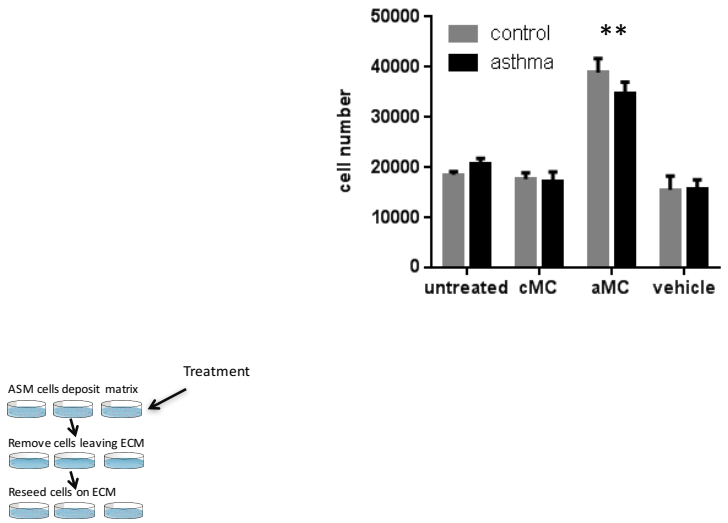


Figure 21. Activated mast cell treatment of ASM generates pro-proliferative ECM. ASM from patients with asthma or control subjects was treated with control or activated mast cell supernatants or mast cell activation vehicle during ECM deposition. ASM cells were removed from ECM preparations and a new generation of ASM cells seeded onto these and incubated for 48 hours in the presence of 1% serum. Cell proliferation was quantitated by cell counting. ASM cell proliferation was enhanced by activated, but not control, mast cell treatment. **p= 0.0001, two-way analysis of variance. aMC = activated mast cell supernatants; cMC = control mast cell supernatants.

Again, both control mast cell supernatants and the activation vehicle did not affect subsequent ASM proliferation on relevant ASM-derived conditioned matrices.

4.3.2 Pro-proliferative ECM is dependent upon tryptase activated MMP-1

In order to determine if the pro-proliferative ECM generated by activated mast cells was dependent on mast cell tryptase activation of MMP-1, a similar experimental model was used as before, except that the mast cell supernatants were pre-incubated with a range of protease inhibitors, prior to their use for the treatment of ASM cultures before derivation of ECM preparations.

The excess ASM proliferation (measured by MTT reduction assay), induced by activated mast cell treatment, was blocked by inhibitors of serine proteases, MMPs inhibitor ilomastat and specific tryptase inhibitor APC366, but not by chymase inhibitor chymostatin (p= 0.029, Figure 22), indicating tryptase mediated

MMP-1 activation as a likely mechanism responsible for ASM-derived ECM modification into a pro-proliferative subtype.

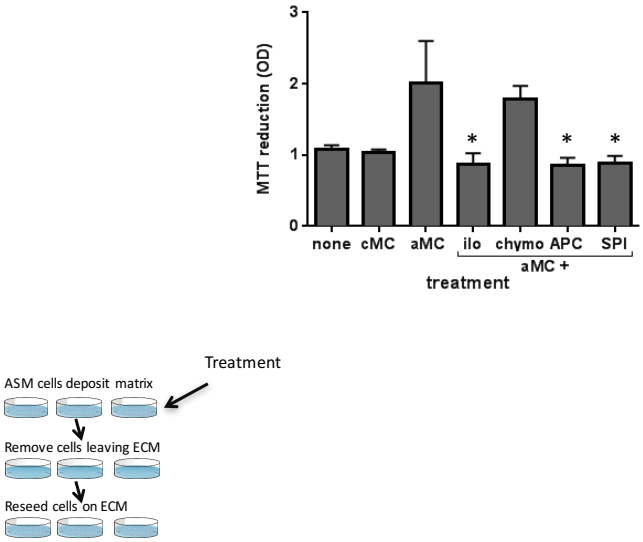


Figure 22. Pro-proliferative matrix is dependent on ASM derived MMP-1 (using protease inhibitors). ASM treated with control or activated mast cell supernatants during ECM deposition were co-incubated with an MMP inhibitor, chymase inhibitor, tryptase inhibitor, or a serine protease inhibitor. Enhanced ASM proliferation generated by aMC treatment was abrogated by inhibitors of MMPs, serine proteases and tryptase, but not chymase. *Difference from aMC, p= 0.029, Mann-Whitney U test. APC = Inhibitor of tryptase; chymo = chymostatin, inhibitor of chymase; ilo = ilomastat, inhibitor of MMPs; aMC = activated mast cell supernatants; cMC = control mast cell supernatants; SPI = Inhibitor of serine protease; MTT = 3-(4,5-dimethylthiaaol-2-yl)-2,5-diphenyltetrazolium bromide; OD = Optical density.

To confirm that MMP-1 was the responsible protease, ASM cell cultures were treated with siRNA targeting MMP-1 or control (scrambled) siRNA during ECM deposition. ECM preparations derived from these cultures were used to seed ASM cells under reduced serum conditions and their proliferation measured after 48 hours as before. MMP-1 siRNA (induced gene knockdown of MMP-1), but not control siRNA, blocked the excess ASM proliferation generated by activated mast cell-treated ECM substrate, as estimated by MTT reduction (p= 0.04, Figure 23).

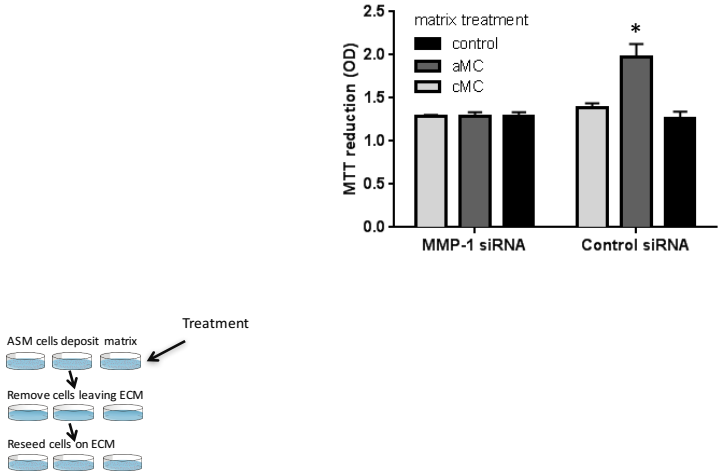


Figure 23. Pro-proliferative matrix is dependent on ASM derived MMP-1 (using siRNA). ECM preparations were generated by ASM cells treated by an MMP-1-specific or control siRNA. During ECM deposition, cells were treated with control or activated mast cell supernatants. The MMP-1-specific siRNA resulted gene knockdown abolished the enhanced proliferation generated by aMC treatment. *p= 0.04 Mann-Whitney U test. aMC = activated mast cell supernatants; cMC = control mast cell supernatants.

Further, direct application of activated recombinant MMP-1 at the concentration of 10ng/ml to untreated ASM derived ECM substrates prior to seeding and incubation of ASM cells onto them, significantly enhanced the proliferative capacity of both asthma and control ASM-derived ECM substrates (p= 0.013 and p= 0.0001, respectively).

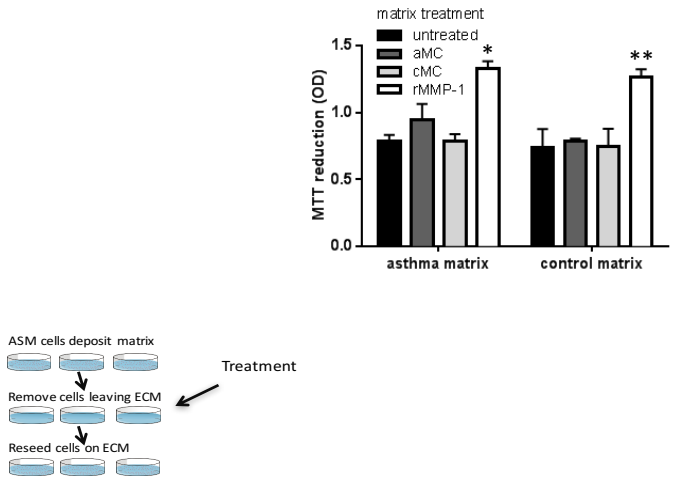


Figure 24. Pro-proliferative matrix is dependent on ASM derived MMP-1(using rMMP-1). Untreated ASM-derived ECM substrates from asthma or control ASM cells were either left alone or incubated with cMC, aMC or rMMP-1. ASM cells were then allowed to grow on these for 48 hours. Mast cell supernatants had no direct effect on the ECM in the absence of ASM but ECM-driven ASM proliferation was enhanced by direct application of rMMP-1. *p= 0.013, **p=0.0001 Mann-Whitney U test. rMMP-1 = recombinant active MMP-1.

In contrast, direct application of either activated mast cell supernatant or control mast cell supernatant to untreated ASM derived ECM substrates, had no effect on subsequent ASM proliferation. Therefore, this implies that MMP-1 expression by ASM cells and collagenase activity is primarily required for facilitation of ECM modulation and its subsequent effect on ASM cell growth (Figure 24).

4.3.3 ASM proliferation is a process that occurs over time

To confirm that the increase in ASM cell number was due to increased proliferation, rather than altered adhesion or cell viability, increased ASM cell number was measured over time from three asthma and control donors at 24 and 48 hours. Again, three separate cell donors were used to generate ECM preparations treated with activated or control mast cell supernatants. Cell number estimations by MTT reduction assay were quantified at 24 and 48 hours. Cell number determined by MTT reduction was increased at both time points on activated mast cell-treated ECM preparations when compared with control ($p = 0.043$, Figure 25).

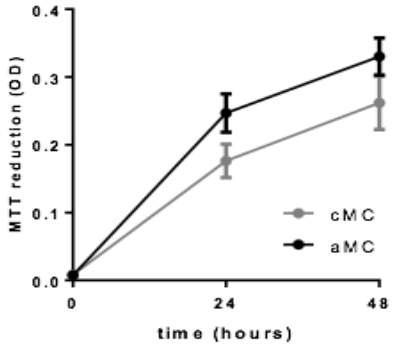


Figure 25. Time course of increase in ASM proliferation (MTT assay). Increase in cell number on ECM generated from primary ASM cells treated with control or activated mast cell supernatants. Graph shows mean (±SEM) of MTT reduction of three asthma and control ASM donors at 24 and 48 hours of incubation. aMC = activated mast cell supernatants; cMC = control mast cell supernatants.

Direct cell counting was also performed to ensure there was a true increase in cell number over the course of the experiment, using the same experimental protocol

as above. Here, equal numbers of ASM cells were seeded on control and activated mast cell-treated ECM substrates and growth arrested in the absence of serum for the first 24 hours. After this, which is time zero, 1% serum was added to the ASM culture medium and cells allowed to proliferate. Consistent with the MTT reduction experiments, after seeding, cell counts showed the initial number of cells to be equal. However, this was followed by a sequential increase in cell number over 24 and 48 hours which was greater on the activated mast cell-treated ECM substrate. Overall these results are consistent with ASM proliferation leading to the previously observed increase in cell number. (Figure 26).

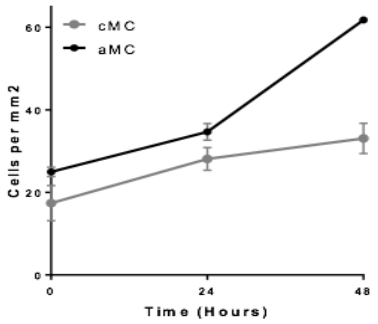


Figure 26. Time course of increase in ASM proliferation (cell counts). Increase in cell number on ECM generated from primary ASM cells treated with control or activated mast cell supernatants. Graph shows mean (±SEM) of cell counts of three ASM donors at 24 and 48 hours of incubation. aMC = activated mast cell supernatants; cMC = control mast cell supernatants.

4.4 Chapter discussion

In summary, this chapter demonstrates that activated mast cell-treated ASM cells produced a matrix substrate that enhanced proliferation of control and asthma-derived ASM in vitro and neither control mast cell supernatants nor the activation vehicle alone affected ASM proliferation. Moreover, pro-proliferative matrix generated by activated mast cells was dependent on mast cell tryptase mediated activation of MMP-1.

The universal properties of ECM proteins are their large and complex structure,

with multiple distinct domains. These domains are highly conserved. The conserved domains are arranged in specific juxtaposition with one another, occasionally controlled by highly regulated alternative splicing, suggesting that the specific domains and architectures of ECM proteins contain information of biological significance and evolutionary value, which may be important for tissue remodelling under disease conditions (Bonnans, C. et al., 2014).

Altered ECM protein deposition contributing to airway wall remodelling is a key feature of asthma and other airway diseases, such as COPD. The molecular mechanisms of this process are not yet well understood. One of the main pathological features of chronic asthma is thickening of the airway wall. This thickening is mostly the result of ASM cell hyperplasia and hypertrophy, as well as increased deposition of ECM proteins around the ASM bundles, eventually contributing to the fixed airflow obstruction and altered airway dynamics (Vignola, A.M., Kips, J. & Bousquet, J., 2000).

MMPs have long been thought to primarily degrade ECM proteins and their ECM substrate specificity has been extensively reviewed (McCawley, L.J. & Matrisian, L.M., 2001) (Egeblad, M. & Werb, Z., 2002). The effects of ECM degradation on cell behaviour are likely to be significantly more complex, due to a combination of changes in the nature of physical interactions between the cell and the degraded ECM proteins, and the release of a host of ECM-sequestered cytokines and growth factors (Cox, T.R. & Erler, J.T., 2011). Several ECM degradation proteins have been shown to display unique biological properties, partially due to exposure of new recognition sites for cell surface ECM receptors that can trigger a variety of cellular signals (Solomonov, I. et al., 2016). For instance, wound healing relies on a number of physiological processes including

cell migration, invasion and ECM degradation. In culture, epidermal keratinocytes involved in wound healing migrate on collagen-1 in a manner that requires specific cleavage of collagen-1 by MMP-1. MMP inhibitors can block this migration and epithelial cells that do not express MMP-1 do not migrate on collagen type I. Moreover, keratinocytes that bear a mutation in the MMP- 1 cleavage site do not migrate on collagen type I (Pilcher, B.K. et al., 1997). Similarly, the ECM protein collagen normally forms a triple helical structure, which when cleaved by collagenases (MMP-1 or MMP-8) exposes normally hidden Pro-Gly-Pro (PGP) strands, unmasking the pro-inflammatory properties of this structural protein (Weathington, N.M. et al., 2006). Normal cutaneous wound healing, as well as healing in essentially all tissues including human airways, involves the systematic development of events to re-establish the integrity of the injured tissue. The initial injury starts an orderly series of interdependent yet isolated responses that include: inflammation, cell proliferation, angiogenesis, ECM deposition and eventually resolution. During each stage in this process, proteases are needed to remove or remodel ECM components in both the cellular and interstitial compartments, thereby accommodating cell growth, migration and tissue repair and remodelling (Mignatti, P. et al., 1988). The interstitial matrix surrounds cells and is mainly composed of collagen and fibronectin, providing structural scaffolding for tissues such as ASM bundles. The composition and altered stiffness of the matrix in which ASM is embedded, promotes a more proliferative and pro-inflammatory ASM phenotype (Lauzon, A.-M. & Martin, J.G., 2016). Both matrix composition and altered deposition influence the programming of ASM cells and play a significant role in modulating histamine-induced calcium responses, and inflammation-induced VEGF secretion (pro-angiogenic functions)

in addition to cell proliferation. The evidence for this comes from in vitro studies, which demonstrate ASM cells cultured on collagen-conjugated polyacrylamide hydrogels of varying elastic moduli increase cellular secretion of VEGF and stimulate cell proliferation, whilst stiff gels reduce VEGF secretion and reduce agonist-induced calcium responses of ASM cells (Shkumatov, A. et al., 2015). In vivo, the amount of collagen III and laminin in the matrix surrounding ASM correlated with reversibility of the FEV_1 (Yick, C.Y. et al., 2012).

Interestingly, several serine proteases released by inflammatory cells, directly or indirectly target and degrade ECM proteins. For example, two plasminogen activators (urokinase and tissue plasminogen activator) target plasminogen to generate plasmin: a protein that degrades fibrin, fibronectin and laminin (Smith, H.W. & Marshall, C.J., 2010). Another serine protease elastase is released by neutrophils and supports the breakdown of fibronectin and elastin (Bonnefoy, A. & Legrand, C., 2000). In addition, serine cathepsins (cathepsins A and G) and cysteine cathepsins (cathepsins B and D) are found both extracellularly and intracellularly in lysosomes; secreted cathepsins degrade ECM proteins (mainly collagen and elastin) (Nomura, T. & Katunuma, N., 2005) (Fonovic, M. & Turk, B., 2014). Tryptase and chymase are the major serine neural peptidases stored and secreted by mast cells. Like other serine proteases, secreted tryptase and chymase support inflammation, matrix destruction and tissue remodelling. The mechanisms include: activating PAR receptors and MMPs, and destroying growth and differentiation factors. They also modulate immune responses by disintegrating chemokines and cytokines (Caughey, G.H., 2007).

Tryptase containing mast cells are the predominant mast cell type in the lung (Irani, A.A. et al., 1986). Tryptase is a tetrameric protease with a molecular size of

134 kDa, composed of four monomers of approximately 34 kDa and one catalytic site each (Schwartz, L.B., 2003). In mast cells, tryptase is present almost exclusively within the secretory granules in a form of heparin proteoglycan complex (Schwartz, L.B. & Bradford, T.R., 1986). The enzymatic activities of tryptase are several and include: the degradation of calcitonin gene-related peptide and airway bronchodilator neuropeptides like vasoactive intestinal peptide (VIP), inactivation of fibrinogen, activation of pro-stromelysins and cleavage of the ECM proteins gelatinase and fibronectin (Tam, E.K. & Caughey, G.H., 1990) (Cairns, J.A. & Walls, A.F., 1997) (Lohi, J., Kesksi-Oja, J. & Harvima, I., 1992). Several studies suggest that the architectural changes of airway remodelling, which may also be associated with some of the physiological disturbances affecting asthmatics, may be directly or indirectly influenced by tryptase. For example, β-like tryptase can directly provoke a variety of responses from fibroblasts and ASM including: proliferation by acting on PAR-2 receptors (Berger, P. et al., 2001), fibroblast chemotaxis and production of collagen (Gruber, B.L. et al., 1997) (Cairns, J.A. & Walls, A.F., 1997). Furthermore, another study revealed that tryptase is an ASM cell mitogen mediated via intracellular MAP kinase pathways (Brown, J.K. et al., 2001). Tryptase is also known to participate in a range of other inflammatory actions, such as being a stimulus for the degranulation of mast cells and eosinophils, and for cytokine release from various other cell types (Walls, A.F., 2000). These findings provide a strong link between mast cell derived products and features of human airway remodelling and AHR in vitro and collectively show that the mast cell tryptase has the potential to be a key mediator of disease progression in bronchial asthma. This is further supported by in vivo observations of others demonstrating tryptase release in asthmatic airways (Wenzel, S.E. et al.,

1988) and the effects of tryptase inhibition on airway remodelling in mouse allergic asthma models (Oh, S.W. et al., 2002). Consistent with these studies, the findings of this chapter show that mast cell tryptase is responsible for ASM-derived ECM degradation and re-conditioning leading to the generation of pro-proliferative matrix, which supports ASM proliferation. The increased ASM mass has been correlated to asthma severity (Benayoun, L. et al., 2003) and ASM cells are known to actively participate in the inflammatory and other remodelling processes by releasing pro-inflammatory cytokines, chemokines and ECM proteins (Panettieri, R.A., Jr., 2002) (Johnson, P.R., 2001) and therefore, may contribute to the pathogenesis of asthma. However, contrary to the direct mitogenic effects of tryptase on ASM cells observed by others, this study affirms an absolute requirement for ASM cell-derived MMP-1 in generation of the pro-proliferative ECM by tryptase and confirmed that the direct action of mast cell-derived tryptase is not sufficient to generate proliferative ECM in the absence of ASM-derived MMP-1.

