Mechanisms of Airway Remodelling

A thesis submitted for the degree of

Doctor of Philosophy

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I hereby declare that I am the sole author of this thesis.

I authorise the University of Sydney to lend this thesis to other institutions or individuals for the purpose of scholarly research.

Abstract

Asthma is an inflammatory disease characterised by tissue remodelling. A prominent feature of this remodelling is an increase in the number and size of the blood vessels- formed from pre-existing capillaries – angiogenesis (Siddiqui *et al.*, 2007; Wilson, 2003). This is triggered by many different endogenous angiogenic stimulators such as vascular endothelial growth factor (VEGF), and inhibited by endogenous angiogenic inhibitors such as tumstatin. Tumstatin is the non-collagenous domain (NC1) of the collagen IV α3 chain which, when cleaved, inhibits endothelial cell proliferation and induces apoptosis. Experiments described in this thesis have for the first time demonstrated the absence of tumstatin in the airways of individuals with asthma and lymphangioleiomyomatosis (LAM) as well as the functional responses to tumstatin as an angiogenic inhibitor, both *in vitro* and *in vivo,* in the airway.

Although tumstatin was absent from the airways of asthmatic and LAM individuals it was present in the airways of individuals with no airways disease, chronic obstructive pulmonary disease, bronchiectasis and cystic fibrosis. No significant difference was seen in the levels of the Goodpasture Binding Protein (GPBP), a phosphorylating protein responsible for the alternate folding of tumstatin, between asthmatic, LAM and individuals with no airways disease. The αvβ3 integrin, reported to be necessary for the activity of tumstatin, as well as the individual αv and β3 sub-units were shown to be equally expressed in the airways of all patient groups. Co-localisation of tumstatin, VEGF and the αvβ3 integrin was seen in the disease free airways, however, a different

pattern of VEGF and the αvβ3 integrin expression was observed in asthmatic and LAM airways with minimal co-localisation.

Tumstatin was detected in serum and bronchoalveolar lavage fluid (BAL-f) samples from asthmatics and individuals with no airway disease, however there was no significant difference in the level of expression between the two groups. It was demonstrated that the tumstatin detected in the serum and BAL-f samples from asthmatics and individuals with no airway disease was part of the whole collagen IV α3 chain and not in its free and potentially active form.

The ability of recombinant tumstatin to inhibit tube formation and proliferation of primary pulmonary endothelial cells was demonstrated for the first time. Further, the functional response of tumstatin was demonstrated *in vivo* in a mouse model of allergic airway disease. Tumstatin inhibited angiogenesis in the airway and decreased airway hyperresponsiveness.

Whether there is potential for tumstatin, or a derivative thereof, to be of therapeutic value in airways diseases in which angiogenesis is a component should be the subject of future studies.

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Publications, presentations and posters arising from this work

ABSENCE OF THE ENDOGENOUS ANGIOGENIC INHIBITOR TUMSTATIN IN ASTHMATIC AIRWAYS. Sarah Boustany MSc, Brian G. Oliver PhD, Lyn M.Moir PhD, Melissa Baraket, Philip M. Hansbro, Nicole G. Hansbro, Paul S. Foster, Judith L. Black MBBS PhD and Janette K. Burgess PhD 2008. Paper submitted.

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Sarah Boustany, Maree.H. Poniris, Brian.G. Oliver, Lyn.M. Moir, Judith.L. Black, Amy McLean and Janette.K. Burgess 2007. Oral presentation at the 12th Asia Pacific Society of Respirology meeting, Gold Coast, Australia, 2007.

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ABSENCE OF AN ENDOGENOUS ANGIOGENIC INHIBITOR IN ASTHMATIC AIRWAY TISSUE. S. Boustany, M.H. Poniris, B.G. Oliver, L.M. Moir, J.L. Black, A.McLean and J.K. Burgess 2007. Poster presentation at the American Thoracic Society annual meeting, San Francisco, USA, 2007.

ANTI-ANGIOGENIC FACTOR TUMSTATIN AND ITS ABSENCE IN LUNG TISSUE FROM PATIENTS WITH LYMPHANGIOLEIOMYOMATOSIS. **S Boustany**, MH Poniris, BG Oliver, JL Black, LM Moir and JK Burgess 2007. Poster presentation at the 6th International Young Investigators' Symposium on Smooth Muscle, Sydney, Australia, 2007.

List of abbreviations

AHR	Airway hyperresponsiveness
Akt	Protein kinase B
Ang-1	Angiopoietin I
ANOVA	Analysis of variance
ASM	Airway smooth muscle
BAL-f	bronchoalveolar lavage fluid
bFGF	basic Fibroblast growth factor
BM	Basement membrane
BSA	Bovine serum albumin
CD31	Cluster of differentiation-31
Cdyn	Dynamic compliance
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic obstructive pulmonary disease
CT	Computed tomography
DAB	liquid 3, 3'- diaminobenzidine
DMEM	Dulbecco's modified eagle's medium
ECG	Endothelial cell growth supplement
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid solution salt
eIF4E/4E-BP1	Eukaryotic translation initiation factor 4E/eukaryotic initiation factor 4E-binding protein 1
eNOS	Endothelial nitric oxide synthase
Eph-B2	Ephrin-B2
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FEV_1	Forced Expiratory Volume in the first second
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FVC	Forced vital capacity
GBM	Glomerular basement membrane
GINA	Global initiative for asthma
GOLD	Global initiative for chronic obstructive disease
GPBP	Goodpasture antigen-binding protein
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IL-1β	Interleukin- 1beta
IN	Intranasally
LAM	Lymphangioleiomyomatosis
MMPs	Matrix metalloproteinase
mTOR	Mammalian target of Rapamycin
NC	Nitrocellulose
NC1	Non-collagenous domain 1
OVA	Ovalbumin
PBS	Phosphate buffered saline
PDGF-BB	Platelet-derived growth factor-BB
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PenH	Enhanced respiratory pause

PI-3 kinase	Phosphatidylinositol-3 kinase
PP2A	Protein phosphatase 2A
PVDF	Polyvinylidene difluoride
PVM	Pneumonia virus of mice
RBM	Reticular basement membrane
RL	Transpulmonary resistance
RSV	Respiratory syncitial virus
SAL	Saline
SDS	Sodium dodecyl sulphate
TGFβ	Transforming growth factor beta
TNFα	Tumour necrosis factor alpha
TSC	Tuberous sclerosis complex
T-TBS	Tween-tris buffered saline
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth receptor
VRI	Viral respiratory infection
vWF	von Willebrand factor

Chapter 1

Introduction

1.1 Chronic respiratory diseases

Chronic respiratory disease was the third leading cause of death in the world in 2002, with 4 million deaths reported worldwide (Yach *et al.*, 2004). Currently, 300 million people are suffering from asthma (Masoli *et al.*, 2004) and approximately 80 million people have moderate to severe chronic obstructive pulmonary disease (COPD) (Lopez *et al.*, 2006). Cystic fibrosis (CF) is the most common autosomal genetic disorder in the Caucasian population with an estimated frequency of 1: 3,400 live births (McColley *et al.*, 2000). Lymphangioleiomyomatosis (LAM) and bronchiectasis are less common, but nevertheless are associated with considerable morbidity and mortality. Although the cause of most respiratory diseases is unknown, there are several contributing factors which may be environmental and/or genetic.

1.1.1 Asthma

Asthma is a chronic respiratory disease characterised by airway inflammation, airflow obstruction, bronchial hyperresponsiveness and airway remodelling. The global prevalence of asthma as well as morbidity and mortality rates have been increasing for the last 40 years (Braman, 2006). The main symptoms of asthma include cough, shortness of breath, tightness in the chest and wheezing. The most common triggers include viral respiratory infections, exercise, inhaled allergens (e.g. pollens, moulds, animal hair and dust mite), cigarette smoke, some foods and food preservatives and some occupations. Diagnosis of asthma requires a physical

examination, assessment of the reversibility of airway obstruction and the exclusion of an alternative diagnosis that mimics asthma. Asthma is associated with an increase in the rate of decline in forced expiratory volume in the first second (FEV_1), however, the effect of asthma is variable and not all subjects have steep rates of decline (Bousquet *et al.*, 2000). Symptoms of asthma are now effectively controlled in most patients with current therapy, which consists of inhaled corticosteroids, long acting or short acting β_2 agonists, anti-cholinergics and monoclonal anti-IgE therapy. However, although symptoms are well controlled, aspects of asthma pathology such as airway remodelling may not be prevented or reversed.

1.1.2 Lymphangioleiomyomatosis (LAM)

Lymphangioleiomyomatosis (LAM) is a rare lung disease of uncertain etiology which is almost exclusively confined to women. However, there have been three cases reported to date of men who have been diagnosed with LAM (Schiavina *et al.*, 2007). It is progressive and often fatal. Pulmonary LAM can occur either independently or in association with tuberous sclerosis complex (TSC), a tumor-suppressor gene syndrome caused by mutations that inactivate either TSC1 or TSC2. Histologically LAM is characterised by proliferation of abnormal smooth muscle cells (LAM cells) in the lungs, lymph nodes and/or other organs. The architecture of the LAM lung is grossly altered, with loss of alveolar structure, thickening of interstitial connective tissue and the development of cystic spaces (Black *et al.*, 2005; Merrilees *et al.*, 2004). Clinical symptoms of LAM include shortness of

breath, chest pain, frequent cough with haemoptysis, fatigue, as well as recurrent spontaneous pneumothorax and progressive respiratory failure (El-Hashemite *et al.*, 2005). Diagnosis of LAM is often difficult, as this disease presents with similar symptoms to those of asthma, emphysema and bronchitis. Chest x-rays, high-resolution computed tomography (CT) scans, lung function testing as well as bronchial biopsies are often required in diagnosing LAM. Currently there are no known effective treatments for LAM, making lung transplantation the only possibility for survival.

1.1.3 Chronic obstructive pulmonary disease (COPD)

Chronic obstructive pulmonary disease (COPD) is a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is progressive and associated with an abnormal inflammatory response (increased inflammatory cell infiltrate) of the lungs to noxious particles or gases such as cigarette smoke and pollutants (Pauwels *et al.*, 2001). COPD is a major cause of morbidity and mortality across the world and its prevalence continues to increase. A population-based study (12 sites worldwide) which identified the prevalence of COPD in stages of severity (stage I- mild, II-moderate and III severe COPD) showed the overall prevalence of stage II COPD or higher was 10.1% and the risk of having stage II COPD or higher increased with age and smoking (Buist *et al.*, 2007). Diagnosis of COPD, according to the global initiative for chronic obstructive disease (GOLD) standards, should be considered in any patient showing symptoms of cough, sputum production or dyspnea. Confirmation of

diagnosis is made by spirometry. The presence of a postbronchodilator FEV_1<80% of the predicted value in combination with an FEV_1/ forced vital capacity (FVC) ratio of <70% confirms the presence of airflow limitation that is not fully reversible. The aim of pharmacological treatment of COPD is to prevent or control symptoms as well as to decrease the frequency and severity of exacerbations. This treatment consists of short-acting bronchodilators and inhaled corticosteroids. However, the current therapeutic regimen does not modify the long term decline in lung function.

1.1.4 Cystic fibrosis (CF)

Cystic fibrosis (CF) is the most common life-shortening autosomal genetic disorder in western society. CF results from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). Pulmonary infection is the dominant clinical feature, however CF manifests as a multiorgan disease which involves the pancreas, salivary glands, genital tubes and liver canaliculi (Moraes *et al.*, 2006). The genetic defect in this disease leads to a greater susceptibility to chronic bacterial infections of the lungs, with *Pseudomonas* species the most common cause. Diagnosis of CF is considered in patients who show manifestations of chronic pulmonary disease and exocrine pancreatic insufficiency. Confirmation of diagnosis is by a positive sweat test which reveals elevated chloride levels. Patients may also present with pancreatic sufficiency or other atypical clinical features sometimes in association with normal or borderline sweat test results. In such cases, detection of CF mutations and measurement of the transepithelial bioelectric

properties can be diagnostically useful (Ware, 2007). Mutation analysis can also be used for carrier and newborn screening, and prenatal diagnosis. Chronic macrolide therapy and inhaled corticosteroids are used as a form of treatment for patients with CF. It is important to note these treatments are only able to reduce the frequency of exacerbations and not necessarily improve lung function (Ware, 2007).

1.1.5 Bronchiectasis

Bronchiectasis is classified as an uncommon disease. Little is known about its prevalence worldwide and its health burden in Australia is unknown. However, bronchiectasis has the potential to cause devastating illness, including repeated infections requiring antibiotics, disabling productive cough, shortness of breath and occasional haemoptysis (Barker, 2002). The pathogenesis of bronchiectasis is not fully elucidated, however this disease is characterised by permanent abnormal dilation of the bronchi, as well as the destruction of the bronchial wall caused by continuous extracellular matrix (ECM) damage. Clinically, bronchiectasis is characterised by recurrent purulent sputum production (Lee *et al.*, 2007; Zheng *et al.*, 2002). Management of this disease is not simple. Therapy includes identification of acute exacerbations and administration of antibiotics to suppress microbial load, promotion of bronchial hygiene (removal of respiratory secretions), control of bronchial hemorrhage (surgical removal of areas subject to uncontrolled hemorrhage) and surgical removal of damaged segments or lobes that may be a nidus for infection or bleeding (Barker, 2002).