The activation of latent collagenase MMP-1 by mast cell tryptase either by direct cleavage as shown in the previous chapter or secondary to activation of the pro-stromelysin (proMMP-3) as shown by others, can degrade various ECM proteins such as aggrecan, proteoglycans, fibronectin, laminin and collagen, thereby modulating ECM to support cell proliferation (Gruber, B.L. et al., 1989) (Whitelock, J.M. et al., 1996). The activation of proMMP-1 is therefore an important step in the initiation of matrix degradation and remodelling. Moreover, besides ECM proteins, MMP-1 can also directly activate or inactivate various bioactive proteins including, PAR-1, IGFBPs, CXCL8 and CXCL12. PAR-1 receptor activation by MMP-1 also provides an additional link between

extracellular proteolytic activity, important for ECM remodelling, with signalling leading to cell invasion and migration (Boire, A. et al., 2005). Similarly, activation of CXCL8 and CXCL12 by MMP-1 reveals an autologous cellular activation mechanism that acts in a feed-forward manner to coordinate the typical inflammatory responses characterised by cell proliferation, migration, ECM modulation and release of chemotactic factors (Tester, A.M. et al., 2007). MMP-1 induced cleavage of IGFBPs secreted by ASM cells, allows increased availability of IGF and synergism between IGF and inflammatory mediators (especially LTD4 and IL1-β) in mediating ASM cell proliferation and hyperplasia of long-standing asthma (Rajah, R. et al., 1995) (Vandenbroucke, R.E. et al., 2011). The MMP-1 mediated pro-proliferative effect of ECM on ASM growth can also be explained by the well-known fact that several ECM proteins such as proteoglycans and glycosaminoglycans (GAGs) undergo limited enzymatic cleavage by different families of proteases during ECM remodelling resulting in the release of growth factors and biologically active fragments such as matrikines and matricryptins. These low affinity ligands like tenascin epidermal growth factor (tEGF) repeats are encrypted within matrix components and modulate cellular responses mediated by growth factor receptors (Ricard-Blum, S. & Salza, R., 2014). The findings of this chapter are therefore in accord with these studies as well as others showing that the ECM functions as a ligand 'reservoir,' by binding numerous growth factors and biologically active molecules that can be proteolytically released via MMPs during the ECM remodelling process and can locally induce cell proliferation, migration and even regulate organ morphogenesis (Hynes, R.O., 2009). Another example of this mechanism come from a study showing VEGF abundance in the ECM in pancreatic neuro-endocrine tumours,

even in non-angiogenic islets. The switch from vascular dormancy to active angiogenesis in pancreatic tumours involves up-regulation of MMPs, which releases sequestered VEGF from the ECM (Bergers, G. et al., 2000), indicating that the ECM and the enzymes that modify it control the bioavailability of growth factors. Many other growth factors and cytokines released from the ECM, such as bFGF-1 and -2, PDGF, TGF-α1 and TGF-β, have the ability to contribute to enhanced ASM proliferation and airway remodelling (Stamatiou, R. et al., 2012). TGF-α1 and FGF-1 also enhance mRNA expression of collagen 1 and 3 in ASM cells, suggesting their additional role in the deposition of ECM proteins by ASM cells in the airways of patients with asthma (Parameswaran, K. et al., 2006). In addition to increased synthesis of ECM proteins, ASM cells are involved in regulation of MMPs and TIMPs, thus eventually contributing to the alteration in overall ECM environment. This signifies the importance of the intricate bi-directional relationship of ASM and ECM and their responses to stimuli such as chronic inflammation, contributing to the increased ASM thickness seen in patients with airway diseases (Burgstaller, G. et al., 2017).

The findings of this study, showing no overall difference in ASM proliferation in situ between those with asthma and control subjects, are consistent with the findings of Ward et al, indicating that the basal proliferation rate was similar in cultured ASM cells from non-asthmatics and patients with asthma. In addition, mitogenic responses to growth factors such as bFGF-2 and thrombin or serum were either decreased or unaffected in cells from patients with asthma. Despite this, the increased cytokine production by the myofibroblasts from patients with asthma, suggests that this contributes to and may sustain ongoing inflammation and related consequences in asthma (Ward, J.E. et al., 2008).

In conclusion, it is obvious from this and other studies, that ECM components are not only functionally diverse and able to affect a wide range of cellular activities, but also extremely dynamic and continuously undergoing remodelling processes. Together with the reciprocal nature of ECM-cell interactions, modification of ECM dynamics is an effective strategy for cells to respond to environmental changes and adjust their behaviours accordingly, in order to maintain tissue integrity and function. Furthermore, due to the crucial importance of the ECM during development and for the maintenance of tissue homeostasis, it is not surprising that dysregulation of ECM components can lead to disease processes characterised by abnormal ECM breakdown and deposition, a process primarily mediated by various proteases including MMPs. Therefore, the prospect of MMP-1 mediated mast cell-ASM interactions playing a role in asthma-associated airway remodelling, in the light of these findings, is significant and offers a new therapeutic avenue for more research in this area.

Chapter 5: Results
Mast cells and MMP-1 interactions and airway remodelling: the clinical implications

5.1 Introduction

The chronic airway inflammation in asthma is associated with AHR leading to recurrent episodes of wheezing, chest tightness, and coughing and is considered a hallmark of airway remodelling (Global Initiative for Asthma Report, 2004). Mast cells produce a range of pro-inflammatory mediators, cytokines and proteases, which are important in the development of fibrotic structural airway changes associated with airway remodelling and AHR (Pickholtz, E. et al., 2011) (Killeen, K. & Skora, E., 2013).

MMPs impact on the function and migration of inflammatory cells and also affect ECM deposition and degradation (Kelly, E.A. & Jarjour, N.N., 2003). Imbalance of the MMPs/TIMPs network and disproportionate secretion of numerous MMPs by stimulated structural or inflammatory cells such as mast cells, eosinophils and neutrophils, is thought to contribute to the pathophysiology of various lung diseases including asthma, COPD, IPF, tuberculosis and lung cancer (Li, J. et al., 2008). Furthermore, altered protease/antiprotease balance is associated with increased susceptibility to respiratory viral infections and asthma exacerbations (Meyer, M. & Jaspers, I., 2015). A number of different cytokines and growth factors involved in the inflammatory processes can interact directly with MMPs, leading either to a deregulation of their expression or changes in their biological activities by proteolytic cleavage. As a consequence, these mediators of

inflammation modulate secretion and activity of MMPs, implementing a complex network of reciprocal interactions. Every MMP seems to play a fairly specific role, with some variations of their expression commonly observed in different lung diseases (Gueders, M.M. et al., 2006). Many MMPs, including MMP-1, -3, and -8, have been implicated in mediating type I collagenolytic activity and can be detected by various methods in the sputum or BAL fluid from patients with asthma (Cataldo, D.D. et al., 2004) (Vandenbroucke, R.E. et al., 2011).

5.2 Hypotheses and aims

5.2.1 Hypotheses

1. In patients with stable asthma, MMP-1 protein levels are related to bronchial reactivity.

2. MMP-1 activation, which is facilitated by the mast cell protease tryptase in vitro, is enhanced during asthma exacerbations and overall MMP-1 activity may be associated with exacerbation severity.

3. Mast cell activated MMP-1 is able to generate pro-proliferative ASM matrix in vitro, therefore, in patients with asthma, mast cell numbers may be associated with airway smooth muscle growth within ASM bundles.

5.2.2 Aims

To test these hypotheses and to understand the relevance of in vitro findings related to MMP-1 activation by mast cell tryptase, leading to ASM derived matrix modulation in human asthma, MMP-1 activation was examined in BAL fluid of patients with asthma who developed exacerbations in response to rhinovirus inoculation. This chapter aims to:

1. Investigate the association between total MMP-1 protein levels and objective

measures of airway responsiveness in patients with stable asthma.

2. Establish the relationship between active MMP-1 levels and clinical parameters of severity of asthma exacerbations.

3. Investigate the fraternity between ASM proliferation markers and mast cell numbers in bronchial biopsies obtained from stable asthma patients and healthy controls.

5.3 Results

5.3.1 MMP-1 levels are associated with bronchial reactivity in stable asthma

The relationship between airway MMP-1 expression and airway contraction induced by methacholine was examined in the first group of 16 patients with asthma (stable asthma cohort). The baseline demographic and clinical characteristics of patients in this cohort are described below (Table 5 and 6).

Characteristics	**Asthma**	**Control**
n (number)	16	11
Age (years)	31 (9.4)	36 (10.2)
Male / Female	9 / 7	6 / 5
Disease duration (years)	16 (9.9)	N/A
Atopy (present/absent)	8 / 8	N/A
ACQ'5 score	2.28 (0.63)	N/A
BMI	25.1 (3.2)	24.9 (2.8)
ICS dose (mcg/day)	412 (296)	0

PC_{20} (mg/ml)		
0-5	2	
5-8	5	
8-16	9	
FEV_1 (% predicted)		
Pre bronchodilator	90.5 (9.5)	98.5 (7.5)
Post bronchodilator	92.5 (11.5)	99.0 (6.0)
FeNO (ppb)	49.6 (30.2)	

Table 5. Demographic and characteristics of patients in the stable asthma cohort. Values represent numbers in the group or mean (±SD) as appropriate. BMI = body mass index; ACQ'5 = Juniper 5 asthma control questionnaire; ICS = Inhaled corticosteroid; PC_{20} = Provocation concentration of methacholine (mg/ml) causing a 20% fall in FEV_1; FeNo= Fractional exhaled nitric oxide levels; ppb = Parts per billion; N/A = Not applicable.

Disease	Severity (GINA)	Atopy status	PC_{20} (mg/ml)	FeNO (ppb)	ICS(mcg /day)	ACQ'5 score
Asthma	Mild	yes	16	15	400	2
Asthma	Moderate	yes	8	59	800	1.8
Asthma	Moderate	no	8	16	400	1.6
Asthma	Mild	no	16	16	200	2.2
Asthma	Severe	no	4	75	200	3.6
Asthma	Severe	yes	2	18	1000	2.4
Asthma	Moderate	yes	8	15	400	3
Asthma	Mild	no	16	119	0	2.2
Asthma	Moderate	yes	6	86	800	1.8
Asthma	Mild	yes	16	51	800	1.4
Asthma	Mild	no	16	45	0	2.6
Asthma	Mild	yes	16	62	400	3.2
Asthma	Mild	no	16	38	400	2.6
Asthma	Mild	no	16	42	200	1.6
Asthma	Mild	yes	16	76	400	1.8
Asthma	Moderate	no	8	60	200	2.6

Table 6. Clinical markers of disease severity and airway inflammation in the stable asthma cohort. PC_{20} = Provocation concentration of methacholine (mg/ml) causing a 20% fall in FEV_1;

ACQ'5 = Juniper 5 asthma control score; ICS = Inhaled corticosteroid; FeNo= Fractional exhaled nitric oxide levels; ppb = Parts per billion.

Increasing airway responsiveness to methacholine was associated with increasing total airway MMP-1 levels, meaning the higher the dose of methacholine required to induce a 20% reduction in FEV_1 (PC_{20}), the lower the levels of total MMP-1 in bronchial fluid samples and vice versa (r2 = 0.256; p = 0.04) (Figure 27).

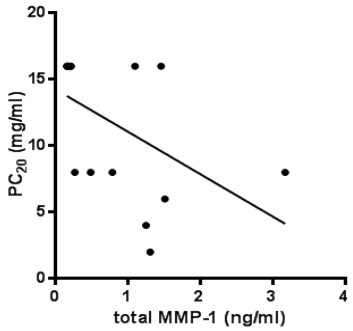


Figure 27. MMP-1 is associated with AHR in stable asthma. In patients with stable asthma, total MMP-1 protein in bronchial fluid samples was associated with enhanced sensitivity to methacholine in bronchial challenge testing. r2 = 0.26, p= 0.045. PC_{20} = Provocation concentration of methacholine (mg/ml) causing a 20% fall in FEV_1. The correlations shown are for subjects with asthma only and are non-parametric (Spearman's correlation).

In these stable asthma patients, modified ACQ'5 scores were not associated with total MMP-1 levels in bronchial fluid samples (r2 = 0.09; p = 0.23, Figure 28A). Studies show that, during inflammation, higher than normal levels of nitric oxide (NO) are released from epithelial cells of the bronchial wall. The concentration of NO in exhaled breath or FeNO, can therefore help identify airway inflammation and support a diagnosis of asthma when other objective evidence is deficient (van den Toorn, L.M. et al., 2001). In stable asthma, there was also no association between FeNO levels and total MMP-1 protein levels in bronchial fluid samples (r2= 0.003; p = 0.8175, Figure 28B). Similarly, there was no correlation between ICS dose and total MMP-1 levels in bronchial fluid in this cohort of stable asthma patients (r2= 0.005; p = 0.77, Figure 28C).

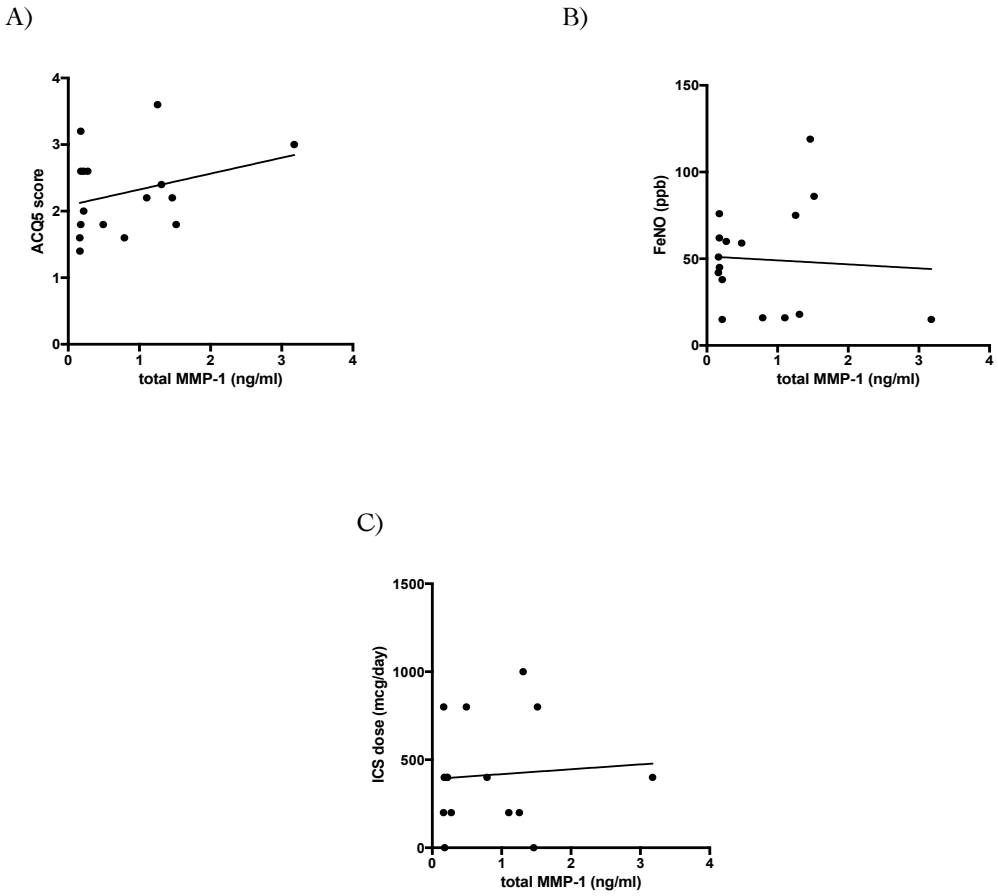


Figure 28. MMP-1 is not associated with symptom scores, FeNO levels and ICS dose in stable asthma. (A) In patients with stable asthma, total MMP-1 protein in bronchial fluid was not correlated with Juniper ACQ'5 scores. r2 = 0.09; P = 0.23. **(B)** In patients with stable asthma, total MMP-1 protein in bronchial fluid was not correlated with FeNO levels. r2 = 0.003; P = 0.8175. **(C)** In patients with stable asthma, total MMP-1 protein in bronchial fluid was not correlated with ICS dose. r2 = 0.005; P = 0.77. ACQ-5 = Modified Juniper asthma control questionnaire; FeNO = Fractional exhaled Nitric Oxide; ICS = Inhaled corticosteroids. The correlations shown are for subjects with asthma only and are non-parametric (Spearman's correlation).

5.3.2 Rhinovirus infection causes more severe LRT involvement and more significant falls in lung function from baseline

Increased levels of MMP-1 are present in asthma airways but mostly found in an inactive form under stable disease conditions (results chapter 3). Most MMPs are transcriptionally up-regulated and activated in response to pro-inflammatory cytokines (Hu, J. et al., 2007). To investigate if asthma exacerbations may increase MMP-1 expression and activation due to up surge of inflammatory

mediators, a second group of mild to moderate asthma patients and healthy control volunteers (asthma exacerbation cohort) underwent rhinovirus inoculation, using a human experimental model of rhinovirus infection to simulate asthma exacerbation. The patient recruitment, study procedures and samples collection for this work was all carried out at Imperial College London/St Mary's Hospital as reported before (Jackson, D.J. et al., 2014) and was a collaboration with Professor Sebastian Johnston's group. A detailed account of study protocols is given in the methods chapter. Briefly, 46 non atopic subjects (32 with asthma and 14 healthy) were inoculated with RV16. Seven subjects failed to develop infections and were excluded. There were no participant withdrawals and there was no requirement for systemic corticosteroids during this study. The baseline characteristics of study volunteers successfully inoculated with virus are shown here (Table 7) and have been reported previously (Jackson, D.J. et al., 2014) (Beale, J. et al., 2014).

Characteristics	Healthy (n = 11)	Asthma (n = 28)	p values
Age (years)	31 ± 12	36 ± 11	NS
Sex			
Female, n (%)	4 (36)	15 (54)	NS
Male, n (%)	7 (74)	13 (46)	NS
Baseline FEV_1, % predicted	104 ± 8	86 ± 12	<0.001
Baseline histamine PC_{20}, mg/ml	>16	1.26 ± 2.01	NS
ICS use, n (%)	N/A	15 (53.6)	NS
ICS daily dose (beclomethasone or equivalent), µg*	N/A	427 ± 71	NS
IgE, IU/ml, median (IQR)	16 (14–19)	139 (70–448)	<0.001
BAL fluid eosinophilia, %, median (IQR)	0 (0)	0.5 (0–1.7)	0.002

Table 7. Baseline characteristics of study volunteers in the asthma exacerbation study.
BAL = Bronchoalveolar lavage; ICS = Inhaled corticosteroid; IgE = Immunoglobulin E; IQR = Interquartile range; N/A = not applicable; NS = non specific. Data shown are mean (±SD) unless otherwise stated. *Mean (±SD) data of steroid-treated subjects, n = 15. p values represent comparison between the groups. Reproduced with permission of American Thoracic Society. Copyright © 2017 American Thoracic Society (Jackson, D.J. et al., 2014. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. American Journal of Respiratory and Critical Care Medicine, 190(12), pp.1373–1382) and Science Translational Medicine (copyright reference: 4215600311423).

Following inoculation with RV16, patients with asthma (11 subjects with mild, 17 with moderate disease) developed significantly greater upper and lower respiratory symptoms and reductions in PEFR and FEV_1(% change from baseline) as compared with 11 healthy subjects. These results have been reported as part of previously published work (Jackson, D.J. et al., 2014) and are reproduced here with copyright permission (Figure 29) to aid the further analyses of BAL samples from these study subjects for MMP-1 activation as a part of this thesis. It is important to note here that because the symptom scores were corrected for baseline and bronchoscopy related symptoms, a small number of subjects had a

negative score.

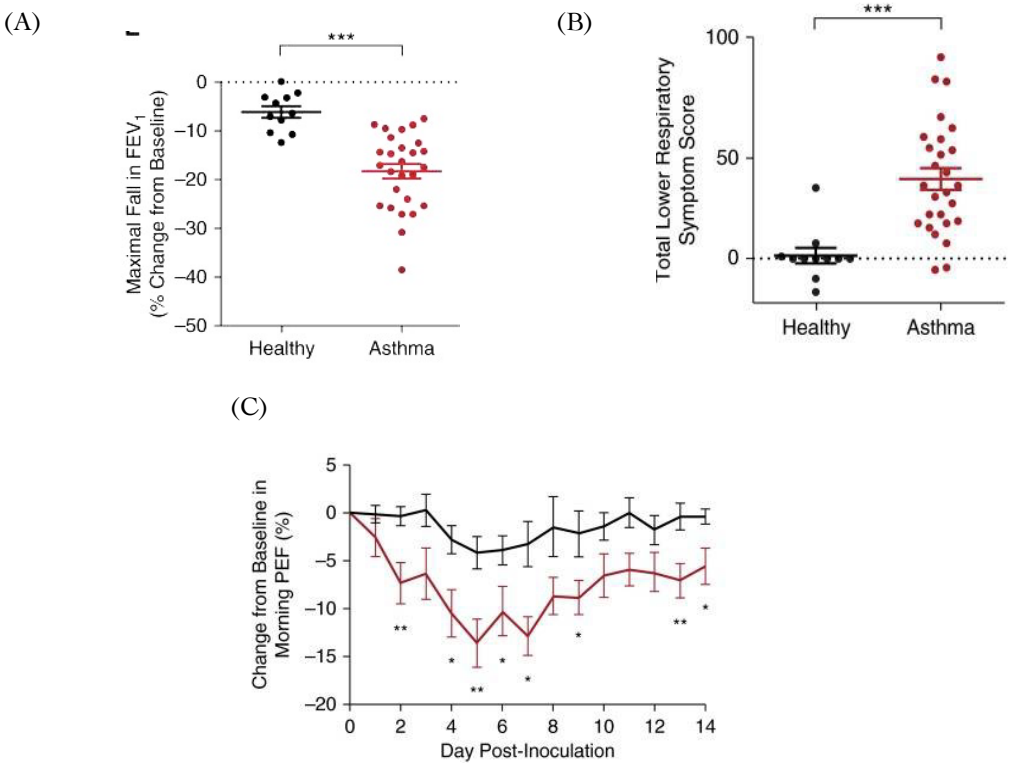


Figure 29. Rhinovirus infection results in more severe upper and lower respiratory tract involvement and increased falls in lung function from baseline. (A) The maximal decline in FEV_1 represents the maximal % change during the infection period for each subject. **(B)** The total LRT symptom score represents summation of daily scores over the 14-day post-inoculation period and represents the severity of the exacerbation. **(C)** Decreases in morning PEF are shown as % changes from baseline following rhinovirus inoculation. Results shown are mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. Reproduced with permission of American Thoracic Society. Copyright © 2017 American Thoracic Society (Jackson, D.J. et al., 2014. IL-33-dependent type 2 inflammation during rhinovirus-induced asthma exacerbations in vivo. American Journal of Respiratory and Critical Care Medicine, 190(12), pp.1373–1382).

5.3.3 Relationship between MMP-1 activation and asthma exacerbation severity

Study volunteers in the asthma exacerbation cohort underwent baseline sampling, including bronchoscopy and BAL collection 2–4 weeks before inoculation with RV16 (Jackson, D.J. et al., 2014) and a second bronchoscopy on Day 4 after inoculation. For measurement of MMP-1 activity, paired BAL samples were concentrated two fold using Vivaspin® 500 ultrafiltration spin columns with 10,000 molecular weight cut off membranes. MMP-1 activity was measured with the

commercially available fluorescent 'Human MMP-1 Fluorokine E activity assay' kit.

Post-viral inoculation, mean MMP-1 activity increased 3.9-fold (n=28. Difference 0.445, 95% CI of difference 0.052 to 0.84, p=0.03) in patients with asthma and 11-fold in healthy controls, although the latter was not statistically significant (n=11. Difference 0.14, 95% CI of difference -0.025 to 0.53. Table 8, Figure 30).

Disease	Severity (GINA)	Treat-ment	ACQ status	Mean total LRT scores	Mean max fall in FEV_1 %	Mean total MMP1 ng/ml (pre-viral)	Mean active MMP1 ng/ml (pre-viral)	Mean total MMP1 ng/ml (post-viral)	Mean active MMP1 ng/ml (post-viral)
Healthy	N/A	None	Healthy	1.45	-6.236	0.724 ± 0.12	0.018 ± 0.008	0.749 ± 0.14	0.196 ± 0.079
Asthma	Mild	SABA	Well/ Partial	23.7	-12.63	0.734 ± 0.48	0.122 ± 0.06	0.980 ± 0.57	0.474 ± 0.08
Asthma	Moderate	SABA/ ICS	Partial/ Poor	43.23	-20.99	0.872 ± 0.54	0.188 ± 0.03	1.475 ± 0.48	0.726 ± 0.32

Table 8. MMP-1 expression and activity in the asthma exacerbations (mean± SEM values). Total LRT symptom scores, maximum fall in FEV_1 from baseline, pre and post viral MMP-1 levels (total and active) in BAL fluid samples measured with the Fluorokine MMP-1 activity assay. ACQ= Juniper asthma control questionnaire; SABA= Short acting β2 agonist; ICS =Inhaled corticosteroids; N/A = not applicable; LRT = Lower respiratory tract; FEV_1 = Forced expiratory volume in I second.

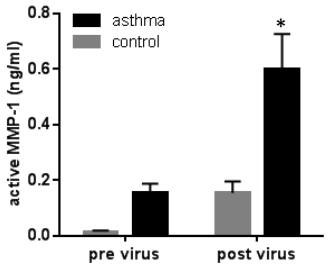


Figure 30. MMP-1 is activated during asthma exacerbations. MMP-1 activity in BAL fluid of healthy control subjects and patients with asthma was measured by MMP-1 fluorescent activity assay at baseline and 4 days post-inoculation with rhinovirus. MMP-1 activity was increased by viral infection and was higher in those with asthma at baseline and in the post-viral inoculation phase. *Pre-viral versus post-viral inoculation for asthma subjects, p< 0.05. Two-way analysis of variance with Tukey's correction for multiple comparisons.

Most importantly, the degree of MMP-1 activation post-inoculation in those with asthma was strongly correlated with more severe falls in FEV_1% from baseline (r2= 0.54; p<0.0001, Table 8, Figure 31A) and total LRT symptom scores (r2= 0.24; p= 0.007, Table 8, Figure 31B).

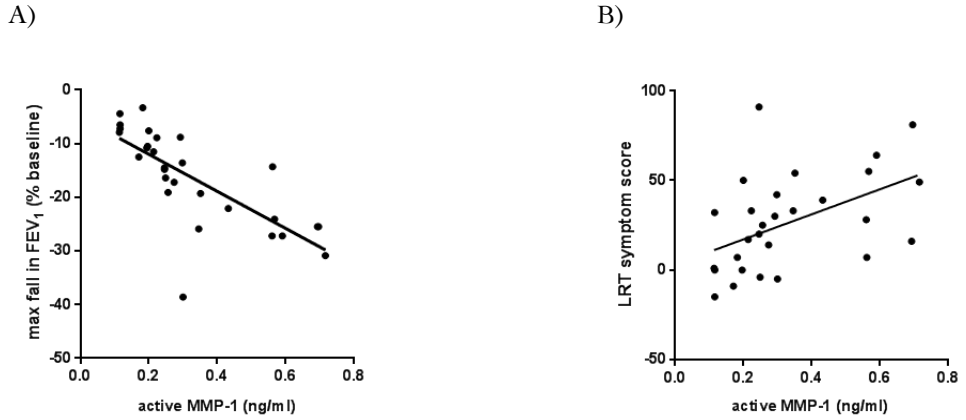
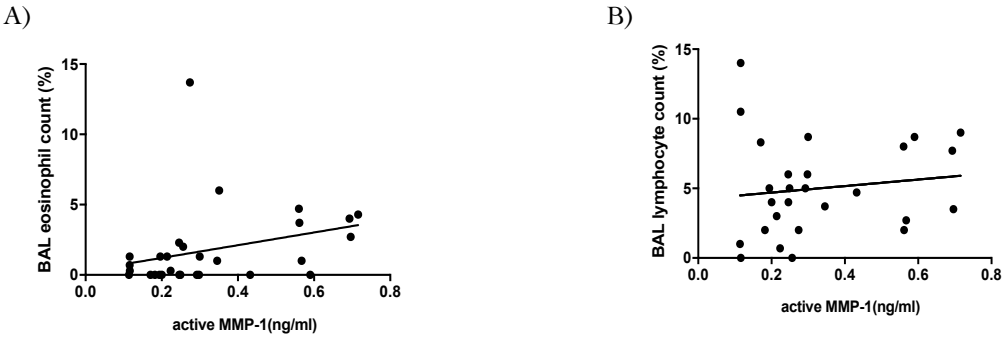


Figure 31. MMP-1 activation is associated with post-viral exacerbation FEV_1 and asthma symptoms during asthma exacerbations. (A) Post-rhinovirus-induced maximal fall in FEV_1 % from baseline in patients with asthma is correlated with the concentration of active MMP-1 in BAL fluid. r2= 0.55, p= 0.0001. **(B)** Increased LRT symptom scores during rhinovirus-induced exacerbations are associated with MMP-1 activation. r2= 0.24, p= 0.007. LRT = Lower respiratory tract. The correlations shown are for subjects with asthma only and are non-parametric (Spearman's correlation).

Interestingly, there was no association between post rhinovirus inflammatory cell counts and active MMP-1 levels in BAL fluid. Percentage proportions of: eosinophils (r2= 0.099; p= 0.0951, Figure 32A); lymphocytes (r2= 0.0005; p= 0.9041, Figure 32B) and neutrophils (r2= 5.038; p= 0.9708, Figure 32C).



C)

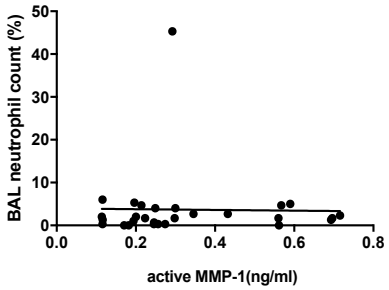


Figure 32. MMP-1 activation is not associated with post-viral exacerbation BAL inflammatory cell counts. (A) Post-rhinovirus eosinophil cell count (%proportion) in patients with asthma is not correlated with the concentration of active MMP-1 in BAL fluid. r2= 0.099, p= 0.0951. **(B)** Post-rhinovirus lymphocyte cell count (%proportion) in patients with asthma is not correlated with the concentration of active MMP-1 in BAL fluid. r2= 0.0005, p= 0.9041. **(C)** Post-rhinovirus neutrophil cell count (%proportion) in patients with asthma is not correlated with the concentration of active MMP-1 in BAL fluid. r2= 5.038, p= 0.9708. The correlations shown are for subjects with asthma only and are non-parametric (Spearman's correlation).

Quantitative determination of mast cell tryptase (MCT) was carried out in BAL samples of the asthma exacerbation study subjects using a ready-to-use Human MCT ELISA kit (Biocompare.com), which applied the competitive enzyme immunoassay technique, utilising a monoclonal anti-MCT antibody and a MCT-HRP conjugate. The intensity of colour at the end of the assay was measured spectrophotometrically at 450nm in a microplate reader and this was inversely proportional to the MCT concentration, since MCT from samples and MCT-HRP conjugate compete with each other for the anti-MCT antibody binding sites on the assay plate. The MCT concentration in each sample is interpolated from the calibration curve, relating the intensity of the colour (optical density) to the concentration of calibrators. Unfortunately, despite following the technique vigorously and concentrating BAL fluid samples x2, MCT was hardly detectable in the majority of BAL samples, both in pre and post virus inoculation phases. MCT levels were detected in only 6 out of the total 28 patients with asthma and were generally higher in the post-inoculation phase. MCT levels were not detected at all in samples from healthy volunteers.

Importantly, there was no statistically significant correlation between MCT levels and the degree of MMP-1 activation post-inoculation in those with asthma in whom MCT was present within detectable limits in BAL samples (r2= 0.53; p= 0.098, Figure 33).

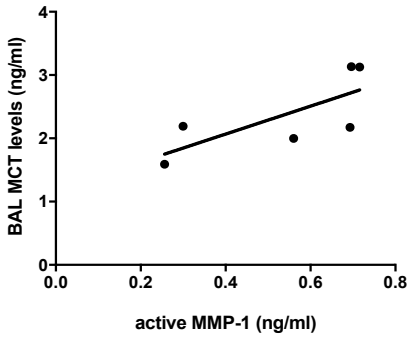


Figure 33. MMP-1 activation is not associated with post-viral exacerbation MCT levels. Post-rhinovirus MCT levels in patients with asthma are not statistically correlated with the concentration of active MMP-1 in BAL fluid. r2 = 0.53, p = 0.098. The correlations shown are for subjects with asthma only and are non-parametric (Spearman's correlation). MCT = Mast cell tryptase.

The lower detection of MCT in BAL samples may be due to low assay sensitivity and may have required switching to a more sensitive detection system (e.g. colorimetric to chemiluminescence or fluorescence) and even from direct to sandwich ELISA. This however was not practical due to limited sample volumes. Another possible reason could be sample incompatibility e.g. bronchial fluid vs. cell extract/serum as this assay has previously been verified for serum, plasma, cell culture supernatants and tissue homogenates, but not for bronchial fluid samples.

5.3.4 Association between ASM proliferation and mast cells in asthma

Having previously shown that the mast cell tryptase dependent MMP-1 activation leads to generation of pro-proliferative ECM, which in turns supports ASM proliferation in vitro, the association between tryptase positive mast cells and ASM proliferation in human airways was further assessed using bronchial biopsy

tissue samples from the stable asthma cohort of 16 asthma patients and 11 healthy controls (Table 5 & 6). Proliferating ASM cells and tryptase positive mast cells within ASM bundles were quantified by immunohistochemistry. Out of 16 asthmatic patients, 2 patients did not tolerate the bronchoscopy to the point of bronchial biopsy and consequently are not included in this analysis.

Endobronchial biopsies were embedded in paraffin blocks and serial tissue sections were cut. ASM bundles were formally identified in tissue sections by immunostaining slides with anti α-SMA antibody and subsequently ASM cell nuclei were counted using DAPI staining. In adjacent sections, proliferating ASM and tryptase-positive mast cells were identified by anti-Ki67 (as a cell proliferation marker) and anti-mast-cell tryptase antibodies, respectively. Images were captured using a Digital Nanozoomer© software (Figure 34).

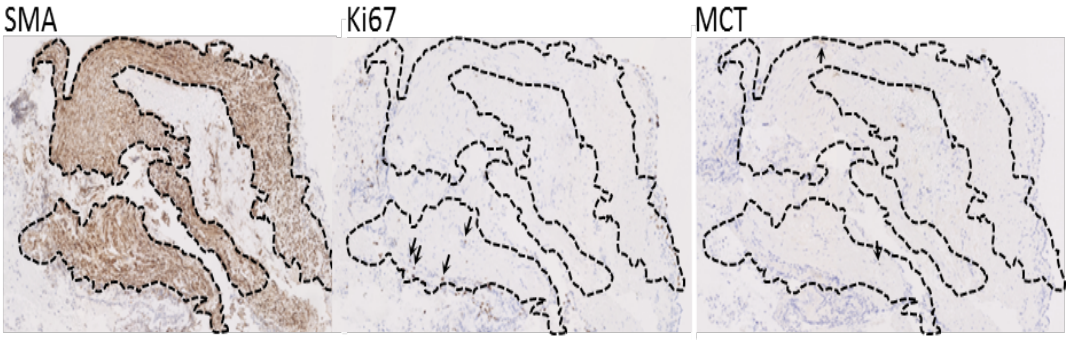


Figure 34. Mast cells are associated with ASM proliferation in stable asthma. Bronchial biopsies from patients with asthma, defined by the GINA criteria, were cut in serial sections, ASM bundles were identified by α-SMA staining and localised in consecutive slides. Proliferating ASM cells and mast cells identified by Ki67 and mast cell tryptase antibody immunostaining, respectively (arrows). MCT = Mast cell tryptase antibody; SMA = α-smooth muscle actin antibody; Ki67 = Ki67 protein (cellular marker of proliferation) antibody.

All tissue slides were also assessed by a respiratory pathologist to verify correct identification of ASM bundles. Proliferating Ki67 positive ASM cells and tryptase positive mast cells co-located within ASM bundles were quantified per every 100 ASM cells by an observer blinded to the clinical details.

In these tissue samples, both proliferating ASM cells and tryptase positive mast

cells tended to be more common in those with asthma, than without asthma. However, the differences between groups were not significant (p=0.41 for Ki67 positive ASM cells and p=0.19 for mast cell tryptase positive cells, Table 9, Figure 35A).

Disease	n	Severity (GINA)	Mean Ki67 positive cells per 100 ASM cells	Mean MCT positive cells per 100 ASM cells
Healthy	11	N/A	1.115 ± 0.358	1.145 ± 0.320
Asthma	8	Mild	2.446 ± 0.753	3.931 ± 1.286
Asthma	4	Moderate	0.677 ± 0.43	1.878 ± 0.840
Asthma	2	Severe	1.805 ± 0.01	2.715 ± 0.02

Table 9. Mast cells are associated with ASM proliferation in stable asthma (mean ± SEM values). The number of proliferating Ki67 positive ASM cells and mast cell tryptase (MCT) positive cells per every 100 ASM cells in endobronchial biopsies.

Across individuals, the number of proliferating ASM cells per every 100 ASM cells was positively correlated with mast cell infiltration in both those with and without asthma (r2=0.49; p<0.0001, Figure 35B).

A) B)

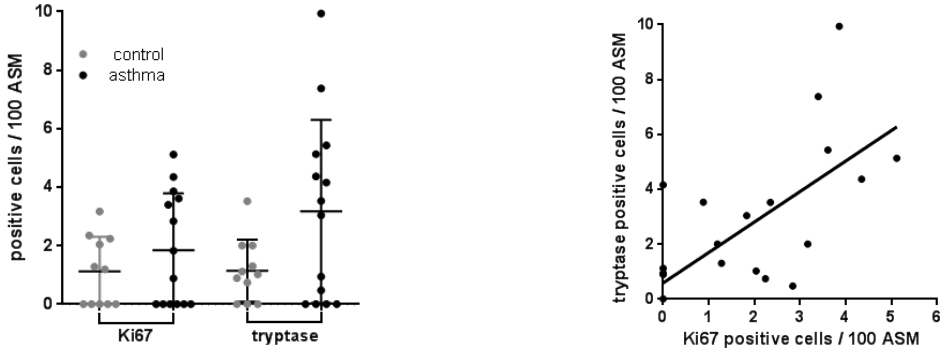


Figure 35. Mast cells are associated with ASM proliferation in stable asthma. (A) Ki67 and tryptase-positive cells in ASM bundles were expressed per 100 ASM cells in healthy control subjects and those with asthma. Those with asthma tended to have more proliferating ASM and mast cells. **(B)** Tryptase-positive mast cells are correlated with proliferating ASM cells. r2 = 0.5, p = 0.0001. The correlations shown are for all subjects and are non-parametric (Spearman's correlation).

5.4 Chapter discussion

In summary, this chapter demonstrates that in patients with stable asthma, MMP-1 expression is directly correlated with airway narrowing in response to a direct bronchoconstrictor stimulus and the presence of tryptase positive mast cells is associated with increased ASM proliferation within ASM bundles. Furthermore, during viral induced asthma exacerbations, MMP-1 activation is directly associated with deteriorating lung functions and worsening asthma related symptoms.

As mentioned before, expression and activity of MMP-1 has been shown to be of great importance for airway and lung health and disease. My work so far (results chapter 3) and that of others show that MMP-1 is minimally present in normal lung but it has robust expression in asthma (Rajah, R. et al., 1999) and many other pulmonary disorders such as emphysema or COPD (Segura-Valdez, L. et al., 2000) (Imai, K. et al., 2001). Furthermore, in BAL fluid and sputum cells from patients with asthma, MMP-1 mRNA transcripts have shown to be associated with airway obstruction, FEV_1 impairment and worse symptoms in asthma (Cataldo, D.D. et al., 2004) (Dolhnikoff, M. et al., 2009).