In this thesis the focus of experiments is asthma and LAM. Tissue obtained from patients with COPD, bronchiectasis and CF has been studied for the purpose of comparison, since these diseases share some, but not all of the features of asthma and LAM.

1.2 Airway remodelling

Airway remodelling is a hallmark feature of both asthma and LAM. Remodelling results from an ongoing response to stimuli such as inflammation, injury or mechanical stress in which an attempt is made to repair the lungs. This leads to permanent structural and functional changes. Remodelling can be defined as an alteration in the size, mass or number of tissue structural components that occur during growth or in response to injury or inflammation (Jeffery, 2001). The concept of remodelling suggests that a 'modelling' process, considered to be normal, must have preceded these events. 'Modelling' in the lungs starts from about 30 weeks of gestation, when alveoli begin to form. At birth, the number of alveoli is between 40-100 million. Up to 8 years of age, the alveoli continue to multiply to an adult number of 300 million. This process is thought of as normal 'modelling'. Wound healing and repair also falls under the category of normal 'modelling', with formation of fluid exudate and oedema, cellular infiltration involving neutrophils, monocytes and lymphocytes, restitution of the epithelium and proliferation of blood vessels and fibroblasts constitute a normal response. Normal 'modelling' turns into abnormal 'remodelling' when that remodelling becomes chronic. Airway remodelling in asthma is characterised by thickening of the basement membrane, an increase in the mass of smooth muscle, ECM

deposition, an increase in the number of blood vessels, mucus gland hypertrophy and epithelial metaplasia (Chiappara *et al.*, 2001; Locke *et al.*, 2007). (Figure 1.1)

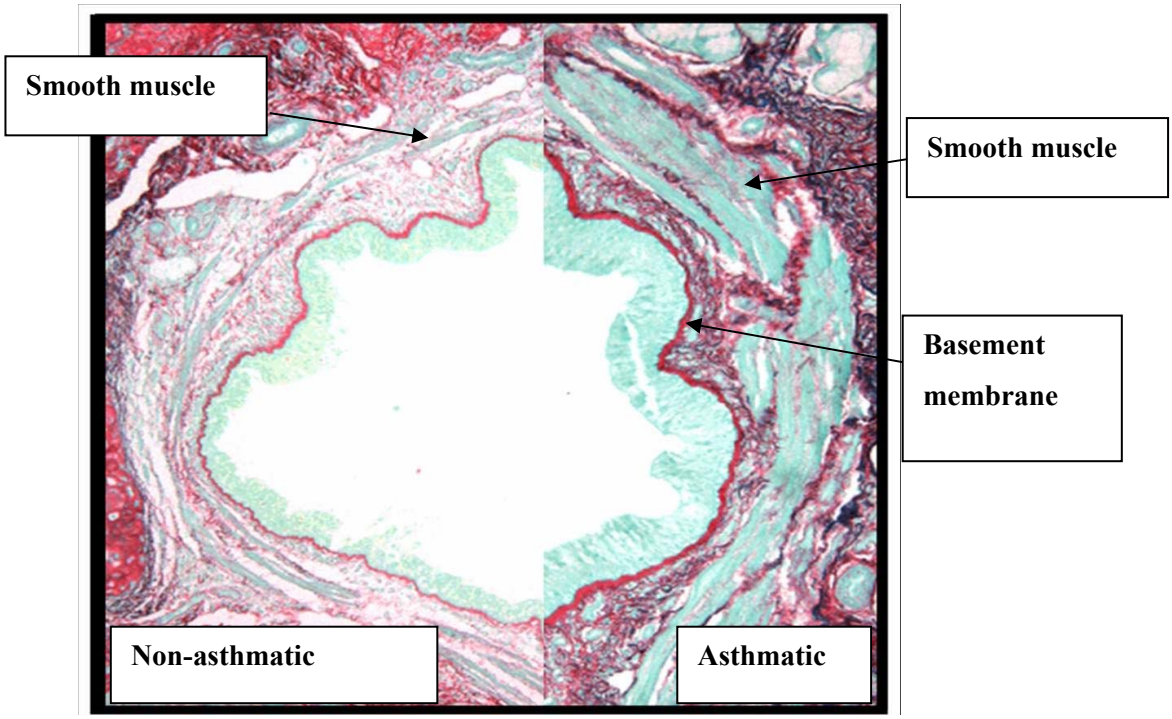


Figure1.1: Section through a bronchus obtained from an asthmatic (right hand side) and a non-asthmatic (left hand side) patient age and sex matched. Sirius red was used to stain collagenous structures and picric green for all non-collagenous structures. Increased smooth muscle and thickened basement membrane are features of the remodelled airway. (Images at X200)

1.2.1 Increased airway smooth muscle (ASM)

A review by James *et al* (James *et al.*, 2000) confirms that all studies conducted to determine the amount of smooth muscle in asthmatic airways, irrespective of the method used, showed an increase in the amount of smooth muscle in asthmatic airways compared with those of non-asthmatic subjects. It is now well established that the increase in airway smooth muscle (ASM) in asthmatic airways is attributed to hyperplasia (increase in cell number) (Hirst *et al.*, 2004; James *et al.*, 2000; Panettieri, 2003; Yamauchi, 2006), although hypertrophy (increase in cell size) is also thought to contribute. A histological study by Ebina *et al.* examined the distribution of hypertrophic smooth muscle cells in the airway. They identified the most pronounced smooth muscle hypertrophy to be localised in the larger bronchi (Ebina *et al.*, 1993). Hyperplasia is an increase in cell number, resulting from an increase in proliferation or decreased rates of apoptosis. Johnson *et al.* showed that ASM cells isolated from asthmatics proliferate *in vitro* at a greater rate than ASM cells from non-asthmatics (Johnson *et al.*, 2001). They further proposed that the enhanced proliferation seen in asthmatic ASM cells may be mediated by the influence of the ECM released by the cells themselves (Johnson *et al.*, 2004). The mechanism of ASM proliferation has been investigated and a number of pathways have been implicated. Polypeptide growth factors that activate receptors were shown to induce smooth muscle cell proliferation through receptor tyrosine kinase and agonists that bind receptors linked to heterotrimeric guanosine triphosphate-binding proteins (Hirst *et al.*, 2004; Trian *et al.*, 2007). Airway narrowing is thought to be induced by smooth

muscle contraction. ASM hypertrophy and hyperplasia which occur in the remodelled airway increase muscle bulk and therefore contribute to airway narrowing and airway hyperresponsiveness (Yamauchi, 2006).