Enhanced MMP-1 expression by epithelial cells overlying intra-alveolar fibrosis has also been observed in BAL fluid samples from patients with pulmonary fibrosis and lung injury (Henry, M.T. et al., 2002). A cytokine TGF-β1 is reported to be increased in the airways of people with asthma and has been proven to induce the expression and activation of MMP-1 in different cell types (Ingram, J.L. et al., 2011). In mice (which lack the gene for MMP-1), the transgenic expression of human MMP-1 generates adult-onset emphysema associated with a loss of lung compliance (Foronjy. R. F, et al., 2003). Moreover, a couple of gene

studies of MMP-1 in human emphysema indicate enhanced expression of MMP-1 by GOLD (Global initiative for chronic obstructive lung disease) stage (Gosselink, J.V. et al., 2010) and correlation of MMP-1 polymorphism with disease severity (Joos, L., 2002). MMPs induced enzymatic degradation of the ECM surrounding the ASM can cause changes in the mechanics of bronchial constriction leading to AHR in asthma (Bramley, A.M. et al., 1995) and also account for the differing effects on the static mechanical properties of the lungs (Karlinsky, J.B. et al., 1976).

Bronchial provocation tests (BPTs) are commonly used in clinical practice to assess the presence of AHR to assist in making a diagnosis of asthma and in research to understand mechanisms of AHR and asthma symptoms (Lougheed, M.D. et al., 1993) (Lougheed, D.M. et al., 1995). Direct BPTs such as the methacholine challenge test refer to the use of a single non-selective muscarinic receptor agonist of the parasympathetic nervous system that acts directly on receptors on the ASM causing airway contraction and therefore is a highly sensitive test for AHR in the absence of the bronchoprotecting effect of deep inhalations (Cockcroft, D.W. & Hargreave, F.E., 1990). A positive response is documented as a 20% fall in FEV_1 and the severity of the response is determined by the dose of methacholine required to induce this positive response measured as PC_{20} (mg/ml). It is now well understood that AHR to direct stimuli have two components of the response that are considered "fixed" and "variable". The variable component is considered to be a rapid change, for example following inhalation of an allergen, possibly resulting from up-regulation of airway inflammation. The fixed component is thought to reflect more persistent and possibly permanent structural and functional changes in the airway that may or

may not be due to airway inflammation, and are commonly referred to as airway remodelling (Cockcroft, D.W., 2010). The findings of this chapter (highlighting a positive correlation between increasing airway responsiveness to methacholine and MMP-1 expression in bronchial fluid of stable asthma patients) therefore suggest that enhanced concentration and activity of MMP-1 in the airways is likely to be detrimental in the long term due to its effects on ECM degradation and remodelling mechanisms, which may in turn be responsible for gradual decline in lung function and AHR in chronic asthma. It is however, interesting to note that in one previous study, within a small cohort of stable mild-to-moderate asthma patients, despite a 3.7-fold increase in MMP-1 protein levels in fibroblasts from patients with asthma vs those from healthy volunteers, no significant correlation between MMP-1 production by airway fibroblasts and FEV_1% predicted or airway responsiveness to methacholine was noted (Todorova, L. et al., 2010). This contrast with the findings of this chapter is likely due to different methodology and populations analysed in both studies. Likewise, these discrepancies can possibly be attributed to real differences in MMPs amount and/or activity, depending on sample type, size or even disease severity. Furthermore, expression and activity of individual MMPs locally may vary in different airway compartments, adding to the complexity of airway inflammatory response (Katainen, E. et al., 2015) (Araujo, B.B. et al., 2008).

The pathogenesis of AHR involves the ultimate event of contact with the trigger, which initiates neurogenic mechanisms. These include: recruitment of neuropeptides, activation of MMPs and cytokine cascades, release of inflammatory mediators such as histamine, prostaglandins, serotonin, leukotrienes and reactive oxygen species, with subsequent increased expression of nuclear

transcription factors and simultaneous airway wall remodelling (Boushey, H.A., 1987). Additionally, AHR may be influenced by the changes in the mechanical (contractile) properties of the ASM (Antosova, M. et al., 2011). Abnormal deposition of ECM proteins has been illustrated in the submucosal and adventitial areas of large and small airways in asthma (Chakir, J. et al., 2003) (de Medeiros Matsushita, M. et al., 2005). Changes in the ECM composition within the ASM are known to affect its proliferative and contractile properties. For example, one study showed that, if the ECM associated with ASM is treated with collagenase, this results in increased force generation and shortening of ASM strips in vitro (Bramley, A.M. et al., 1995). In murine lung slices, when incubated with collagenase, acetylcholine induced airway contraction and contraction velocity were enhanced. This was attributed to collagen degradation and resultant reduction in airway wall stiffness (Khan, M.A. et al., 2010). These observations suggest that degradation of the ECM by collagenases such as MMP-1, may allow excessive bronchoconstriction by decreasing the resistive load the muscle must overcome to shorten in disorders like asthma. Also consistent with these findings are in vitro studies of ASM collagen gels, suggesting that MMP-1 can remodel the structure of collagen fibrils and contribute to ASM contraction in a non-superfluous manner (Ohta, Y. et al., 2008) (Rogers, N.K. et al., 2014). In addition to this and also conforming with the results of previous chapter, ECM substrates are known to differentially modulate both proliferation and contractile phenotype of human ASM cells in vitro (Hirst, S.J. et al., 2000). Both these features of airway remodelling (increased ASM proliferation and contraction) can also have varied effects on the loads opposing ASM shortening, with parallel consequences for AHR (Matsumoto, H. et al., 2007) (Bentley, J.K. & Hershenson, M.B., 2008).

This would therefore suggest that MMP-1 is a potential cause of AHR and its production and the resulting collagen redistribution affecting cell behaviour, with or without inflammation, may serve as a novel mechanism of airway remodelling in asthma.

Asthma exacerbations are exaggerated lower airway responses to environmental exposure and associated with the acute production of high levels of a number of endogenous inflammatory mediators and cytokines (Rennard, S.I., 2004). Rhinovirus infections are the most common trigger for asthma exacerbations. Research shows that rhinovirus infection leads to induction of IL-33 and type 2 cytokines IL-4, IL-5, and IL-13 in vivo in asthma and that the levels of these mediators are related to the severity of asthma exacerbations (Jackson, D.J. et al., 2014). Exacerbations likely lead to structural alterations of airway remodelling in the lung and possibly to permanently worsened irreversible airflow obstruction (Wark, P.A.B., 2006). Exacerbations promote airway remodelling by altering MMP mediated ECM homeostasis. There are plenty of exacerbation studies concerning MMP-9, verifying it as a mediator of airway inflammation and tissue remodelling and linking its activity with the pathophysiology of asthma exacerbations. A few studies however have also studied other MMPs, including MMP-2, -3, or -12. It is clear from these studies that MMPs are elevated in asthma exacerbations and genetic analysis shows evidence of at least one MMP gene (ADAM 33) closely associated with AHR and severity of asthma exacerbations (Holgate, S.T. et al., 2003). Consistent with these findings, the results presented in this chapter also show that the asthmatic airways harbour, not only increased levels of MMP-1, but also increased levels of MMP-1 activity, during an exacerbation. In addition, it was also demonstrated that MMP-1

activation is directly associated with exacerbation severity. It is important to note that substrate specificity of individual MMPs entirely enable their self-sustained work with the full range of ECM components. It is therefore reasonable to speculate that MMP-1 not only contributes to ECM breakdown, but is also capable of disrupting cell-cell/cell-matrix interactions, leading to the putative effects of airway inflammation. These effects, which are particularly important in an exacerbation setting, may include: airway epithelium detachment from basement membrane, degradation of components of vascular endothelium, cell migration due to the release of chemokines from ECM stores and degradation of ECM generating rather unconventional chemotactic fragments and growth factors leading to ASM hyperplasia (Kelly, E.A. & Jarjour, N.N., 2003). In this context, MMP-1 mediated ECM modification may be a potential target for the management of exacerbations of asthma and its role in asthma exacerbation related airway remodelling should be a subject of further research. The observation that exacerbation severity is significantly associated with increased MMP-1 activity adds further weight to the case that this pathway is functionally related to exacerbation pathogenesis.

Mast cells are important players in the pathophysiology of asthma. Although tryptase containing mast cells predominate in lung tissue (Andersson et al., 2009), it is their location in the ASM and airway epithelium that is thought to be of significant importance in AHR in asthma (Brightling et al., 2002) (Dougherty et al., 2010) (Berger et al., 1999). A few asthma studies have alluded to the fascinating finding of infiltration of ASM bundles by mast cells, sometimes even termed as 'mast cell myositis,' a consistent and perhaps unique feature of asthmatic bronchi (Brightling, C.E. et al., 2002). There is also evidence that the mast cells

within the ASM bundles in asthma demonstrate features of activation when examined under electron microscopy, appearing as partially or completely degranulated, a phenomenon not seen in ASM bundles from the control group (Begueret, H. et al., 2007) (Broide, D. et al.,1991). Mast cells localised within the ASM bundles are capable of releasing a plethora of pro-inflammatory mediators such as cytokines (including IL-4, IL-5, and IL-13) and other serine proteases, through their activation by IgE allergens and a variety of other non-immunological stimuli, thereby closely regulating eosinophilic inflammation in atopic asthma (Bradding, P. et al., 2006) (Cruse, G. et al., 2005). Mast-cell specific serine proteases can potentially induce bronchoconstriction, airway remodelling and AHR through a variety of mechanisms (Hauck, R.W. et al., 1999) (Tam, E.K. & Caughey, G.H., 1990) (Berger, P. et al., 2001). Several mast-cell products also have the ability to unfavourably affect the growth and function of ASM (Berger, P. et al., 1999) (Berger, P. et al., 2001) and their co-localisation within the ASM bundles would probably promote this interaction. For example, the mast-cell–derived mediators: histamine, PGD2 and the cysteinyl leukotrienes, are not only pivotal to the genesis of an inflammatory response (Krishnaswamy, G. et al., 2005), but also act as potent spasmogens of ASM (Page, S. et al., 2001). Similarly, tryptase not only plays a central role in inflammatory and allergic reactions, but is also an effective stimulator of DNA synthesis and cell proliferation in ASM cells (Berger, P., 2003). In another related study, K Sekizawa et al showed that purified mast cell tryptase added to isolated dog bronchi greatly enhanced responses to histamine and other bronchoconstriction agonists, while not affecting bronchial smooth muscle contraction itself (Sekizawa, K. et al., 1989). Furthermore, both animal and human mast cell tryptase potentiates contraction in sensitized bronchi

via a calcium-related mechanism (Johnson, P.R.A. et al., 1997) (Tanaka, R.D. et al., 2004). Chymase, another mast cell protease, has a profound effect on ASM cell functions and ASM-matrix interactions (Lazaar, A.L. et al., 2002). Infiltration of ASM bundles by mast cells is therefore an important determinant of the asthma phenotype and interactions between these two cell types promote ASM proliferation and differentiation into a more contractile phenotype (Bradding, P. & Holgate, S.T., 1996). On the other hand, ASM provide the right sort of microenvironment for the differentiation, activation and survival of mast cells (Page, S. et al., 2001). By using the specific marker of the asthma phenotype (resident mast cell numbers in ASM bundles), to test hypotheses set out in this chapter, an association of this was observed with ASM proliferation in the stable asthma cohort. This is in keeping with various other studies, which have demonstrated increased mast cell numbers in the airways of asthma patients and have found mast cell products such as histamine, PGD2, and tryptase in BAL fluid, both in symptomatic asthma and after allergen airway challenge, suggesting degranulation of mast cells (Wardlaw, A.J. et al., 1988) (Gibson, P.G. et al., 1993) (Zhang, M.-Q. & Timmerman, H., 1997). Moreover, mast cell infiltration of ASM has been reported in post-mortem studies of both fatal and non-fatal asthma (Carroll, N.G. et al., 2002) and also in isolated bronchial tissue sections from patients with asthma (Ammit, A.J. et al., 1997). Mast cell numbers in BAL fluid and airway biopsies were related to AHR in asthma studies (Gibson, P.G. et al., 1993) (Wardlaw, A.J. et al., 1988) (Brightling, C.E. et al., 2002). The findings of the presence of mast cells and their correlation with AHR have been confirmed by several independent studies as well (Begueret, H. et al., 2007) (Amin, K. et al., 2005) (Carroll, N.G. et al., 2002). It is important to note here that most

inflammatory mediators are rapidly inactivated after being released from the cell, so they only act across distances of a few micrometres. Therefore, co-localisation is a basic adaptive principle of inflammatory responses and is important in the development of tissue remodelling (Kaur, D. et al., 2012) (Brightling, C.E. et al., 2002). In conclusion, the widely accepted theory of infiltration of mast cells into ASM bundles being functionally important in asthma, is further supported by the observations of this chapter that the number of mast cells in the smooth muscle bundles of patients with asthma positively correlates with the proliferating ASM cells. This suggests that specific aspects of the asthma endotype drive ASM proliferation, rather than enhanced ASM proliferation being a feature of all patients with asthma at all times in the course of their disease. Moreover, these observations highlight a mechanism driving asthma severity that is mainly independent of Th2-mediated inflammation and is poorly responsive to current asthma therapies (Fahy, J.V., 2015).

Chapter 6: Results
Gelatinase expression and their functional significance in asthma

6.1 Introduction

As the gelatinase subgroup of MMPs, MMP-2 and MMP-9 are also called gelatinase A and B, respectively (Murphy, G. & Docherty, A.J.P., 1992). MMP-2 degrades: gelatin (type IV, V, VII, X, and XI collagens), fibronectin, elastin and laminin (Nagase, H., 1997). MMP-9 degrades: gelatin (type IV, V, VII, and X collagens), elastin, proteoglycan, entacin, fibronectin and laminin (Murphy, G. & Docherty, A.J.P., 1992) (Nagase, H., 1997). Like MMP-1, MMP-2 and -9 are also expressed at a low level in normal adult lung tissues, and their up-regulation appears to play an important role in the development of various pathological processes relevant to bronchial asthma including pulmonary inflammation, fibrosis and airway remodelling featuring sub-epithelial basement membrane thickening and hyperplasia of ASM (O'Connor, C.M. & Fitzgerald, M.X., 1994) (Kay, A.B., 1991). Like other MMPs, both MMP-2 and MMP-9 are also released in their pro/inactive form (Nagase, H. et al., 1991). MMP-2 can be activated by a MT-MMP–dependent pathway, which involves proMMP-2, TIMP-2 and MT-MMP-1. These three molecules form a complex on the cell surface that removes the pro-peptide from proMMP-2, thereby initiating a cascade of events that is completed by auto-proteolysis to yield the truncated active form of MMP-2 (Zucker, S. et al., 2004). MMP-9 is activated by proteolytic cleavage of its pro-peptide domain by a variety of serine proteases and other MMPs such as MMP-2 and MMP-3 (Nakamura, H. et al., 1998). Moreover, there is now growing evidence that free

radicals may directly activate MMP-9 in a non-proteolytic manner (Maeda, H. et al., 1998).

MMP- 2 is produced by tissue structural cells including fibroblasts, endothelial and epithelial cells, whereas MMP-9 is mainly produced by inflammatory cells such as macrophages, neutrophils and eosinophils (Murphy, G. & Docherty, A.J.P., 1992) (Hoshino, M. et al., 1998). Although many intrinsic lung cells are also capable of producing MMP-9, inflammatory cells are thought to be the primary source of MMP-9 in disease (Farhat, A.A. et al., 2014). Therefore, MMP-9 is considered to be the link between inflammation and remodelling processes in asthma.

Both MMP-2 and MMP-9 contain fibronectin type II - like repeats within their catalytic domains, which are responsible for the enzymes' high affinity for gelatin and elastin (Doyle, G.A.R., 1996). MMP-2 is secreted as a 72 kDa pro-form that is cleaved into a 64 kDa active form; the corresponding pro and active forms of MMP-9 have masses of 92kDa and 83kDa, respectively (Chakrabarti, S. & Patel, K.D., 2009). MMP-9 is mostly expressed by bronchial mucosa in all asthmatics and approximately half of COPD patients (Han, Z., Junxu & Zhong, N., 2003). It is found to be abundantly present in bronchial biopsies from asthma patients compared with normal subjects (Ohno, I. et al., 1997). In airways, under inflammatory conditions, MMP-9 transcription is enhanced by cells such as fibroblasts. On the other hand, the gene for MMP-2 lacks binding sites for pro-inflammatory transcription factors and as a result, most pro-inflammatory stimuli in isolation may not increase MMP-2 transcription (Frisch, S.M. & Morisaki, J.H., 1990). Normally the levels of the gelatinases return to baseline, once the inflammation is settled. However, several chronic pulmonary diseases such as

asthma and COPD are characterised by sustained, increased expression of MMP-2 and/or MMP-9 as well as altered expression of the TIMPs (Cataldo, D. et al., 2000). Although there are many reports of altered gelatinase expression in pulmonary pathologies, a detailed understanding of the role of gelatinases in disease pathology is still emerging.

6.2 Hypotheses and aims

6.2.1 Hypotheses

1. MMP-2 and -9 expression and activity are increased in bronchial fluid from asthma airways as compared to healthy controls.
2. Gelatinase expression is characterised by prominent neutrophil inflammation in BAL samples of patients with asthma exacerbation and associated with the severity of exacerbations.

6.2.2 Aims

To test our hypotheses and to understand the relevance of gelatinase expression in human asthma pathogenesis, MMP-2 and -9 protein levels and activity were measured both in bronchial wash fluid of patients with asthma in the stable asthma cohort and BAL fluid of patients with asthma who developed exacerbations in response to rhinovirus inoculation in the asthma exacerbation cohort . This chapter therefore aims to:

1. Compare MMP-2 and -9 protein expression in bronchial fluid of stable asthma patients with their healthy counterparts.
2. Investigate the effects of asthma exacerbation on MMP-2 and -9 protein expression and activity in paired BAL fluid samples.
3. Establish the correlation between gelatinase expression, differential BAL

inflammatory cell counts and exacerbation severity.

6.3 Results

6.3.1 MMP-9 expression is increased in the airways of patients with asthma

MMP-9 expression and activity were evaluated in bronchial fluid samples using gelatin zymography (a substrate specific, fairly sensitive and relatively inexpensive method for protein analysis). Gelatin zymography was performed on bronchial fluid samples from the stable asthma cohort and on paired BAL samples obtained from asthma exacerbation cohort, before and during induced viral exacerbations, to characterise the changes in airway gelatinolytic activity.

A unique property of MMP-9 is its ability to exist in monomeric (homodimer) and disulphide-bonded dimeric (heterodimer) forms; the latter involves the C-terminal hemopexin-like domain of the enzyme. The homo- and many of the hetero-dimer forms are reduction-sensitive and can be easily detected in the SDS-PAGE electrophoresis system under non-reducing conditions (Goldberg, G.I. et al., 1992). One of the commonly identified MMP-9 heterodimers is neutrophil gelatinase-associated lipocalin (NGAL), as seen in zymogram gels here in Figure 36A. It is secreted as a 25kD protein, which can bind to proMMP-9 via a covalent bond, to form a 130kDa disulphide-linked heterodimer. NGAL is also mainly expressed by activated epithelial and inflammatory cells such as neutrophils and monocytes (Bouchet, S. & Bauvois, B., 2014). It is important to note that pro MMP-9 levels in this chapter represent the measurements of both NGAL - MMP-9 complex (referred to as MMP-9 heterodimer) and proMMP-9 proteins. The densitometry of both MMP-9 heterodimer complex (130kDa) and proMMP-9 (92kDa) bands was measured and expressed as arbitrary units (AU). A mean total

value was then calculated to represent total pro MMP-9 expression in bronchial fluid and BAL samples.

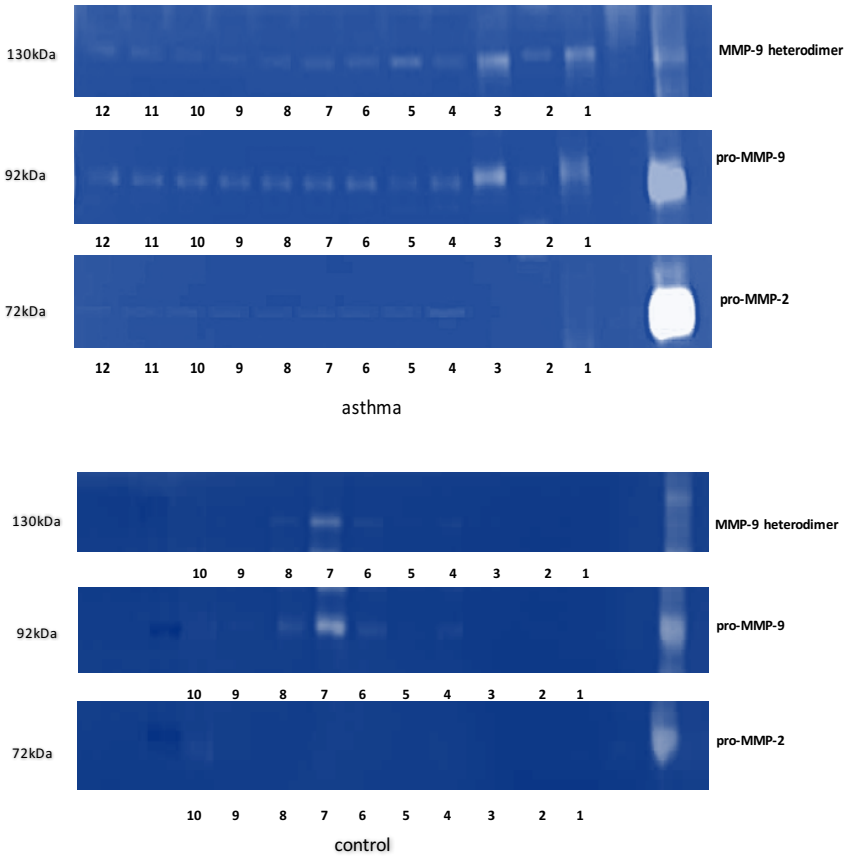
First, bronchial wash fluid samples (from 12 patients with asthma and 10 healthy controls in the stable asthma cohort) were analysed by gelatin zymography, to quantify the presence of both active and latent forms of MMP-2 and MMP-9 in the bronchial secretions (Table 10, Figure 36).

Disease	**n**	**Severity (GINA)**	**Mean pro MMP9 (AU)**	**Mean pro MMP2 (AU)**
Healthy	10	N/A	18.24 ± 7.814	Not detected
Asthma	7	Mild	45.68 ± 1.443	11.57± 5.72
Asthma	4	Moderate	52.49 ± 13.34	9.19 ± 3.86
Asthma	1	Severe	59.61 ± 0.04	Not detected

Table 10. Gelatinase expression in stable asthma (mean ± SEM values). Pro MMP-9 and pro MMP-2 values measured in bronchial wash fluid by gelatin zymography. AU = Arbitrary units.

These results showed that the expression of pro MMP-9 protein was 2.7 fold greater in bronchial washings from patients with stable asthma than those from healthy controls (n=22, control mean 18.24, 95% C.I. 0.5636 to 35.92; asthma mean 49.11, 95%C.I. 39.61 to 58.61, p=0.001, Figure 36B). There was no significant MMP-2 expression seen in healthy controls or patients with asthma in the stable asthma cohort. None of the activated 82 kDa (MMP-9) and 62 kDa (MMP-2) species was present in the bronchial fluid samples of patients with asthma or healthy controls.

A) Representative zymogram gels for gelatinase expression (stable asthma cohort)



B) MMP-9 expression in stable asthma cohort

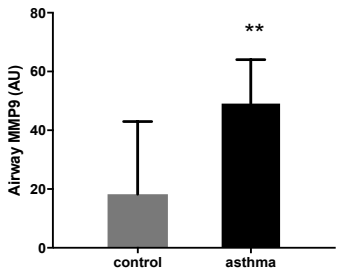


Figure 36. MMP-9 expression in stable asthma. (A) Representative zymogram gels showing proMMP-2 and -9 expressions in patients with stable asthma and healthy controls (Table 10) **(B)** MMP-9 protein is low in healthy control subjects and significantly elevated in those with stable asthma **p= 0.001. No statistically significant MMP-2 expression was seen in healthy controls or patients with asthma in stable asthma cohort. Mann-Whitney U test. AU = Arbitrary units.

Next, paired BAL fluid samples from the asthma exacerbation cohort were analysed by gelatin zymography to quantify the presence of both latent and active forms of MMP-2 and MMP-9. This group comprised of 19 patients with mild to

moderate asthma who had an asthma exacerbation induced by rhinovirus inoculation and 11 healthy control subjects. The study recruitment has been described in detail previously in chapters 2 and 5 (Jackson, D.J. et al., 2014). For measurement of gelatinase expression by zymography, BAL fluid samples were concentrated two fold using Vivaspin® 500 ultrafiltration spin columns with 10,000 molecular weight cut off membranes.

Pre-virus inoculation basal pro MMP-9 expression was higher in the asthma group as compared to healthy controls (n= 30, control mean 6.7, 95% C.I. -0.3484 to 13.75; asthma mean 26.15, 95% C.I. 16.61 to 35.69, p=0.01) (Table 11, Figure 37). After viral inoculation, pro MMP-9 expression increased 1.7-fold (n=19, difference 30.54, 95% C.I. of difference 17.89 to 43.20, p=0.002) in patients with asthma and 3.4-fold in healthy controls (n=11, difference 20.14, 95% C.I. of difference 7.49 to 32.78, p=0.02, Figure 37). The activated 82 kDa species was not detected in patients with asthma, or healthy controls, pre or post-viral exacerbation.

Disease	Severity (GINA)	n	Treatment	ACQ status	Mean proMMP9 pre-viral (AU)	Mean proMMP9 post-viral (AU)
Healthy	N/A	11	None	Healthy	6.7 ± 3.16	15.91± 2.78
Asthma	Mild	8	SABA	Well/Partial	25.46 ± 6.97	54.53 ± 11.16
Asthma	Moderate	11	SABA/ICS	Partial/Poor	26.65 ± 6.26	37.71 ± 6.64

Table 11. MMP-9 expression in asthma exacerbations (mean ± SEM values). ProMMP-9 values measured in BAL by gelatin zymography, 14 days before (pre-viral) and 4 days post-viral inoculation in asthma exacerbation cohort. SABA = short acting β2 agonist; ICS = Inhaled corticosteroids; AU = Arbitrary Units.

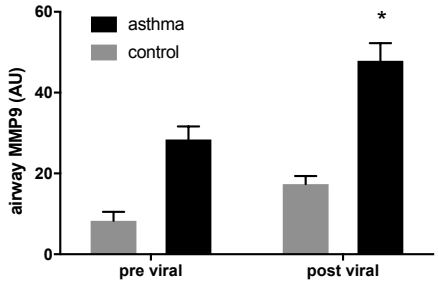


Figure 37. MMP-9 expression in asthma exacerbations. ProMMP-9 expression in BAL fluid of patients with asthma, measured by gelatin zymography, 14 days before and 4 days post-viral inoculation in asthma exacerbation cohort (Table 11). ProMMP-9 expression was increased by viral infection and was higher in those with asthma at baseline and in the post-viral inoculation phase. *Pre-viral versus post-viral inoculation for asthma subjects $p < 0.05$. Two-way analysis of variance with Tukey's correction for multiple comparisons.

6.3.2 Relationship between MMP-9 expression and asthma exacerbations

The relationship between asthma exacerbations and MMP-9 expression was examined in the asthma exacerbation cohort of 19 mild to moderate asthma patients, who had asthma exacerbations induced by rhinovirus inoculation, compared with 11 healthy control subjects as before. All asthma patients and healthy volunteers underwent bronchoscopy, spirometry and daily respiratory symptoms diary cards before and after viral inoculation. Nasal washes were collected at each visit and evaluated using quantitative polymerase chain (PCR) reaction to measure viral load. All this data was available to us through collaboration with Imperial College, London (Jackson, D.J. et al., 2014). The statistical relationship between MMP-9 expression and laboratory/clinical parameters of asthma exacerbation was analysed using logistic regression with Spearman's correlation coefficient.

6.3.2.1 Relationship between MMP-9 expression and bronchial fluid inflammatory cells during asthma exacerbations

MMP-9 expression in both asthma and healthy groups pre and post-viral exacerbation was significantly correlated with percentage proportions of

eosinophils in BAL fluid (pre-viral % proportion of eosinophils r2= 0.14; p= 0.04, Figure 38A and post-viral % proportion of eosinophils r2= 0.256; p= 0.004. Figure 38B, respectively).

A) B)

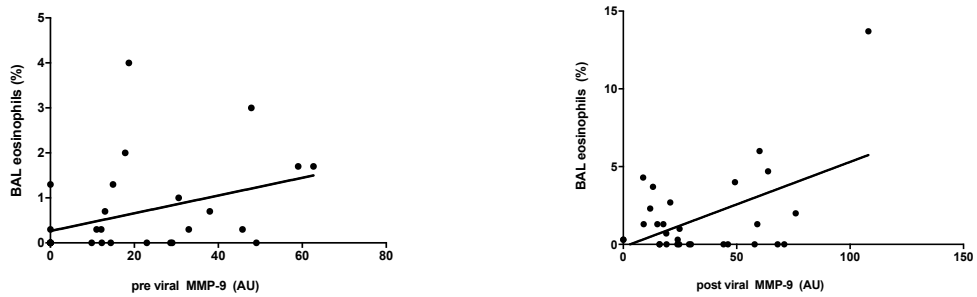


Figure 38. MMP-9 expression is associated with pre and post-viral exacerbation BAL eosinophils percentage. (A) Pre-rhinovirus %proportion of BAL eosinophils in all study participants was correlated with the MMP-9 levels in BAL fluid. r2= 0.14, p= 0.04. **(B)** %proportion of BAL eosinophils during rhinovirus-induced exacerbation was also associated with MMP-9 expression in bronchial fluid. r2= 0.256, p= 0.004. The correlations shown are for all study subjects and are non-parametric (Spearman's correlation).

However, there was no association between pre and post rhinovirus MMP-9 expression and the percentage proportion of neutrophils in BAL fluid in both asthma and healthy groups (pre-viral % proportion of neutrophils r2= 0.003; p= 0.74, Figure 39A and post-viral % proportions of neutrophils r2= 0.01; p= 0.58, Figure 39B, respectively).

A) B)

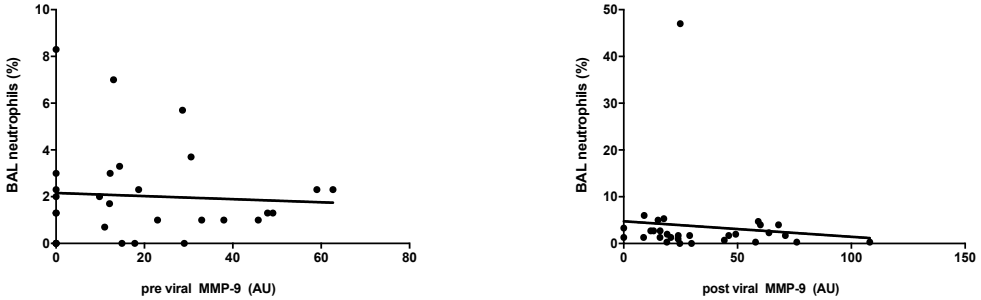


Figure 39. MMP-9 levels are not associated with pre and post- viral exacerbation BAL neutrophils percentage. (A) Pre-rhinovirus neutrophils (%proportion) in all study subjects were not correlated with the MMP-9 expression in BAL fluid. r2= 0.003, p= 0.74. **(B)** Post-rhinovirus neutrophils (%proportion) were also not correlated with the concentration of MMP-9 in BAL fluid. r2= 0.01, p= 0.58. The correlations shown are for all study subjects and are non-parametric (Spearman's correlation).

Interestingly, MMP-9 expression in all study participants in the pre-exacerbation phase was positively correlated with percentage proportions of lymphocytes in BAL fluid (r2= 0.15; p= 0.02, Figure 40A), but this association was lost in the post-viral exacerbation phase (r2= 0.06; p= 0.17, Figure 40B).

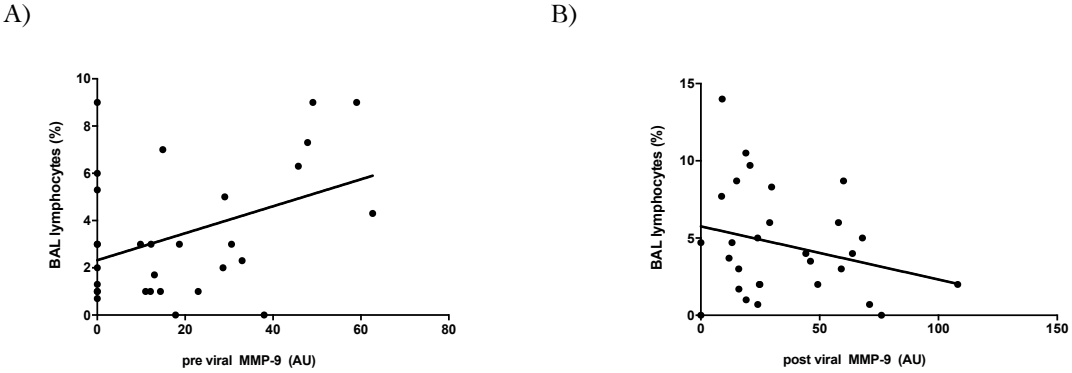


Figure 40. MMP-9 levels are associated with pre exacerbation BAL lymphocytes percentage. **(A)** Pre-rhinovirus lymphocytes (%proportion) in all study subjects were positively correlated with the MMP-9 expression in BAL fluid. r2= 0.15, p= 0.02. **(B)** Post-rhinovirus lymphocytes (%proportion) were not associated with the concentration of MMP-9 in BAL fluid. r2= 0.06, p= 0.17. The correlations shown are for all study subjects and are nonparametric (Spearman's correlation).

6.3.2.2 Relationship between MMP-9 expression and nasal viral loads during asthma exacerbations

Some previous in vitro cell based viral infection studies have suggested that the asthmatic airway may be more susceptible to rhinovirus infection and therefore more permissive of increased viral replication as well as defective viral clearance (Wark, P.A.B. et al., 2005) (Durrani, S.R. et al., 2012). Despite this, no significant differences in viral loads in nasal washes from the adults with asthma challenged experimentally with rhinovirus and infected individuals without asthma has been detected (Kennedy, J.L. et al., 2014). Consistent with these studies, in the post-viral inoculation phase, there was no statistically significant difference between nasal viral loads of asthma patients and healthy controls in the asthma exacerbation cohort (n=30, control mean 5989200, 95% C.I. -2889411 to

14867811; asthma mean 20640493, 95% C.I. 4818259 to 36462727, p=0.12, Figure 41A). There was also no significant correlation between MMP-9 levels and peak nasal viral load in all study participants in post-viral inoculation phase (r2= 0.05; p= 0.20, Figure 41B).

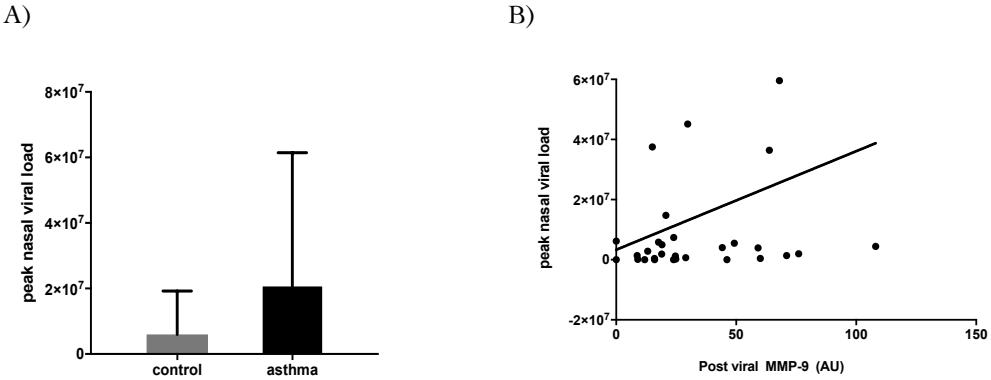


Figure 41. MMP-9 expression is not associated with nasal viral load in asthma exacerbations. **(A)** Post-viral exacerbation, there was no significant difference between nasal viral loads of asthma patients and healthy controls. p=0.12. Mann-Whitney U test. **(B)** There was also no significant correlation between MMP-9 levels and peak nasal viral load in all study participants. r2= 0.05; p= 0.20. The correlations shown are for all study subjects and are non-parametric (Spearman's correlation).

6.3.2.3 Relationship between MMP-9 expression and clinical parameters of asthma exacerbation severity

Although total LRT scores increased and more severe reductions in lung function were reported in the asthma group in the post virus inoculation phase in the asthma exacerbation cohort as described in chapter 5 (Jackson, D.J. et al., 2014), MMP-9 expression in both the asthma and healthy groups was not correlated with maximum fall in FEV_1 described as % change from baseline in this cohort (r2= 0.094; p= 0.097, Figure 42A). Similarly, there was no association between post-viral exacerbation BAL MMP-9 levels in asthma and total LRT symptom scores (r2= 0.004; p= 0.71, Figure 42B).

A) B)

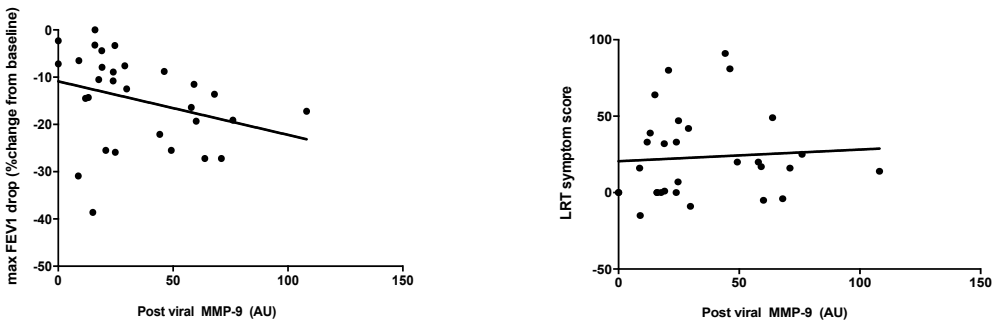


Figure 42. MMP-9 expression is not associated with post-viral exacerbation FEV_1 and asthma symptoms in asthma exacerbations. (A) Post-rhinovirus-induced maximal fall in FEV_1 in both healthy volunteers and asthma patients was not correlated with the MMP-9 levels in BAL fluid. r2= 0.094; p= 0.097. **(B)** Total LRT symptom scores during rhinovirus-induced exacerbations were also not associated with MMP-9 expression. r2= 0.004; p= 0.71. LRT = Lower respiratory tract. The correlations shown are for all study subjects and are non-parametric (Spearman's correlation).