1.2.2 Thickening of the basement membrane

Thickening of the reticular basement membrane (RBM) is one of the first changes to occur in the remodelling process seen in asthma. It is a well established characteristic feature of asthma. The RBM is not present in the foetus but develops later in normal healthy individuals during infancy. Remodelling of the RBM occurs early in asthma. Jeffery examined biopsies taken from children with a persistent wheeze, who had been unresponsive to high doses of corticosteroids. There was a significant increase in the thickness of the RBM in asthmatic compared with non-asthmatic children (Jeffery, 2001). Niimi *et al.* measured the RBM from 81 asthmatic and 28 non-asthmatic patients and concluded that airway wall thickening occurs in patients with asthma and is not limited to those with severe asthma (Niimi *et al.*, 2000). However, a study by Chetta *et al.* showed that the degree of thickening of the RBM was positively correlated with asthma severity (Chetta *et al.*, 1997). Several studies have shown thickening of the RBM to be due to collagen deposition and an increase in subepithelial myofibroblasts. An excess of interstitial collagens beneath the RBM has been demonstrated in asthmatic subjects, with collagen type III and V specifically elevated (Roche *et al.*, 1989). A study by Brewster *et al.* showed a significant correlation between the depth of collagen deposition and the number of

myofibroblasts in the basement membrane (Brewster *et al.*, 1990). The thickening of the RBM has clinical implications for asthmatic patients. It was concluded by Hoshino *et al.* that the thickening of the RBM was due to an increase in fibroblasts and an increase in subepithelial collagen, and this appeared to be linked to an increase in bronchial responsiveness and exacerbation of symptoms (Hoshino *et al.*, 1998b).

1.2.3 Altered extracellular matrix protein deposition

The ECM in the airway acts as a mechanical support to surrounding tissues. The ECM plays an important role in the maintenance of airway structure and has the ability to influence different cellular functions. Overproduction of matrix molecules is a major contributor to the permanent loss of normal tissue structure and function. Alteration in airway ECM protein deposition has been reported in asthma and is one of the prominent features of remodelling. Histological studies of the ECM in asthmatic airways have shown an increase in collagen I, III and V as well as fibronectin, tenascin, versican and laminin (Laitinen *et al.*, 1997; Laitinen *et al.*, 1996; Roche *et al.*, 1989). In contrast, Bousquet *et al.* described an abnormal superficial elastic fibre network in the asthmatic airways. They reported not only a decrease in elastin, but also an abnormal elastolytic process (Bousquet *et al.*, 1996). In addition, the levels of collagen IV were decreased in asthmatic airways compared to non-asthmatics (Bousquet *et al.*, 1992). The significance of the altered ECM and its role in the pathogenesis of asthma is not well understood. Three dimensional models of the bronchial wall are

being studied to examine the importance of mechanical strain on ECM remodelling and the potential consequences in the airway (Choe *et al.*, 2006).

1.2.4 Increased angiogenesis

Angiogenesis is the growth of new capillary blood vessels from pre-existing vasculature. It is a vital process required for embryogenesis, growth, tissue repair after injury, and a normal function in the female reproductive cycle. Bronchial vasculature is essential for maintaining homeostasis, which includes the provision of oxygen and nutrients, temperature regulation and humidification of inspired air, as well as providing the primary portal for the immune response to inspired organisms and antigens (Wilson *et al.*, 2002). Under physiological conditions, angiogenesis is regulated by a balance of pro- and anti-angiogenic factors within the vascular microenvironment (Puxeddu *et al.*, 2005).

Vascularity is an important component of the remodelling process in airway disease and increased vascularity, angiogenesis, is likely to occur in response to chronic inflammation. Increased angiogenesis in the airways of asthmatics is well reported (Hashimoto *et al.*, 2005; Li *et al.*, 1997) and is now considered to be one of the major components of airway remodelling in asthma. In healthy individuals, up to 10% of a bronchial biopsy section may be covered with vessels and therefore there are over 500 vessels/mm^2, whereas, in an asthmatic biopsy, up to 17% of the area is vascular with over 700vessels/ mm^2 (Li *et al.*, 1997). Wilson *et al.* suggested that the angiogenic process in asthma could be due to recurrent inflammatory episodes, or a

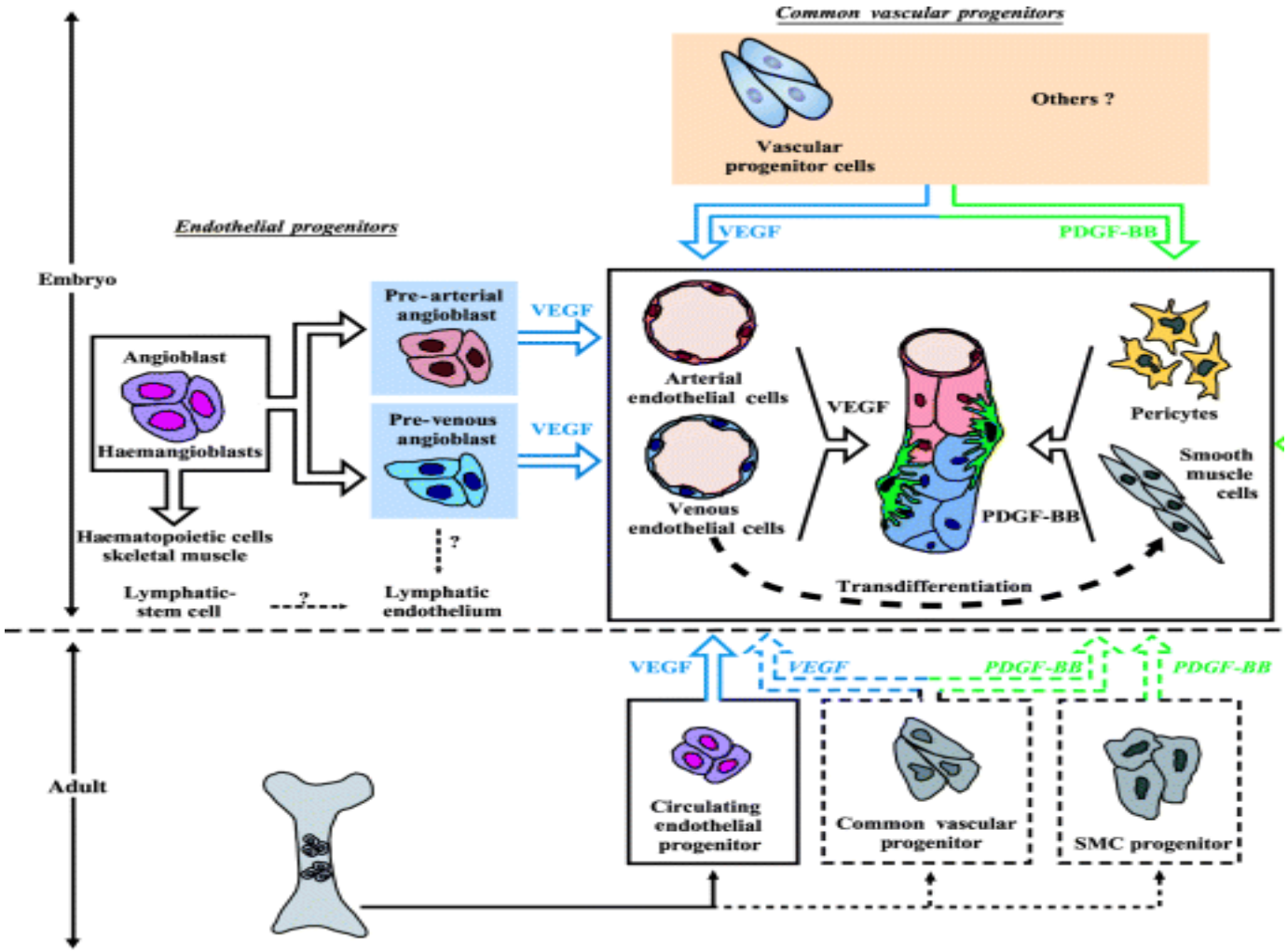
response to the two- to threefold increase in tissue volume that occurs due to remodelling (Wilson *et al.*, 1999). Blood vessel size has also been shown to be larger in the asthmatic airway (Kuwano *et al.*, 1993; Li *et al.*, 1997). Cross-sectional measurements of blood vessels showed over 19% of blood vessels in the asthmatic airway to have an area greater than 300μm^2, compared to 12% in non-asthmatic airways (Li *et al.*, 1997). Increased angiogenesis is also reported to be a pathological feature of remodelling in COPD, CF and bronchiectasis (Jeffery, 1998; McColley *et al.*, 2000; Shi *et al.*, 2007).