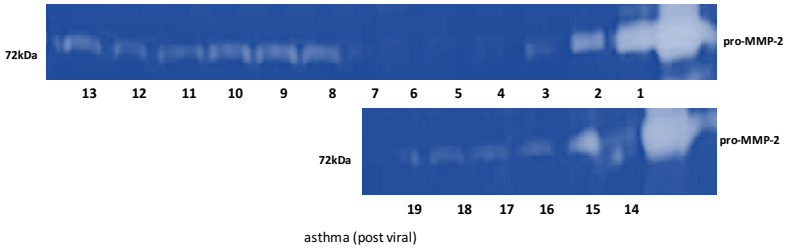
6.3.3 MMP-2 expression is increased during asthma exacerbations

As mentioned before, in the stable asthma cohort of 12 asthma patients and 10 healthy controls, there was no significant expression of MMP-2 (Figure 36A). In the asthma exacerbation cohort of 19 mild to moderate asthma patients (who had induced asthma exacerbation) and 11 healthy controls, significantly enhanced MMP-2 expression was seen only in the post-viral exacerbation phase in the asthma group (n=19, pre-viral vs. post-viral for asthma, difference 45.44, 95% C.I. of difference 33.76 to 57.12, p=0.0004) (Table 12, Figure 43), whereas MMP-2 expression was not significant in the healthy group before or after exacerbation (n=11, difference 0.5942, 95% C.I. of difference -11.09 to 12.27) (Table 12, Figure 43). The activated 62 kDa species was not detected in patients with asthma or healthy controls in either pre or post-viral exacerbation phase.

Disease	Severity (GINA)	n	Treatment	ACQ status	Mean proMMP2 pre-viral (AU)	Mean proMMP2 post-viral (AU)
Healthy	N/A	11	None	Healthy	0.55 ± 0.37	0.46 ± 0.17
Asthma	Mild	8	SABA	Well/Partial	1.36 ± 0.92	46.76 ± 17.56
Asthma	Moderate	11	SABA/ICS	Partial/Poor	0.84 ± 0.80	38.71 ± 6.53

Table 12. MMP-2 expression in asthma exacerbations. Mean (± SEM) proMMP-2 values measured in BAL by gelatin zymography, 14 days pre-viral and 4 days post-viral inoculation in asthma exacerbation cohort. SABA = Short acting β2 agonist; ICS = Inhaled corticosteroids; AU = Arbitrary units.

A) Representation zymogram gel for MMP-2 expression (asthma exacerbation cohort)



B) MMP-2 expression in the asthma exacerbation cohort

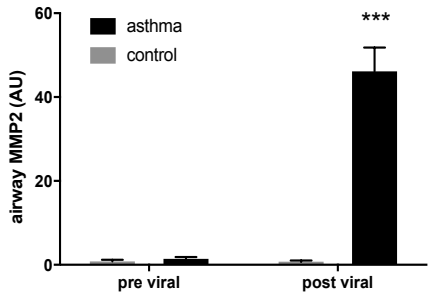


Figure 43. MMP-2 expression in asthma exacerbations. A) Representative zymogram gels showing proMMP-2 expression in patients with asthma post-viral exacerbation in the asthma exacerbation cohort. B) ProMMP-2 expression was increased by viral infection and was higher in those with asthma in the post-viral inoculation phase. ***Pre-viral versus post-viral inoculation for asthma subjects p= 0.0004. Two-way analysis of variance with Tukey's correction for multiple comparisons. AU = Arbitrary Units.

Despite increased expression, MMP-2 levels in asthma patients in the post-viral exacerbation phase were not correlated with maximum fall in FEV_1 from baseline (r2= 0.025; p= 0.5, Figure 44A) in the asthma exacerbation cohort.

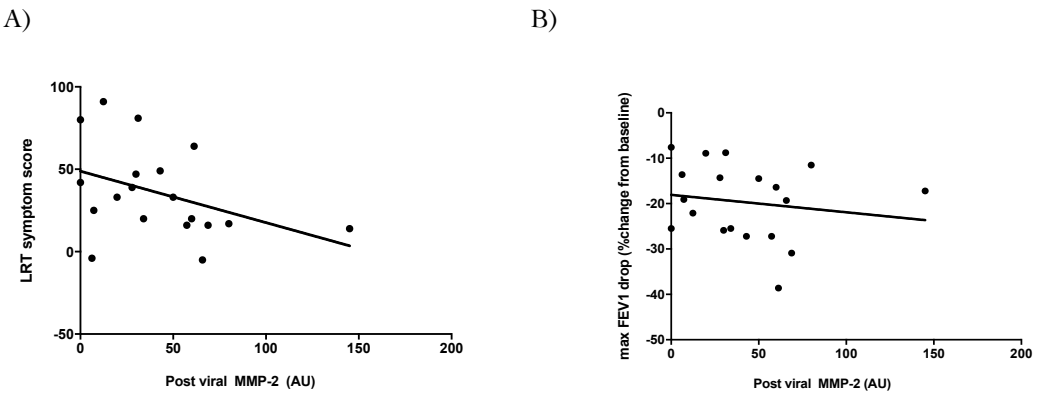


Figure 44. MMP-2 expression is not associated with post-viral exacerbation FEV_1 and asthma symptoms in asthma. (A) Post-rhinovirus-induced maximal falls in FEV_1 in asthma patients were not correlated with the MMP-2 levels in BAL fluid. r2= 0.024; p= 0.5. **(B)** Total LRT symptom scores during rhinovirus-induced exacerbations were also not associated with MMP-2 expression in asthma. r2= 0.015; p= 0.09. LRT = Lower respiratory tract. The correlations shown are for patients with asthma only and are non-parametric (Spearman's correlation).

6.4 Chapter discussion

In summary, this chapter has demonstrated that in patients with stable asthma, there is increased expression of MMP-9. Moreover, MMP-9 expression is further enhanced during an asthma exacerbation and directly correlated with the percentage proportion of eosinophils (but not with neutrophils) in BAL fluid of both asthmatics and healthy volunteers in pre and post-viral exacerbation phases. MMP-2 on the other hand is very minimally expressed in stable asthma, but is present in BAL fluid during an asthma exacerbation. However, MMP-2 expression during an asthma exacerbation is not associated with the severity of exacerbation.

Gelatinases, MMP-2 and MMP-9 not only proteolytically degrade components of ECM (Nagase, H. et al., 2006) (Kumagai, K. et al., 1999) but are also capable of processing a variety of non-ECM proteins. For example, MMP-9 (and to a lesser

extent MMP-2) can cleave certain chemokines, myelin basic protein, amyloid β peptide and substance P (Opdenakker, G. et al., 2001).

Airway inflammation in asthma is characterised by the accumulation of activated inflammatory cells (including neutrophils, eosinophils, lymphocytes and mast cells/basophils) in airway lumen and walls (Arm, J.P. & Lee, T.H., 1992). Both MMP-2 and -9 are implicated in multiple aspects of inflammation including: accumulation of inflammatory cells, tissue injury and repair. However, the role of these enzyme in the pathogenesis of chronic asthma and asthma exacerbations is largely unknown. The importance of inflammation in the pathophysiology of asthma is related to the fact that the intensity of inflammation is associated with the degree of airway remodelling (Chetta, A. et al., 1997) and the severity of disease (Louis, R. et al., 2000). The inflammatory processes underlying asthma involve a complex series of cell reactions, resulting in an increase in overall airway wall thickness such as an increase in vascularity, airway wall oedema, ASM hyperplasia and excessive mucus secretion (Dunnill, M.S. et al., 1969). These changes are believed to be related to the severity of the disease and are also part of the repair process to restore tissue integrity in response to tissue injury (Busse, W. et al., 1999). The acute inflammation that occurs in asthma exacerbation is most commonly manifested as a sudden symptomatic deterioration due to bronchoconstriction and increased AHR in clinical terms. It is therefore important to highlight the role of exacerbations on the long term complications of asthma in the form of airway remodelling. Recent animal studies have shown that repeated antigen challenges provoking episodes of acute inflammation can promote airway remodelling in a murine asthma model, by modifying the homeostasis of ECM components (Tanaka, H. et al., 2001). MMPs cleave ECM proteins which in turn

have significant effects on the development, migration and metabolic function of inflammatory cells. MMP-2 and MMP-9, in particular, has been shown to mediate capillary vascular leakage by degrading components (mainly collagen IV) of vascular basement membrane and inducing the migration of inflammatory leukocytes such as neutrophils, lymphocytes and dendritic cells, through basement membrane into the sites of inflammation such as asthma airways (Keck, T. et al., 2001) (Okada, S. et al., 1997) (Leppert, D. et al., 1995) (Delclaux, C. et al., 1996). Circulating MMP-2 and -9 levels in bronchial fluid may therefore reflect a "spill over" of these MMPs produced in the airway tissue (Wenzel, S.E. et al., 2003). Although such migratory changes in the influx of inflammatory cells can be explained by the effects of MMP-2 and -9 on the ECM components of basement membrane, recent studies suggest that these may also reflect direct effects of MMPs on certain cytokines and chemokines. For example, MMP-2 breaks down the pro-inflammatory molecule monocyte chemoattractant protein (MCP)-3 into the truncated anti-inflammatory molecule, which sometimes helps in the attenuation of inflammatory processes (McQuibban, G.A. et al., 2002). MMP-9, however has many more diverse effects. It cleaves IL-8 into the 10-fold more potent form, and is also able to cleave the inactive membrane-bound forms of TNF-α and TGF-β to generate their active forms, which then help to recruit inflammatory cells (Mohan, M.J. et al., 2002) (Yu, Q. & Stamenkovic, I., 2000). Similarly, both gelatinases are able to generate the active form of the cytokine IL-1β from its inactive pro-form (Schönbeck, U. et al., 1998). Another study showed that the effects of IL-13 (a major cytokine factor driving airway inflammation that leads to airway remodelling) overexpression are mediated by TGF-β; TGF-β activation was in turn MMP-9 dependent (Lee, C.G. et al., 2001). Thus, the

cleavage of cytokines and chemokines by gelatinases can have varying effects on their biological properties, such as: potentiation, inactivation and antagonist formation. These effects consequently play a pivotal role in a variety of acute and chronic inflammatory states and the overall migration of inflammatory cells across different compartments of airway tissue (Turner, M.D. et al., 2014). Through these mechanisms, gelatinases and more specifically MMP-9 is believed to be involved in several important pathways responsible for asthma exacerbations, such as airflow obstruction (Lemjabbar, H. et al., 1999), increased vascular permeability, AHR (Beeh, K.M. et al., 2001) and interstitial fibrosis (Oshita, Y. et al., 2003) (Corbel, M. et al., 2001). Additionally, increased levels of MMP-9 may lead to excessive mucus production by goblet cells, which is also associated with asthmatic exacerbations (Oshita, Y. et al., 2003). Several studies implicate increased MMP-9 expression and activity during exacerbations compared with subsequent convalescence (Lee, Y.C. et al., 2001) (Oshita, Y. et al., 2003). It is therefore likely that asthma exacerbations support airway remodelling by modifying MMP-9 mediated tissue homeostasis. The up-regulation of MMP-9 expression in BAL fluid from asthma patients observed in two cohorts (stable and asthma exacerbation) in this chapter, is consistent with these studies and others reporting that gelatinase content in BAL fluid from untreated asthma patients is increased when compared with that from healthy controls and steroid-treated asthmatics (Mautino, G. et al., 1997). Moreover, findings described in this chapter are compatible with studies demonstrating increased MMP-9 expression in sputum and BAL fluid, under stable conditions (Vignola, A.M. et al., 1998), during spontaneous exacerbations of asthma (Suzuki, R. et al., 2001) (Lemjabbar, H. et al., 1999) and in response to airway allergen challenge (Maisi, P. et al.,

2001).

The finding of increased expression of MMP-2 during asthma exacerbation in this chapter is also in accord with a study demonstrating that ASM cells, fibroblasts and other parenchymal cells contribute significantly to the total production of MMP-2, the expression of which in asthma is controlled through inflammatory cytokines IL-4 and IL-13 (Corry, D.B. et al., 2002). Although MMP-2 is the major ASM derived MMP and is closely controlled by TIMP-2, bronchial and alveolar epithelial cells also constitutively express MMP-2, and its expression by these cells may be enhanced by stimulation with pro-inflammatory cytokines like TNF-α and IL-1 (Yao, P.M. et al., 1997), which are increased in asthmatic inflammation (Broide, D. et al., 1992). These findings therefore provide an essential link between the pulmonary mesenchyme (a source of MMP-2) and cytokine-producing inflammatory cells that underlie acute asthma (Grunig, G. et al., 1998) (Corry, D.B., 1999) and therefore are relevant in understanding of asthma exacerbation pathophysiology.

Thrombin, a serine protease, is present in sputum and BAL fluid of patients with asthma and rises after allergen exposure and antigen challenge (Gabazza, E.C. et al., 1999) (Terada, M. et al., 2004). In asthma, thrombin and other components of the asthmatic airway may lead to gelatinolytic activity and could contribute to airway remodelling (Nguyen, M. et al., 1999). Moreover, type I collagen and activated protein C (APC) can also enhance gelatinase activity (Nguyen, M., 2000). ASM derived MMP-2 is selectively activated by the ECM protein collagen-1, which is also increased in the airways of patients with asthma (Roche, W. et al., 1989) (Henderson, N. et al., 2007). In the current study, there was no significant MMP-2 or -9 activity detected by gelatin zymography of bronchial fluid samples

in either the cohort of stable asthma patients or those with asthma exacerbations. A few reasons for this failure could be limitation of the assay sensitivity or over-dilution of BAL fluid samples. It has been previously observed that localisation of MMP-9 to the cell surface is a key event in facilitating its proteolytic activity with regards to the ability of this enzyme to promote tumor invasion (Yu, Q. & Stamenkovic, I., 2000). Similarly, cell surface localisation of proteolytically active MMP-2 mediated by integrin αvβ3 has been shown to promote angiogenesis and tumour invasion (Brooks et al. 1996). It would therefore seem that retention of MMPs on the cell surface by ECM adhesion receptors may provide a general mechanism for cellular regulation of MMP activity with important consequences for cell-matrix interactions. This offers another possible explanation for the absence of gelatinase activity in bronchial fluid samples from both cohorts; that MMP-2 and -9 may be activated only at the cell surface and the MMP content of airway secretions might just be a reservoir waiting to be activated locally. Nevertheless, the possibility of the presence of active forms of MMP-2 and -9, especially during asthma exacerbations, cannot be ruled out.

It is important to mention here that several research groups have explored the roles of MMP-2 and MMP-9 using animal (especially murine) models of asthma. Most of these models imitate the changes seen in acute asthma rather than chronic asthma. For example, in atopic asthma murine models, the levels of gelatinases increase in sputum and BAL fluid quite quickly following allergen exposure (Blyth, D.I. et al., 1996). Inhibition of these MMPs seems to reduce the migration of leukocytes into the bronchial mucosa and subsequently AHR (Lee, Y.C. et al., 2004). A few other studies using mice deficient in either MMP-9, MMP-2, or both have also suggested a role for these mediators in asthma. Interestingly, one study

has shown that MMP-2 null mice have compensatory increased expression of MMP-9, predisposing them to the tissue abnormalities of remodelling (Esparza, J. et al., 2004). However, in sharp contrast to some of these studies, MMP-2 knockout mice also manifest a defect in clearance of allergic inflammatory cells and demonstrate severe AHR upon allergen challenge leading to increased risk of hypoxic death than their wild-type counterparts (Corry, D.B. et al., 2002). Similar to MMP-2 deficient mice findings, MMP-9 knockout mice were also shown to have an increased inflammatory cell infiltrate in both bronchial mucosa and BAL fluid on allergen challenge (McMillan, S.J. et al., 2004). These observations indicate an additional role for MMP-2 and -9 in 'trapping' and possibly facilitating the clearance of pre-apoptotic inflammatory cells in the lung parenchyma of the allergic lung (Corry, D.B., 2004). Gelatinases are post-translationally regulated by TIMP-1 and TIMP-2, which make complexes with active forms of either MMP-2 or -9 and with latent forms of MMP-9 and -2, respectively (Nagase, H. et al., 2006). In acute exacerbations of asthma, higher than normal levels of MMP-9 and a high MMP-9 to TIMP-1 ratio are found in both sputum and BAL fluid obtained from patients during viral exacerbations (Tonnel, A.B., Gosset, P. & Tillie-Leblond, I., 2001) and allergen challenge (Becky Kelly, E.A. et al., 2000) and are decreased when asthma control improves (Hoshino, M. et al., 1999) and after therapy with corticosteroids (Tanaka, H. et al., 2000) (Mattos, W. et al., 2002). Moreover, MMP-9/TIMP-1 ratios have been implicated in predicting subsequent responsiveness to oral corticosteroids in asthma. Patients with asthma who do not respond to corticosteroids are found to have a lower MMP-9/TIMP ratio prior to treatment compared with those who did respond to treatment (BOSSÉ, M. et al., 1999). TIMP-1 is known to have fibrogenic properties that are believed to result

from inhibition of MMP-9 (Hayakawa, T., 1994) (Matrisian, L.M., 1990). TIMP-1 expression is differentially regulated in inflammatory degenerative disease and has been correlated with an increase in cytokine levels such as interferon-gamma (IFN-δ), TNF-α and IL-6 (Khuth, S.T. et al., 2001). In the view of these findings, it is reasonable to speculate that the conflicting results from animal studies may have resulted from the abnormalities of complex and tightly regulated balance between gelatinases and TIMPs driving different aspects of asthma inflammation and pathology.

Since the majority of free MMP-9 is conjugated to the protein NGAL, the primary source of MMP-9 is believed to be the neutrophils which infiltrate bronchial mucosa (Lemjabbar, H. et al., 1999). NGAL is also capable of protecting MMP-9 from degradation and thereby preserving MMP-9 enzymatic activity in a dose dependent manner (Yan, L. et al., 2001). Although a large number of studies support that MMP-9 originates both from activated chemo-attracted neutrophils , in response to in situ lung injury during acute severe asthma, it is the eosinophils and activated respiratory epithelial cells that are thought to be the main sources of gelatinases in chronic inflammatory asthma (Possa, S.S. et al., 2013).

Furthermore, there is evidence that MMP-9 is important in the in vitro transmigration of basement membrane components by eosinophils and that MMP-9 is overexpressed by eosinophils accumulating in the airway walls of asthmatics (Ohno, I. et al., 1997). Eosinophils are thought to exert a beneficial role in protecting the host from parasitic infection, while at the same time playing a detrimental role in a variety of acute and chronic airway disorders. In asthma, strong evidence suggests that eosinophils may be responsible for damage to the bronchial epithelium, by releasing cytotoxic granule proteins and other pro-

inflammatory mediators (Frigas, E. & Gleich, G., 1986) (Wardlaw, A.J. et al., 2000). The eosinophil count is significantly elevated in BAL fluid in the symptomatic asthmatics and the concentration of eosinophil products has been found to correlate with disease severity (Wardlaw, A.J. et al., 1988). Enhanced expression of MMP-9 mRNA has been observed in airway biopsy tissue obtained from asthmatic subjects. Interestingly, the majority of cells expressing MMP-9 mRNA in these biopsies were eosinophils (Ohno, I. et al., 1997). In keeping with these studies, findings of this chapter also demonstrate positive correlation between MMP-9 expression and the percentage proportion of eosinophils (but not of neutrophils or lymphocytes) in BAL fluid samples of asthma patients before and after rhinovirus inoculation. Cytokines and chemokines play a central role in the induction of allergic and non-allergic airway inflammation in bronchial asthma (Busse, W.W., 1998). Among the cytokines involved in MMP regulation, there is evidence to suggest that IL-5 may be crucial and its expression is increased in BAL fluid and bronchial biopsy tissue samples from asthma patients (Ying, S. et al., 1995). IL-5 is considered as one of the main cytokines promoting eosinophil accumulation and MMP-9 expression in asthmatic airways (Hamelmann, E. et al., 1997) (Lee, E.-J. et al., 2012). Therefore, overexpression of MMP-9 by eosinophils in the airways of asthmatic individuals and the subsequent participation of MMP-9 in the maintenance of chronic airway inflammation in asthma possibly as a result of up-regulation of IL-5, could serve as an important mechanism of airway remodelling in chronic asthma. Perhaps the most compelling findings to support these observations are the results of the recent drug trials targeting IL-5 monoclonal antibodies involving mepolizumab (Pavord, I.D. et al., 2012).