1.3 Angiogenesis

When endothelial cells attach to the basement membrane of an intact capillary, which is composed of collagen IV, laminin, heparin sulphate proteoglycans, perlecan, collagen XVIII and other molecules, they receive signals that promote cell-cell adhesion and inhibit proliferation. At the onset of angiogenesis, enzymes such as matrix metalloproteinases (MMPs), which are produced by smooth muscle cells, endothelial cells or immune cells recruited to the site of inflammation, disassemble the highly cross-linked basement membrane and release pro-angiogenic growth factors. The degradation of the basement membrane enables the endothelial cells to migrate and proliferate and exposes different domains on the ECM proteins that would not normally be available for interaction with the endothelial cells (Carmeliet, 2004) (figure 1.2). Within a given microenvironment, the

angiogenic response is determined by a net balance between pro- and anti-angiogenic regulators

Figure 1.2: Vascular progenitors in the embryo and adult. In the embryo, endothelial, smooth muscle and common vascular progenitors contribute to vascular development. Smooth muscle cells have different origins, as indicated. The effect of VEGF and platelet-derived growth factor BB (PDGF-BB) on these progenitors is indicated. Figure has been adapted from Carmeliet (2004).



released from activated endothelial cells, monocytes, smooth muscle cells and platelets.

1.3.1 Angiogenic promoters

Angiogenic promoters are responsible for triggering and sustaining angiogenesis. The list of pro-angiogenic cytokines and growth factors is extensive and these factors are secreted by inflammatory cells (mast cells and macrophages), pericytes, keratinocytes (during epidermal wound healing), tumor cells, smooth muscle and fibroblasts. Table 1.1 lists some of the known angiogenic promoters and their role in angiogenesis (D'Andrea *et al.*, 2006; Rundhaug, 2005).

Several angiogenic promoters have been reported to be increased in asthma, including vascular endothelial growth factor (VEGF), transforming growth factor β (TGFβ), Tumor necrosis factor α (TNFα) and Basic fibroblast growth factor (bFGF) (D'Amore, 1992).

Table 1.1: List of angiogenic promoters and their role in angiogenesis

Angiogenic promoters	Role in angiogenesis
Vascular endothelial growth factor (D'Amore, 1992).	Induces proliferation, migration and tube formation of endothelial cells
Transforming growth factor β (D'Amore, 1992).	Up-regulation of VEGF and proteinases
Tumor necrosis factor α (D'Amore, 1992).	Stimulates migration of endothelial cells
Basic fibroblast growth factor (D'Andrea *et al.*, 2006).	Induces endothelial cell replication, migration and extracellular proteolysis
Angiopoietin I and II (Rundhaug, 2005)	Stimulates vessel and endothelial cell growth and capillary tube formation Stimulates the growth of immature tumour vessels
Platelet-derived growth factor (Rundhaug, 2005)	Stabilisation of nascent blood vessels via coverage with smooth muscle

1.3.1.1 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) induces proliferation, migration and tube formation of endothelial cells. It is a homodimeric protein belonging to the cystine knot growth factor family. The VEGF gene is expressed as four different isoforms, $VEGF_{121}$, $VEGF_{189}$, $VEGF_{206}$ and $VEGF_{165}$ of which $VEGF_{165}$ is the most abundant and most intensely studied. $VEGF_{165}$ is a soluble protein although approximately half of the secreted amount remains bound to the cell surface heparin sulphate proteoglycan and the ECM (D'Andrea *et al.*, 2006). The effects of VEGF on endothelial cells are mediated through signals generated by binding to receptor tyrosine kinases (RTKs). Several RTKs for VEGF have been identified, vascular endothelial growth receptor (VEGFR)-1, VEGFR-2 and VEGFR-3. VEGFR-1 and VEGFR-2 are mostly expressed on endothelial cells, VEGFR-2 is associated with integrin-dependent endothelial cell migration because it forms a complex with integrin αvβ3 (D'Andrea *et al.*, 2006; Puxeddu *et al.*, 2005). VEGFR-1 and VEGFR-2 are the two main receptors for VEGF-signalling in human airways (Yancopoulos *et al.*, 2000).

VEGF plays a role in vascular remodelling (Lee *et al.*, 2004). It is produced by ASM cells, and TGFβ, TNFα and interleukin-1 β (IL-1β), all of which are increased in asthma, have been shown to increase VEGF expression (Burgess *et al.*, 2006; Kazi *et al.*, 2004; Knox *et al.*, 2001). Feltis *et al.* reported an increase in VEGF concentration in bronchoalveolar lavage fluid (BAL-f) in asthmatics (Feltis *et al.*, 2006). In addition, increased VEGF levels were also reported in induced sputum of asthmatics (Kanazawa *et al.*, 2004; Lee *et al.*,

2001). Bronchial biopsies from asthmatic subjects express higher levels of VEGF, VEGF mRNA and VEGF receptors (Hoshino *et al.*, 2001a). The increased vessel numbers seen in asthmatic airways are associated with increased VEGF expression. Chetta *et al.* demonstrated an association between increased numbers of VEGF positive cells and a thickened basement membrane in bronchial biopsies from asthmatic patients (Chetta *et al.*, 2005). What is the significance of VEGF expression in the airways of asthmatics? It has been suggested that VEGF may contribute to airway remodelling by altering the ECM composition (Chetta *et al.*, 2005). Knox *et al.* proposed two theories regarding the significance of the increase in VEGF; increased airway wall thickness would cause enhanced airway narrowing on stimulation, thereby contributing to airway hyperresponsiveness; increased bronchial vasculature could also increase airway hyperresponsiveness by supporting the increased airway smooth muscle mass which is the hallmark feature of asthma (Knox *et al.*, 2005).

1.4 Angiogenic inhibitors

Endogenous angiogenic inhibitors are naturally present in body fluids and tissue. They are potentially able to offer a counterbalance to angiogenic promoters (Sund *et al.*, 2005). Anti-angiogenic factors work through many different mechanisms. Angiogenic inhibitors have been reported to antagonise angiogenic activity induced by growth factors or inhibit the proteolytic activity of angiogenic proteinases, endothelial cell proliferation, migration or microtube formation (Grant *et al.*, 2005). Endogenous inhibitors of angiogenesis include various peptides, hormone metabolites and apoptosis modulators, many of which are fragments of naturally occurring ECM and BM proteins (Grant *et al.*, 2005; Nyberg *et al.*, 2005). A list of some of the known endogenous inhibitors of angiogenesis, both matrix derived and non-matrix derived are described in table 1.2.

Table 1.2: List of identified matrix derived and non-matrix derived angiogenic inhibitors (Nyberg *et al.*, 2005)

Matrix Derived	Non-Matrix Derived
Arresten	Interferons
Canstatin	Interleukins
Endostatin	Platelet factor – 4
Fibronectin fragments	Angiostatin
Fibulin	Chondromodulin
Thrombospondin -1 and -2	TIMPs
Tumstatin	Troponin -1
	Vasostatin
	Prolactin fragments
	Prothrombin Kringle -2

As described in section 1.2.3, the composition and deposition of the ECM is altered in asthma, with collagen being one of the altered proteins. Collagen derived angiogenic inhibitors have been identified as having a crucial role in the inhibition of angiogenesis (Sund *et al.*, 2004) (see table 1.3).

Table 1.3: Collagen derived angiogenic inhibitors

Collagen Derived Angiogenic Inhibitor	Collagen Type	Mechanism of Action
Endostatin (Folkman, 2004b)	Collagen XVIII	Inhibits endothelial cell migration, proliferation. Induces endothelial cell apoptosis. Causes G1 arrest of endothelial cells
Restin (Sund *et al.*, 2004)	Collagen XV	Inhibits endothelial cell migration
Canstatin (Sund *et al.*, 2004)	Collagen IV α2 chain	Inhibits endothelial cell migration, proliferation. Induces endothelial cell apoptosis
Arresten (Sund *et al.*, 2004)	Collagen IV α1 chain	Inhibits endothelial cell proliferation, migration, tube formation and neovascularisation
Tumstatin (Maeshima *et al.*, 2000)	Collagen IV α3 chain	Induces apoptosis of proliferating endothelial cells

1.4.1 Endostatin

Endostatin, an internal fragment of the matrix protein collagen XVIII, was the first endogenous angiogenesis inhibitor described. Folkman and colleagues originally described it in 1997 (Folkman, 2004b) as a 20-kDa fragment from the carboxyl-terminal non-collagenous domain 1 (NC1) of collagen XVIII. Enzymes, such as pancreatic elastase-like enzyme, cathepsins and MMPs cleave endostatin from collagen XVIII (Grant *et al.*, 2005). This cleavage is necessary for endostatin to be active.

The anti-angiogenic activity of endostatin works via inhibition of endothelial cell proliferation and migration, inducing apoptosis of proliferating endothelial cells and causing G1 arrest (Folkman, 2004a). Endostatin has been well characterised to inhibit tumour growth and metastasis (O'Reilly *et al.*, 1997) however it does not influence pre-existing vessels, but prevents revascularisation and can prevent vessel growth during wound healing (Boehm *et al.*, 1999).

One of the mechanisms of action which has been reported for endostatin is the interaction between endostatin and VEGF signalling. Endostatin prevents migration and angiogenesis via inhibition of VEGF-induced endothelial nitric oxide synthase (eNOS) phosphorylation (Kim *et al.*, 2002; Urbich *et al.*, 2002). Schmidt *et al.* demonstrated that the key signalling events for endostatin-induced morphogenesis are activation of protein phosphatase 2A (PP2A) and subsequent dephosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), resulting in the retraction of newly formed vessels (Schmidt *et al.*, 2006).

1.4.1.1 Endostatin in asthma

To date, endostatin is the only angiogenic inhibitor that has been studied in asthma. Although there have been no studies examining asthmatic lung tissue, levels of endostatin were measured in asthmatic sputum. Asai *et al.* showed levels of VEGF and endostatin to be significantly increased in asthmatic compared to non-asthmatic sputum. They also showed that there was an imbalance in the VEGF/endostatin ratio in asthmatics compared to non-asthmatics and that the increase reflected in the asthmatic sputum was due to an increase in the levels of VEGF and not an increase in endostatin (Asai *et al.*, 2002). Suzaki *et al.* reported that administration of endostatin/Fc to ovalbumin sensitised mice inhibited airway hyperresponsiveness, pulmonary allergic inflammation, production of ovalbumin-specific IgE and markers of lung inflammation. These investigators examined the expression of CD31 (an endothelial cell marker) mRNA expression and found it was reduced in the endostatin treated mice. Other markers of angiogenesis were not examined in this study (Suzaki *et al.*, 2005).

1.4.2 Collagen IV

Asthmatic airways express lower levels of collagen IV compared to normal airways (Bousquet *et al.*, 1992). The reduction in collagen IV may have profound effects on angiogenesis as three different angiogenic inhibitors have been identified within the collagen IV molecule.

Collagen IV is one of the major components of all basement membranes, and is found in the kidney, testis, oesophagus and the lung. It is crucial for the stability and assembly of the basement membrane (Timpl *et al.*, 1981). Type IV collagen is crucial in endothelial cell proliferation (Madri, 1997) as well as the regulation of cell adhesion and migration (Netzer *et al.*, 1998).

Six different collagen IV α chains, α1-α6, encoded for by three sets of genes form collagen IV heterotrimers (Hostikka *et al.*, 1990; Kalluri, 2003; Maeshima *et al.*, 2001a; Zhou *et al.*, 1994). Each α chain is composed of three domains, a cysteine-rich N-terminal 7S domain, a central triple-helical domain and a globular C-terminal NC1 domain. The NC1 domain is involved in the initiation and assembly of the α-chain heterotrimers (Soder *et al.*, 2004) and the 7S domain is involved in the covalent assembly of four heterotrimers in a web-shaped structure (Boutaud *et al.*, 2000). Out of 56 possible combinations of assembly, collagen IV α chains only assemble into 3 specific trimeric molecules; α1:α1:α2; α3:α4:α5 and α5:α5:α6 (Khoshnoodi *et al.*, 2006). Distribution of the six α chains is variable in that α1 and α2 are ubiquitous to all basement membranes, whereas there is a temporal and spatial regulation of α3-α6 expression in physiological processes (Sund *et al.*, 2004). The collagen IV α3 chain has been shown to be abundant in the lung,

kidneys and testis (Zeisberg *et al.*, 2001). The α5 chain is mainly expressed in the kidneys and the α6 chain in the oesophagus and lung (Ries *et al.*, 1995).