In conclusion, the widespread view is that sustained elevation of MMP-2 and MMP-9 levels in chronic asthma could lead to airway tissue remodelling. An increased gelatinase presence may predispose asthma patients to many of the tissue abnormalities of remodelling characteristic of chronic asthma such as thickening of the basement membrane, reduction in epithelial thickness, increased inflammatory cell migration and increased vascularity of the mucosa. (Hoshino, M. et al., 1999). Contrary to this, some animal studies have shown that MMP-2 and -9 may be essential for coordinating lung cellular trafficking and the resolution of allergic inflammation. Despite these caveats, MMP-2 and MMP-9 knockout mice studies have also provided imperative evidence that these two gelatinases play important roles in asthma inflammation. Moreover, the increase in gelatinase production suggests a process of ECM degradation in asthma exacerbations and proposes MMP-2 and -9 as non-invasive systemic markers of inflammation in asthma. These inconsistent observations are interesting and reflect a level of complexity involved in evaluating the roles different MMPs play in airway inflammation and remodelling.

Chapter 7: Final discussion

7.1 Overview of results

This study has expanded on the current knowledge of the role of MMP-1 in asthma. Previous studies show that MMP-1 and mast cells are minimally expressed in normal airways but both are present in patients with asthma (Rajah, R. et al., 1996) (Cataldo, D.D. et al., 2004). The results described here demonstrate a likely mechanism linking MMP-1 activation by airway mast cells with ECM remodelling, ASM growth, AHR and asthma severity. They show that MMP-1 can be activated by the mast cell protease tryptase (by direct proteolysis) and that this activation further remodels ECM to generate a pro-proliferative substrate for ASM cells. The presence of tryptase positive mast cells within ASM bundles is associated with increased ASM proliferation in patients with stable asthma. Moreover, MMP-1 expression is directly correlated with airway contraction in response to a bronchoconstrictor stimulus methacholine. During asthma exacerbations, MMP-1 activity is correlated with exacerbation severity. Findings in this study also show that ASM-derived MMP-1 is definitely required for the generation of the pro-proliferative ECM by mast cell tryptase and that the direct action of mast cell derived proteases does not suffice to generate proliferative ECM in the absence of ASM derived MMP-1. Collectively, these findings suggest that the MMP-1/mast cell interactions and ECM remodelling may contribute to airway narrowing, remodelling and worsening symptoms in patients with asthma.

In active form, MMP-1 is capable of breaking down triple helical fibrillar collagens as part of ECM turnover. Through collagen processing, this pathway

can generate bioactive mediators and growth factors, including the neutrophil chemo-attractant matrikine PGP, which may be able to cause ASM growth and sustain inflammation by recruiting neutrophils in the airway (Weathington, N.M. et al., 2006). As well as collagens, MMP-1 has other matrix substrates including aggrecan, proteoglycan, versican and perlecan (Murdoch, A.D., 1996) and can activate or inactivate bioactive proteins including IGFBPs , PAR-1, CXCL8 and CXCL12, which could also have lasting effects on airway remodelling (Boire, A. et al., 2005). The advantage of using biosynthesised ASM-derived matrix in our study is the presence of multiple protein substrates in the preparation, making this system more physiological and robust than individual ECM substrates. This does mean inability to define which ECM proteins have been proteolytically processed by active MMP-1 and are therefore responsible for the pro-proliferative effect on subsequent ASM growth. It is possible that generation of bioactive neo-epitopes, or processing of ECM related growth factors, alter signalling to ASM cells to enhance their proliferation (Ricard-Blum, S. & Salza, R., 2014).

Although the ECM remodelling by MMP-1 and ASM growth seen in vitro cannot be directly linked to humans with asthma, the study findings, using primary asthma-derived ASM cells, are consistent with this mechanism acting in asthma. Tryptase mediated MMP-1 activation is one pathway by which mast cells could contribute to airway remodelling. Mast cells and mast cell degranulation are often associated with severe and sometimes fatal asthma (Carroll, N.G. et al., 2002). The primary and secondary mediators generated by degranulation increase vascular permeability, ASM constriction, mucous secretion and leukocyte chemo-attraction (Elliot, J.G. et al., 2009). Sub-acute responses include increased cellular infiltrates, mucosal oedema, desquamation, basement membrane thickening and

goblet cell hyperplasia. These responses may occur because of the continued release of primary and secondary mediators as well as effects caused by the mast cell granule matrix-derived factors (Hart, P.H., 2001). It is therefore likely that in vivo, mast cell mediators including prostaglandins and tryptase, by activation of PAR-2 receptors (as previously observed by others in vitro) or other mechanisms (as observed in this study), may not only stimulate ASM proliferation, but also affect ASM contraction to worsen asthma symptoms (Balzar, S. et al., 2012) (Berger, P. et al., 2001). Previously, it has been shown that MMP-1 supports ASM contraction in vitro (Rogers, N.K. et al., 2014) and others have shown that collagenase treatment enhanced contraction of bronchial rings by reducing the forces of parenchymal tethering (Khan, M.A. et al., 2010). Consistent with others, this study also showed airway MMP-1 is associated with airway narrowing in response to methacholine in patients with stable asthma. Also, during exacerbations, activation of MMP-1 is associated with exacerbation severity, as evidenced by increasing LRT symptom scores and fall in FEV_1 from baseline. The underlying mechanisms of airway narrowing and the relationship between airway remodelling, airway contraction and AHR are complicated. The findings in this study and those of others, associating ECM remodelling, ASM growth (Freyer, A.M. et al., 2001) and contraction (Freyer, A.M. et al., 2004) as well as ASM mass with AHR (Woodruff, P.G. et al., 2004), are befitting with MMP-1 activation contributing to changes in cell-matrix coupling leading to excessive shortening of ASM due to altered resistive ASM loading, resulting in increased airway narrowing (An, S.S. et al., 2007).

Several longitudinal asthma studies are generally consistent with the presence (but not progression) of airway obstruction, with the small number of studies

suggesting airflow obstruction increases with disease duration (Lange, P. et al., 1998). However, none of the longitudinal studies examined the ASM numbers or ECM mass over time in patients with asthma (McGeachie, M.J. et al.,2016) (Sears, M.R. et al., 2003) (James, A.L. et al., 2012). There are conflicting data on rates of ASM proliferation in asthma, with some studies suggesting that ASM proliferation itself is not increased, but instead enhanced rates of cellular proliferation have been identified in situ in the asthmatic airway, occurring outside the expanded smooth muscle compartment (Ward, J.E. et al., 2008). However, others have demonstrated that ASM proliferation in bronchial smooth muscle biopsies is both elevated in asthma and related to asthma severity (Hassan, M. et al., 2010) indicating that airway remodelling is not an end-stage phenomenon, but an active and continuous process in longstanding asthma. These inconsistent findings, both in vitro and in vivo, may be due to a combination of methodological and study population differences. The study findings, showing no overall difference in ASM proliferation in situ between those with asthma and healthy control subjects, may be due to the exclusion of those with moderate to severe asthma.

Additionally, this study shows increased levels of MMP-9 in the BAL fluid of patients with stable asthma and enhanced expression of both MMP-2 and -9 in asthma exacerbations. The current evidence suggests that sustained elevation of gelatinases (MMP-2 and MMP-9) in chronic asthma could lead to airway remodelling (Chakrabarti, S. & Patel, K.D., 2009). Gelatinases have been widely implicated in asthma pathogenesis. For example, in allergen challenge murine models of asthma, the levels of gelatinases dramatically increase in sputum and BAL fluid over a 24- hour period following allergen exposure (Blyth, D.I. et al.,

1996). MMP-9 in particular, is also thought to be involved in several important pathways responsible for asthma exacerbations including sustained airway inflammation, ECM breakdown and increased mucous production leading to airflow obstruction and AHR (Kumagai, K. et al., 1999). It is therefore likely that asthma exacerbations promote airway remodelling by altering MMP-2 and -9 mediated processes such as cell growth and activation, vascularization and ECM protein degradation.

7.2 Study strengths and limitations

7.2.1 Endobronchial biopsies and primary cell culture

The experiments in this study were done using an in vitro primary ASM cell culture system, with cells originating from endobronchial biopsies. These biopsies were obtained through fibreoptic bronchoscopy of healthy volunteers and patients with asthma. This entailed ASM bundle dissection from surrounding airway connective tissue and subsequent enzymatic dispersion to achieve a single cell suspension. It is debatable that such a technique may not yield a pure ASM population and may be contaminated by fibroblasts and myofibroblasts (Wenzel, S.E. & Balzar, S., 2006). However, cells used in these experiments adopted a characteristic "hills and valleys" appearance as viewed by electron microscopy during growth cycles and exhibited filamentous actin staining (Figure 6, chapter 2). Using current available techniques, some contamination of ASM cultures with other cell types is almost unavoidable and therefore affects all research pertaining to smooth muscle tissue. Assuming that this contamination is very minimal and contaminating cells do not form the predominant cell culture population, it would be fair to say that this is a close representation of the in vivo situation. Obviously, even if the ASM cells utilised in experiments were a pure population, this still

would not completely recreate the multi-cellular three dimensional airway environment.

Endobronchial biopsies also come with innate variability. The size and quality of the biopsy specimens cannot only have major effects on the assessment of morphological and histopathological features (Jeffery, P. et al., 2003), but also significant consequences for the primary ASM cell culture (Ma, X. et al., 2002) and for genetic studies (Laprise, C. et al., 2004). Many factors are involved in determining the quality of the biopsy samples, such as: the type of forceps, the sampling sites, number of samples taken and the experience of the operator. This could explain why some biopsy derived-cells cultured for this study became archaic, even at a relatively lower passage, as if they were generated from small tissue samples, they would have effectively gone through a higher number of passages. As passage number is known to affect ASM cell characteristics (Kwist, K. et al., 2015), cells in our experiments were used between P3-P5 to minimise this effect. P3 was used for ASM cells showing slower growth rate and P5 for the rest. It is important to note that, although no statistical differences were noted between early and late passage cells, this was not subject to analysis in this study. From the quantitative measurements, significant differences between asthmatic and healthy controls were observed for smooth muscle area and percentage of intact epithelium within bronchial biopsies. However, when normalising the smooth muscle area for the total surface of the biopsy, no statistical difference was observed.

7.2.2 ASM derived ECM preparations

The composition of the ECM is complex and tissue-specific. It is difficult to obtain an in vitro ECM model that recreates the in vivo ECM composition by simple

mixing of single ECM molecules. To solve this problem, decellularised ECM preparations were used in this study as in vitro ECM models, to assess effects of collagenase on ASM growth. Decellularised ECM substrates were derived from the tissue and ECM proteins deposited by cultured primary ASM cells. First reported in 1948, decellularisation aims to isolate the ECM from its inhabiting cells (Poel, W.E., 1948). The process of decellularisation removes cell residues, with only minimal disruption of other components, including group-specific antigen (GAG), growth factors and collagen, and helps maintain ECM ultrastructure (Reing, J.E. et al., 2010). The major advantage of using a decellularised ECM is the presence of a multi-dimensional fibrous and porous cell substrate structure, which represents a more physiological model, particularly with regards to cell behaviour (Ott, H.C. et al., 2010). Decellularised ECM is expected to exhibit physical and mechanical properties of native ECM and by mimicking the natural ECM, it is possible to control the effect of these properties on cell growth and behaviour (Ott, H.C. et al., 2010). Additionally, decellularised ECM substrate not only acts as a biophysical template, providing a suitable space for the adhesion and growth of seeded progenitor cells, but also has an ability to become a biochemical reservoir of cell-generated bioactive signalling molecules, such as TGF-β and IGF (Kim, I.G. et al., 2015) (Zhang, W. et al., 2016). In short, many researchers agree on the fact that decellularised ECM substrates provide a more physiological, rapid and highly informative platform to explore reciprocal feedback between cells and stromal ECM proteins (Green, J. & Yamada, K., 2007), as they not only act as matrix scaffold for cell–ECM adhesion, but also incorporate various signalling pathways involving growth factors and cytokines. Despite this, ultrastructural analyses of cell-derived ECM models prepared in-

vitro have shown a few disadvantages of this method. One drawback of the process of decellularisation (which involves several chemical, physical and biological treatments and their combinations), is that it can potentially damage cell structural elements. For example, one study claimed that the PGs and GAGs, which connect cells to collagen bundles or elastic fibres and induce a variety of cellular responses, are mainly depleted from decellularised ECM, leading to a somewhat reduced ECM integrity (Hinderer, S. & Schenke-Layland, K., 2013). The other slight disadvantage is that the composition of cell-derived ECM depends considerably on the cell type and the cell culture conditions. The composition varies between decellularised ECM derived from primary versus passaged cell lines, leading to different cellular functions (Hoshiba, T. et al., 2011). It is therefore important to carefully consider the cell source for the preparation of decellularised ECM and the composition of the decellularised ECM should be directly comparable with the composition of the target ECM in vivo (Hoshiba, T. et al., 2011). For that reason, only primary ASM cells of consistent early passages, obtained from patients with asthma and healthy controls were used to prepare experimental ECM substrates in all experiments. The seeding cell numbers and culture conditions were also strictly controlled to minimise variability between ECM preparations. It is also important to understand that in decellularised ECM, the effects of various ECM molecules on cellular function are integrated to regulate subsequent cell behaviour and it is almost impossible to differentiate the effects of individual ECM molecules on cellular function using this model alone, making it less specific than commercially available ECM preparations (Hill, R.C. et al., 2015). Nevertheless, decellularised ECM is still one of the best physiological ECM models mimicking native ECM composition and structure and is considered

a powerful tool for studying the comprehensive roles of ECM in cell growth and differentiation in vivo (Hoshiba, T. et al., 2016).

7.2.3 Methods for analysis of MMP regulation

Most studies examining MMP regulation in the airways or other tissues tend to evaluate a single marker of expression: protein, mRNA or activity. This approach fails to characterise the mechanisms by which different MMP regulators work to change MMP expression and activity under pathological conditions. Although various preclinical studies suggest that MMP activity plays an important role in tissue remodelling, specific enzymatic activities of individual MMPs have not been described. Part of the reason for the difficulty in establishing the role of particular MMPs in tissue remodelling, is the lack of specific assays for measuring activity of individual proteases in vivo or ex vivo (Acker, M.G. & Auld, D.S., 2014). Instead, previous investigators have focused on the detection of MMP mRNA or protein using in situ hybridisation, immunohistochemistry, zymography or immunoblotting. These techniques are semi-quantitative in nature. Moreover, they do not take into account the presence of enzymatic inhibitors in the tissue environment and thus may be lacking in clinical relevance. For example, high molar levels of TIMPs might negate high molar levels of proteases with respect to net enzymatic activity. In this study, both the latent and active forms of MMP-1 protein were assessed at both secreted and cell/matrix level, owing mainly to its ability to bind to cell surface receptors, matrix or to localise intracellularly, a factor rather poorly appreciated. Therefore, specific enzymatic assay designed to measure net activity of MMP-1 was used such as substrate-specific ELISA technique (DuoSet and Fluorokine E activity kits by R&D Systems) to quantify the total protein expression and net enzymatic activity of human MMP-1 in

bronchial fluid and primary ASM cell culture supernatants. The specificity for the assay was provided by the anti-MMP-1 antibody as described in the methods chapter. Additionally, presence or absence of latent or active species of MMP-2 and -9 in bronchial fluid were monitored by substrate specific gelatin zymography. A significant up-regulation of net MMP-1 activity was shown during asthma exacerbations and in those with asthma this was strongly correlated with more severe falls in lung function. In contrast, net MMP-2 and MMP-9 activity in the bronchial fluid remained low and unchanged during asthma exacerbations, when analysed by gelatin zymography. This data suggest that assays measuring the activity of individual MMPs could be clinically relevant and this approach may have a potential therapeutic value in targeting individual enzymes in treating asthma.

MMP-1 activation, like that of other MMPs, is regulated at multiple levels including: transcription, post-translation and by inhibitors including TIMP-1 (Hinks, T.S.C. et al., 2016). It is also important to note here, that due to time constraints, this piece of research was not originally aimed at investigating if and how activity of MMP-1 is regulated by TIMPs in asthma. Instead, the Fluorokine MMP-1 activity assay was used as a direct measure of net MMP-1 activity, covering the different mechanisms of activation and also taking into account the suppressive effect of MMP-1/TIMP complexes. The scope of this study was eventually increased to measure TIMP-1 protein in the bronchial fluid samples after it was reviewed for publication. This work was carried out by my colleagues at the University of Nottingham and is not part of this thesis. Briefly, the results showed that there was no significant difference in BAL TIMP-1 protein levels between patients with asthma and healthy controls; BAL TIMP-1 protein levels

did not change significantly during asthma exacerbations. This would suggest that the increase in MMP-1 activity is likely driven by MMP-1 activation, rather than suppression by TIMP-1 (Naveed, S.-U.-N. et al., 2016).

7.3 Significance of the study

This study set out to advance our understanding of the pathophysiology of airway remodelling. The term "airway remodelling" refers to a range of progressive structural changes occurring within the airway walls of patients with asthma and other obstructive lung diseases. These changes seems to be associated with airway narrowing and may eventually lead to fixed airflow obstruction (Bergeron, C.L. et al., 2010) (Phelan, P.D. et al., 2002). Airway remodelling is closely related to airway inflammation and it has its own consequences with regards to asthma symptoms, exacerbations, decline in lung function and response to treatment (James, A.L. & Wenzel, S., 2007). Treatment with bronchodilators results in transient improvements in symptoms, but only long term treatment of inflammation results in continued improvements in symptoms and AHR. The decrease in AHR directly relates to an increase in lung function and overall better asthma control (Haahtela, T. et al., 1991). However, in mild to moderate asthma, continuous daily treatment with anti-inflammatory agents such as ICS had no effect on longitudinal change in lung function as measured by the FEV_1 after bronchodilator treatment (The Childhood Asthma Management Program Research Group, 2000) (Paschall, V.L., 2001). The other clinical parameters of airway remodelling are also associated with disease severity and duration (Larsen, G.L., 1999), indicating a clear and essential need to elucidate the underlying mechanisms of airway remodelling and uncover new treatment targets.

It is important to refer back to the literature to assess: whether the mechanisms

demonstrated by this study conform to prevailing paradigms, how these findings may impact airway remodelling research and if there is a potential to translate this information into therapeutic intervention in the future. In brief, this study combines in vitro cell and mediator analysis with clinical assessment of asthma symptoms and exacerbation severity. The immunohistochemistry data linking mast cell number with ASM proliferation (within ASM bundles), suggest that this feature of airway remodelling may be physiologically relevant in asthma.

It has been noted that increased thickness of the ASM layer in asthma results from hyperplasia or hypertrophy of muscle cells or increased ECM deposition, and these changes are in turn related to the severity (but not necessarily the duration) of asthma (James, A.L. et al., 2012). Decline in lung function in patients with asthma may therefore present very early in life, continue into childhood and adulthood (Tai, A. et al., 2014) (James, A.L. et al., 2005) (Lange, P. et al., 1998), and even increase with age (James, A.L. et al., 2005) (Lange, P. et al., 1998). Mast cells are known to be the predominant cell within the ASM layer in both those with or without asthma; they are more often degranulated in the poorly controlled asthma patients (Carroll, N.G. et al., 2002). During an acute episode of asthma, activation of mast cells may lead to abnormal release of chemical mediators, resulting in excessive stimulation of ASM and mucous glands (Connell, J.T., 1971). Tryptase, a prominent mast cell mediator, is a marker of mast cell burden and degranulation when detected in extracellular fluids, and it is known to be elevated in the BAL fluid from patients with uncontrolled asthma (Jia, G. et al., 2015). These observations, along with the pro-inflammatory nature of mast cell granule contents, make them an interesting subject when investigating the link between airway inflammation and remodelling. This study has suggested a

mechanism by which ASM remodelling might be affected by short-term inflammatory events, such as those seen during an exacerbation of asthma. Through a series of in vitro experiments, demonstrating the effects of MMP-1 on ASM-derived ECM from cases of stable asthma and from control subjects without asthma, a link was uncovered between activation of mast cells (through Th1 or Th2 pathways) and increased ASM proliferation, mediated by MMP-1 derived from ASM cells. Next, experimental rhinovirus inoculation, simulating an exacerbation of asthma, was associated with MMP-1 activation, reduced lung function from the baseline and increased respiratory symptom scores, all of these being greater in patients with asthma. Thus, these findings make a strong argument that mast cell activation, which is not always dependent on airway inflammation, could account for the increased ASM proliferation in asthma. Perhaps in the long term this mechanism adds to the overall ASM layer thickness and fixed airway structural abnormalities and associated airflow obstruction. The increased baseline MMP-1 expression observed in patients with stable asthma has been seen by others (Cataldo, D.D. et al., 2004), but not in all studies (Araujo, B.B. et al., 2008) (Mautino, G. et al., 1999) and may reflect a modified phenotype of ASM in patients with asthma. Equally, the activation of MMP-1 after rhinovirus infection may be just one of many acute phase dynamic events happening during an asthma exacerbation. There is little knowledge about whether structural changes of airway remodelling are permanent or not. Some studies in equine models of asthma, suggest that ASM proliferation and lung function improve considerably, but do not return to normal, after avoidance of antigen exposure and treatment with corticosteroids (Herszberg, B.R.N. et al., 2006) (Leclere, M. et al., 2012). By comparison, in humans, corticosteroids are

found to have both positive and neutral effects on airway remodelling (Bergeron, C.L. et al., 2010). Therefore, it is reasonable to speculate that exacerbations of asthma may contribute to semi-permanent or permanent cumulative structural changes constituting airway remodelling over time. The findings of this study support this hypothesis by indicating that MMP-1 may contribute to such a mechanism, given the association between MMP-1 activation and lung function during asthma exacerbations. Another retrospective study has indeed shown that intermittent periods of worsening airway inflammation during asthma exacerbations can enhance airway remodelling and lead to excess decline in lung function (Bai, T.R. et al., 2007). Structural changes of airway remodelling are an overall result of many processes including ASM proliferation/apoptosis and ECM deposition/resorption. The association of ASM proliferation and mast cells in vitro and in vivo is consistent with the findings of others, suggesting that, in the asthmatic airway, there is development of an environment that alters the balance between these processes and sustains airway remodelling (Chernyavsky, I.L. et al., 2014). Importantly, results described here suggest a mechanism driving asthma severity which is generally independent of Th2 mediated inflammation and is of relevance in developing therapies for the rather poorly understood Th2-low asthma endotype, which is present in many if not all asthmatics and is poorly responsive to current asthma treatments including ICS (Fahy, J.V., 2015). Further research is however required to determine the importance of this pathway in specific asthma endotypes.

More specifically, the results of this study support recently published double-blind, placebo-controlled trial of Imatinib (an inhibitor of the SCF receptor KIT required for mast cell development and survival in tissues), that suggests that mast

cell dependent processes contribute to airway obstruction in the patients with asthma who fail to respond to the maximum asthma treatment. Imatinib significantly reduced airway mast cell counts, AHR and mast cell activity as measured by serum and airway tryptase levels in these patients with refractory asthma (Cahill, K.N. et al., 2017). With this in view, the observations of current study provide a clear demonstration of the relevance of mast cell/ASM interactions mediated by tryptase induced MMP-1 activation as a potential novel target for the prevention and/or treatment of airway remodelling. Indeed, this alternate approach may be more effective than directly inhibiting MMPs, which is associated with many off-target effects (Cathcart, J. et al., 2015).

The findings of increased expression of MMP-2 and MMP-9 during asthma exacerbations are also quite interesting. It is well known that the most common trigger for an asthma exacerbation is usually a viral respiratory tract infection (Johnston, S.L. et al., 1995) and the lower airway inflammation induced by experimental rhinovirus infection in asthma patients is not reduced by ICS (Grünberg, K. et al., 2001). Studies have also shown that MMP-9 expression and activity are enhanced in the lower airways by allergic inflammation and that ICS have no effect on this (Cataldo, D.D. et al., 2002) (Mattos, W. et al., 2002). It is therefore likely that viral/allergic lower airway inflammation during asthma exacerbation and increased airway MMP-9 expression go hand in hand, promoting airway remodelling. Therefore, better treatments for asthma exacerbations need to be established to tackle factors involved in lower airway inflammation. In this context, MMP-9 and possibly MMP-2 may be potential targets for the management of asthma exacerbations and to achieve better disease control. However, to devise therapies directed against gelatinases, a fine balance

needs to be achieved between an excess (leading to tissue destruction) and a shortfall (causing fibrosis) of gelatinase activity in lung tissue and this remains a biggest challenge in developing MMP inhibitors for clinical use (Chakrabarti, S. & Patel, K.D., 2009). The role of MMPs, especially gelatinases, in tissues other than the lung also have to be considered to prevent undesired effects of anti-gelatinase therapy (Chakrabarti, S. & Patel, K.D., 2009). By elucidating the roles of individual MMPs in asthma and airway remodelling, it is hoped that in the future, more selective MMP inhibitor drugs can be designed.

Pharmacological inhibition of MMP activity has been explored previously for its potential in the treatment of a variety of diseases. Clinical trials examining the effects of MMP inhibitors have mainly focused on diseases such as cancer, arthritis and periodontal disease (Cathcart, J. et al., 2015) (Close, D.R., 2001) (Ozmeric, N. & Gokmenoglu, C., 2016). However, results from clinical trials of MMP inhibitors have been generally disappointing (Cathcart, J. et al., 2015). MMP inhibitors mainly consist of two basic features: a zinc chelating ligand and a chemical component which binds to the substrate recognition cleft of the enzyme, rendering the protease inactive (Cathcart, J. et al., 2015). Just like most enzyme-targeting drugs, early MMP inhibitors were devised to bind within the catalytic domain of the proteases (Hu, J. et al., 2007). The initial therapeutic inhibitors were peptidomimetics, or compounds derived from the amino acids sequences of the endogenous MMP ligands (Dorm N, G.R. et al., 2010). During extensive clinical trials in cancer, most MMP inhibitors exhibited moderate efficacy in delaying disease progression. The significance however could not be established due to dose-limiting toxicity causing significant musculoskeletal pain and inflammation (Coussens, L.M., 2002) (Cathcart, J. et al., 2015). Fewer trials of

MMP inhibitors have targeted pulmonary pathologies, and the evidence regarding the efficacy of these compounds as anti-inflammatory and anti-neoplastic agents has been limited (Belvisi, M.G. & Bottomley, K.M., 2003) (Zucker, S. et al., 2000). More recently, design of MMP inhibitors has focused on development of compounds with selectivity and increased potency for a particular MMP (rather than a broad-spectrum approach) and unique delivery mechanisms to only target the diseased tissue (Fisher, J.F. & Mobashery, S., 2006). Because of the structural and sequence homology between catalytic domains of MMPs, efforts have shifted to investigating compounds which mediate non-competitive inhibition (Devy, L. et al., 2009) (Zarrabi, K. et al., 2011). Alternate ways of blocking MMP activity in the absence of highly specific MMP inhibitors are slowly gaining popularity and one way of doing this is emphasized by this study, which is to target the mediators causing up-regulation of MMPs under pro-inflammatory and pathological conditions.

7.4 Future work

Functional studies in asthma suggest significant regional heterogeneity of airway narrowing in asthma (Altes, T.A. et al., 2001), indicating variability of the distribution of ASM remodelling within and between asthma cases. It is also widely believed that clinical heterogeneity of asthma in terms of disease severity may be reflected by the degree of airway inflammation (Bousquet, J. et al., 1994). This lead to a number of outstanding questions and uncertainties regarding airway remodelling and require future studies to adequately address them. These questions especially include:

1. Whether airway/ASM remodelling in asthma is a transient or a permanent process?

2. How does ASM remodelling relate to asthma outcomes over time?

One way of answering these important questions might be to collaborate with major asthma research centres locally to access archived asthma bronchial biopsies from different asthma phenotypes at different stages of disease progression, immunostain them for mast cells and ASM proliferation markers and correlate these findings with sequential lung function measurements and clinical asthma outcomes to generate some preliminary data. This preliminary data can then be used to secure funding to set up a longitudinal study to check robustness and association of mast cell derived ASM remodelling with long term clinical and functional airway abnormalities in asthma of varying severity and endotype.

Furthermore, our current understanding of exacerbation-induced airway remodelling is largely limited to mouse models of disease and is in its infancy. A study could specifically look for induction of a range of potential remodelling factors in bronchial fluid and biopsy samples following experimental rhinovirus infection in a group of asthma patients with normal lung function compared to those with evidence of significant airflow obstruction or airway remodelling. Analyses of remodelling factors following RV infection of cultured bronchial epithelial and ASM cells can also be performed in parallel, and would allow inclusion of asthma patients with severe disease, who might be otherwise unsuitable for experimental rhinovirus inoculation. This approach can provide a comprehensive in vitro and in vivo model to evaluate the significance of exacerbation related airway remodelling and its relationship with asthma morbidity.

A future experimental study can aim to identify the more specific role of ECM components in ASM remodelling, especially with regards to uncovering the

pathways involved in collagenase mediated ECM modulation. The fact that ECM proteins mediate directional signalling and can affect cell behaviour, has serious implications in understanding the different disease processes involved in airway remodelling. Therefore, targeting the ECM, the enzymes that remodel it and the receptors that transduce their signals, can offer promising therapeutic opportunities for treatment of chronic asthma.

Last but not least, further research is also required to look at the ways of using MMP-1 as a biomarker to assess presence and progression of airway remodelling in asthma and to better characterise Th2 low asthma endotypes. Asthma phenotyping is now thought to be an effective way of predicting clinical response to various treatments (Corren, J., 2013) and it is hoped that this approach will improve asthma outcomes and quality of life for a vast number of asthma patients: a major step forward in the treatment of chronic asthma.

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Appendix

Materials

Consumables

Consumables	Supplier
Haemocytometer	Fisher Scientific, Loughborough, UK
T25 and T75 cell culture flasks	Costar Corning, SchipholRijk, Netherlands
6, 12, 24, 48 well plates	Fisher Scientific, Loughborough, UK
Corning® Carbo-BIND™ 96 well plates	Sigma-Aldrich, UK
Plain microscope slides	Sigma-Aldrich, UK
Plastic cover slips	Sigma-Aldrich, UK
Vivaspin® 500 ultrafiltration spin columns (10,000 MW)	Sartorius, UK

Ready to use kits

Kits	Catalogue No	Supplier
Colloidal blue kit	LC6025	Invitrogen, Paisley, UK
Fluorokine® E human active MMP-1 fluorescent assay	F1M00	R&D Systems
Human Total MMP-1 DuoSet ELISA kit	DY901	R&D Systems
VECTASTAIN elite ABC HRP kit, R.T.U.	PK-7200	Vector Laboratories
VECTOR VIP Peroxidase (HRP) substrate kit	SK-4600	Vector Laboratories
Silver Stainlus™ kit	16104	Bio-Rad

Software

Software	Supplier
Adobe Photoshop® CS5 for Windows XP	Adobe Systems, Inc.
Adobe Acrobat Reader DC for Mac	Adobe Systems, Inc.
GeneSnap 6.00.22© 1993-2002	Synoptics Ltd.
Microsoft® Excel for Mac (version 15.30)© 2017 Microsoft	Microsoft® United Kingdom
Prism 7 for Windows and Mac (version 7.01)©	GraphPad Software Inc.
Softmax® Pro© 1999-2009	MDS Analytical Technologies Inc.
SPOT Advanced imaging ™ software	SPOT Imaging ™ Solutions Inc.
NDP NanoZoomer © Digital (view 2)	Hamamatsu Photonics
ImageJ software	Open Source

Equipment

Equipment	Supplier
Envair BIO2+ microbiological safety cabinet class II	Envair Ltd.
Flexstation® 3 benchtop multi-Mode microplate reader	Molecular Devices Inc.
GeneGenius Gel Doc	Syngene Ltd.
MicroLab MK8 spirometer	MicroMedical
Mini Trans-Blot® electrophoresis transfer cell	Bio-Rad
Mistral 3000i refrigerated centrifuge	MSE (UK) Ltd.
NIOX MINO® single breath FeNO measurement kit	NIOX (Circassia) Ltd.
Olympus IX51/71/81 microscope	Olympus Inc.
Nikon Diaphot 300 inverted microscope	Diagnostic Instruments Inc., MI, USA
TRIO thermocycler	Biometra GmbH Ltd.
Fibreoptic bronchoscope (PF260)	Olympus Inc.
Bronchial biopsy forceps 1.8mm radial jaw (1523)	Boston Scientific, USA

Antibodies

Antibodies	Host	Clone	Isotype	Catalogue no	Concentration	Supplier
α-SMA	Mouse	B4	IgG2a	A2547	1:500	Roche Diagnostics
α-SMA-Cy3™	Mouse	1A4	IgG2	C6198	1:250	Sigma-Aldrich
Ki67	Rabbit	43008	IgG	790-4286	3-25 µg/mL	Roche Diagnostics
Mouse IgG HRP	Goat	Poly	IgG	HAF007	1:1000	R&D Systems
Mouse IgG HRP	Horse	Poly	IgG (H&L chain)	PI-2000	1:500	Vector Laboratories
Human lung tryptase	Mouse	AA1	IgG1	Ab2378	1:100	Roche Diagnostics
Recombinant human MMP-1	Mouse	3665	IgG1	MAB901	8-25 µg/mL	R&D Systems
Rabbit IgG HRP	Horse	Poly	IgG	MP-7401	RTU	Vector Laboratories

Reagents

Reagents/Chemicals	Catalogue no	Supplier
100 bp ladder	15628-019	Invitrogen, Paisley, UK
1KB ladder	15615-016	Invitrogen, Paisley, UK
Acetic acid (glacial)	A-0400-PB-17	Fischer Scientific, UK
4-Aminophenylmercuric acetate (APMA)	A9563	Sigma-Aldrich, UK
Ammonium hydroxide solution	221228	Sigma-Aldrich, UK
Antibiotic antimycotic solution	A5955	Sigma-Aldrich, UK
APC 366 (tryptase inhibitor)	2511	Tocris Bioscience
BEGM (Basal epithelial growth medium)	CC2547	Lonza, Cambridge, UK
BEGM bullet-kit	CC2552	Lonza, Cambridge, UK
Budesonide (powder, ≥99%)	B7777	Sigma-Aldrich, UK
BSA (Bovine serum albumin)	A-3059	Sigma-Aldrich, UK
Calcium ionophore (A23187)	C9275	Sigma-Aldrich, UK
Chymostatin (specific chymase inhibitor)	230790	Calbiochem (Merck Millipore)
Collagen -1 (human placental)	C7774	Sigma-Aldrich, UK
Collagenase A	1088793	Roche Diagnostics, UK
cOmplete™ Mini, EDTA-free protease inhibitor Cocktail	1183617001	Roche, West Sussex, UK
Coomasie Blue	443283M	BDH Chemicals
Colour Reagent A (stabilised hydrogen peroxide)	895000	R&D Systems
Colour Reagent B (stabilised tetramethylbenzidine)	895001	R&D Systems
DAPI (4',6-diamidino-2-phenylindole)	32670	Sigma-Aldrich, UK
Deoxyribonuclease I (DNase I)	1.11285E+11	Roche Diagnostics, UK
Developer and replenisher (for autoradiography)	P7042	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO >99.9%)	154938	Sigma-Aldrich, UK
DMEM (Dulbecco's modified eagle's medium)	D5796	Sigma-Aldrich, UK
DMEM: nutrient mixture F-12 (phenol red free)	21041-025	Sigma-Aldrich, UK
Dried skimmed milk (340g pack)	N/A	Marvel
ECL western blotting detection reagent	RPN-2232	Amersham, GE Healthcare
EDTA (Ethylenediaminetetraacetic acid)	100935-V	BDH Chemicals
Eosin Y	230251	Sigma-Aldrich, UK
Ethanol (95%)	10107	BDH Chemicals
Fixer and replenisher (for autoradiography)	P8307	Sigma-Aldrich, UK
Foetal calf serum (FCS)	A15-152	Invitrogen, Paisley, UK
Formaldehyde (37%)	533998	Sigma-Aldrich, UK
Glycine	G8790	Sigma-Aldrich, UK
Glycerol	BPE299-1	Fischer Scientific, Loughborough, UK
Goat serum	G6767	Sigma-Aldrich, UK
Haematoxylin (Mayer's)	H3136	Sigma-Aldrich, UK

HBSS (Hank's balanced salt solution)	14175095	Fischer Scientific, UK
Histoclear	C78-G	Raymond A. Lamb
Horse serum (2.5%)	S-2012	Vector Laboratories
Hydrochloric acid	10125	BDH Chemicals
Hyperfilm	28906837	GE Healthcare
Hydrogen peroxide (30%)	H1009	Sigma-Aldrich, UK
IMDM (Iscove's modified dulbecco's medium) GlutaMAX™	31980030	Thermo Fischer Scientific
Immersion oil	10976	Sigma-Aldrich, UK
INTERFERin® siRNA transfection reagent	409-10	Polyplus Transfection
Isobutanol	15828-0010	Fischer Scientific, UK
Magnesium chloride	M-8266	Sigma-Aldrich, UK
Methacholine (Provocholine®)	46841	Methapharm Inc. UK
Methanol	M/4000/17	Fisher Scientific, UK
Mineral oil	M-3516	Sigma-Aldrich, UK
Mini-PROTEAN® TGX™ gels (4–15%)	456-1083S	Bio-Rad
MMP-1 specific siRNA sequence	104015	Thermo Fischer Scientific
MMP-1 Silencer® negative control no. 1 RNA sequence	AM4611	Thermo Fischer Scientific
MMP inhibitor ilomastat (GM6001)	CC10	Sigma-Aldrich, UK
MTT (powder, ≥97.5%, cell culture grade)	M-5655	Sigma-Aldrich, UK
Opti-MEM® reduced serum medium	31985070	Thermo Fischer Scientific
Penicillin & Streptomycin (10x)	P0781	Sigma-Aldrich, UK
Phosphate buffered saline (non-sterile tablets)	BR0014	Oxoid, UK
Phosphate buffered saline (sterile solution)	14200-067	Sigma-Aldrich, UK
Phorbol-12-myristate-13-acetate (PMA)	P1585	Sigma-Aldrich, UK
Platelet derived growth factor (PDGF)	100-14B	PeproTech, UK
Propan-2-ol	10224	BDH Chemicals
Protein reagent dye concentrate	5000-0006	Bio-Rad
Polyvinylidene di-fluoride (PVDF) P- 0.2 membrane	10600021	Amersham Hybond, GE Healthcare
Precision plus kaleidoscope standards	161-0375	Bio-Rad
Recombinant human mast cell tryptase	G7061	Promega, Southampton, UK
Recombinant Human MMP-1	SRP3117	Sigma-Aldrich, UK
Recombinant Human MMP-9	WBC018	R&D Systems
See Blue plus-2 prestained standard	LC5925	Invitrogen, Paisley, UK
Serine protease inhibitor cocktail	BPE9721-1	Fischer Scientific, UK
Sodium dodecyl sulphate (SDS)	L5750	Sigma-Aldrich, UK
Sodium thiosulfate (99%)	217263	Sigma-Aldrich, UK
Streptavidin-horseradish peroxidase	4800-30-06	R&D Systems
Sulphuric acid (2N)	895032	R&D Systems
Thrombin	T6884	Sigma-Aldrich, UK
Tris-Gly SDS zymogram running buffer	LC2675	Invitrogen, Paisley, UK

Tris-Gly SDS zymogram sample buffer	LC2673	Invitrogen, Paisley, UK
Triton X-100	T8787	Sigma-Aldrich, UK
Trypsin-EDTA solution (x10)	T3924	Sigma-Aldrich, UK
Tween®	P7949	Sigma-Aldrich, UK
UltraPure Tris	15504-020	Invitrogen, Paisley, UK
VectaMount permanent mounting medium	H-5000	Vector Laboratories
Xylene cyanol	4126	Sigma-Aldrich, UK
Zymogram developing buffer (x10)	LC2674	Invitrogen, Paisley, UK
Zymogram pre-cast Gelatin gels (Novex™ 10%)	EC61752BOX	Invitrogen, Paisley, UK
Zymogram renaturing buffer (x10)	LC2671	Invitrogen, Paisley, UK