Normal assembly of type IV collagen is essential for basement membrane function. This is highlighted in diseases such as Alports syndrome, a hereditary progressive renal disorder caused by mutations in the type IV collagen α3, α4 or α5- chain gene (Heidet *et al.*, 2001). Patients with Alports syndrome present with sensorineural hearing loss and less commonly with ocular defects such as lenticonus and macular flecks. Pathologically, the glomerular basement membrane displays a characteristic ultrastructural alteration consisting of diffuse thickening with splitting of the lamina densa into multiple interwoven strands (Sasaki *et al.*, 1998). These pathological changes are a consequence of the α3 and α5 chain mutations (Wei *et al.*, 2006). Although levels of collagen IV are reported to be decreased in asthmatic lung (Bousquet *et al.*, 1992), it is unknown as to which α chain or chains of collagen IV is decreased.

Proteolytic cleavage of the α-chains of collagen IV leads to the release of the NC1 domains. Anti-angiogenic activity has been reported for the α1, α2 and α3 chain NC1 domains of type IV collagen. Arrestin, the NC1 domain of the collagen IV α1 chain, was originally isolated from human placental basement membrane (Colorado *et al.*, 2000). It has been shown to inhibit the proliferation of bFGF stimulated human endothelial cells and inhibit neovascularisation (Colorado *et al.*, 2000). Canstatin, the NC1 domain of the collagen IV α2 chain, was shown to inhibit the proliferation of foetal calf

serum-stimulated human endothelial cells and induce apoptosis in these cells (Kamphaus *et al.*, 2000). Tumstatin, the NC1 domain of the collagen IV α3 chain, has received the most interest in the collagen IV group, which has led to its characterisation.

1.4.3 Tumstatin

Tumstatin was identified as the bioactive NC1 domain of the collagen IV α3 chain (Maeshima *et al.*, 2000). It is a 28kDa, 245 amino acid molecule which is liberated from the basement membrane of the lung, kidney and testis. Circulating physiological levels of tumstatin in the blood have been shown to be between 300-350ng/ml and absence of normal physiological levels of circulating tumstatin facilitates pathological angiogenesis and increased tumour growth (Hamano *et al.*, 2003; Sund *et al.*, 2005).

Tumstatin is active as an angiogenic inhibitor when it is cleaved from the whole collagen IV α3 chain. Hamano *et al.* found that active MMP-9 was most effective in liberating the NC1 domain from the remaining α3 chain, however MMP-2, 3 and 13 were also able to release tumstatin but were significantly less efficient (Hamano *et al.*, 2003).

Recombinant human tumstatin inhibits the proliferation of human, bovine and mouse endothelial cells, suppresses tumour growth of renal cells and prostate carcinoma cells in xenograft mouse models. It also causes G1 arrest of VEGF- and bFGF-stimulated endothelial cells and induces apoptosis of proliferating endothelial cells (Maeshima *et al.*, 2000; Maeshima *et al.*, 2001a; Maeshima *et al.*, 2001b).

Using tumstatin knock-out mice, Hamano *et al.* showed that tumstatin is not involved in normal embryogenesis, development and wound healing. Tumstatin knock-out mice showed no alterations in litter size or development. Closure and repair in skin wounds of these mice progressed at the same rate as the wild-type mice, and the regeneration of liver after partial hepatectomy was the same as in the wild-type mice. Tumstatin knock-out mice developed renal failure and died at around 40 weeks (Hamano *et al.*, 2003). The effect of this knock-out on the lungs was not examined.
Using deletion mutagenesis, Maeshima *et al.* showed tumstatin's anti-angiogenic activity to be localised to amino acids 54-132 (tum5), however tum5 was only able to inhibit proliferation of endothelial cells and it had no effect on proliferating tumor cells. This region was further defined using overlapping synthetic peptides. From these studies, T3 peptide (69-88 amino acids) and T7 peptide (74-98 amino acids) were identified as having the sequences which possess anti-angiogenic activity. T3 and T7 peptides were shown to inhibit proliferation and induce apoptosis in bovine pulmonary arterial endothelial cells, human umbilical vein endothelial cells (HUVECs) and human prostate adenocarcinoma cell lines (Maeshima *et al.*, 2001b).

The collagen IV α3 chain is associated with a complex folding process which results in multiple heterotrimer conformers. The non-assembled conformers are structures which require specific activation by phosphorylation to enable their assembly into a triple helical molecule. The molecular organisation of the collagen IV molecule is determined by the NC1 domain. The good

pasture antigen-binding protein (GPBP) is a non-conventional Serine/Threonine kinase that targets the NC1 domain of the collagen IV α3 chain and phosphorylates it (Raya *et al.*, 2000). GPBP exists as two isoforms generated by alternative splicing, GPBP and GPBPΔ26. Both are preferably expressed in skeletal muscle and poorly expressed in the placenta, lung and liver (Raya *et al.*, 2000). GPBP is associated with the glomerular basement membrane (GBM) collagen organisation. GPBP has been shown to be more efficient in phosphorylating collagen IV α3 NC1 than GPBPΔ26 and to have a higher binding affinity to the α3 chain. In kidney and pancreas cancer cell lines GPBP expression is lower than GPBPΔ26 (Granero *et al.*, 2005; Raya *et al.*, 2000), but its expression is higher in apoptotic cell bodies suggesting that GPBP is involved in signalling pathways induced during programmed cell death.

Goodpastures disease is an autoimmune disease characterised by the formation of autoantibodies against the heterotrimeric basement membrane type IV collagen, affecting the kidneys and lungs. The pathogenic antibody response is directed to the NC1 domain of the α3 chain of collagen IV. Increased expression of GPBP is linked to the induction of the pro-autoimmune inflammatory response and the disorganisation of collagen IV in the GBM (Granero *et al.*, 2005), suggesting that the GPBP plays a role in the GBM collagen organisation. The levels of the GPBP in the airways of asthmatic individuals have not been previously investigated.

The mechanism of action of tumstatin is through its interaction with αvβ3-integrin on endothelial cells, an interaction that is pivotal for its angiogenic activity. In β3-integrin deficient mice tumstatin is unable to suppress neovascularisation of matrigel plugs, emphasising the importance of this interaction (Hynes, 2002). The binding of tumstatin to the αvβ3-integrin on endothelial cells prevents VEGF from binding to the endothelial cells thus reducing the survival rate and increasing the rate of apoptosis in the endothelial cells (Hutchings *et al.*, 2003). Through its interaction with αvβ3-integrin, tumstatin inhibits activation of focal adhesion kinase (FAK), phosphatidylinositol (PI)-3 kinase, protein kinase B (Akt) and mammalian target of rapamycin (mTOR) and prevents the dissociation of the eukaryotic translation initiation factor 4E/ eukaryotic initiation factor 4E-binding protein 1 (eIF4E/4E-BP1) complex, resulting in the inhibition of cap-dependent protein translation in endothelial cells (Yamamoto *et al.*, 2004).

1.5 Aims

Asthma and LAM are characterised by airway remodelling. Angiogenesis is a major feature of this remodelling in asthma but has not been described in LAM to date. Angiogenic promoters are elevated in the airways of asthmatics. However, the role of angiogenic inhibitors in the airway has yet to be characterised.

The specific aims of this thesis are:

1) to confirm the presence of angiogenesis in asthmatic and and determine if it is present in LAM airways;

2) to examine the expression of the six collagen IV α chain NC1 domains in asthmatic and LAM airways;

3) to examine the expression of tumstatin in other known chronic respiratory diseases;

4) to investigate the presence and level of expression of the GPBP in asthmatic and LAM airways;

5) to examine the expression of the αvβ3 integrin in asthmatic airways;

6) to investigate levels of tumstatin in asthmatic individuals;

7) assess the activity of tumstatin on primary pulmonary endothelial cells;

8) to identify the functionality of tumstatin in the airway *in vivo* and to test the effectiveness of tumstatin to inhibit AHR and angiogenesis in a mouse model of airway hyperresponsiveness.

Chapter 2

Collagen IV α3 NC1 domain "Tumstatin" an endogenous angiogenic inhibitor is absent in the airways of asthmatic and LAM individuals.

2.1 Introduction

Angiogenesis, a vital process for embryogenesis and wound healing, is the formation of new blood vessels. When blood vessel supply is impaired tissue ischaemia results. However, when there is excessive blood vessel formation and growth there is excessive growth of inflamed tissue therefore chronic inflammatory disorders are aggravated (Carmeliet, 2004). Asthma is an inflammatory disease characterised by airway remodelling. One of the major features of this remodelling is an increase in the number and size of blood vessels in the airways (Hashimoto *et al.*, 2005; Li *et al.*, 1997; Wilson *et al.*, 2006).

Angiogenesis is triggered by many different endogenous angiogenic stimulators such as vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) (D'Andrea *et al.*, 2006; Rundhaug, 2005), and is inhibited by endogenous angiogenic inhibitors including thrombospondin-1, a secreted glycoprotein, endostatin, the non-collagenous domain (NC1) of collagen XVIII, and tumstatin, the NC1 domain of the α3 chain of collagen IV (Nyberg *et al.*, 2005). Under normal conditions this system exists as an equilibrium with both endogenous stimulators and inhibitors being switched "on" and "off" when necessary.

Collagen IV is a major component in the basement membrane (BM) of many tissues, including the kidneys, testis and lungs (Timpl *et al.*, 1981), that forms a complex branch network necessary for stability and assembly of the BM

(Timpl *et al.*, 1981). Levels of collagen IV have been shown to be decreased in the airways of asthmatics. Histological studies of airway biopsies from asthmatics and non-asthmatics showed a decrease in the level of collagen IV in the asthmatic airway compared to the non-asthmatic (Bousquet *et al.*, 1992). Johnson *et al.* demonstrated *in vitro* that asthmatic airway smooth muscle (ASM) cells release less collagen IV than non-asthmatic ASM cells (Johnson *et al.*, 2004).

Collagen IV has six isoforms refered to as α chains (α1-α6), encoded for by three sets of genes (Ortega *et al.*, 2002). Each chain consists of a 7S domain at the amino terminus, a central collagenous domain and a NC1 domain at the carboxyl terminus (Heidet *et al.*, 2001) as represented in figure 2.1.

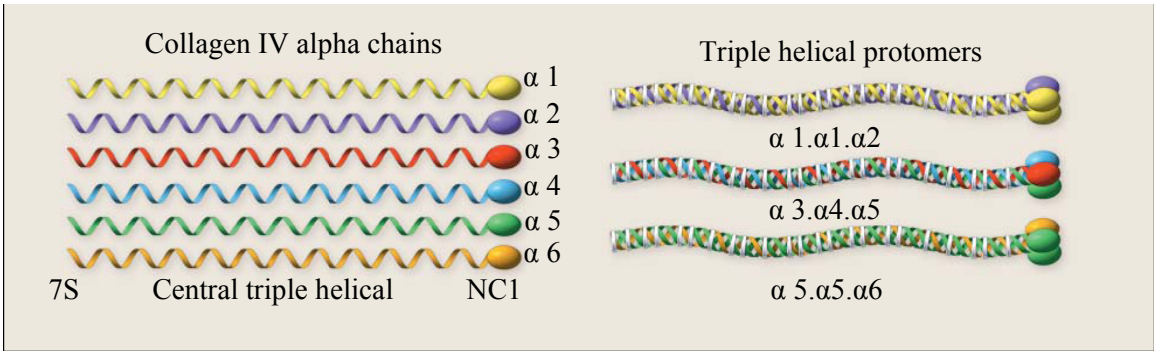


Figure 2.1: Triple helical organisation of collagen IV chains. Figure has been adapted from Hudson BG 2003.

Six α chains are arranged into three triple helical protomers. The selection of α chains for triple helical formation is controlled by the molecular recognition sequences encoded within the NC1 domains of the α chains.

The macromolecular structure of collagen IV is a network in which molecules are connected via like ends, the NC1 domains of two molecules become aggregated to form a hexameric complex that is stabilised by the intermolecular disulfide bonds. The NC1 domain is crucial in linking two molecules via C-terminal ends. Before an intact triple helical molecule is generated the NC1 domain is responsible for chain selection and assembly (Ries *et al.*, 1995). The six α chains combine into three specific trimeric molecules; α1:α1:α2, found in the basement membrane of all tissues, α3:α4:α5, occurs in the basement membrane of the kidney, lung, testis, cochlea and eye, and α5:α5:α6, found in the skin, smooth muscle, oesophagus and kidney (Ries *et al.*, 1995; Sund *et al.*, 2004). The exact distribution of the collagen IV α chains in the lung is unknown although α1-α4 have been identified in human alveolar basement membrane (Derry *et al.*, 1994).

Fragments of the Collagen IV molecule have anti-angiogenic properties. Tumstatin, an angiogenic inhibitor, is the NC1 domain of the α3 chain of Collagen IV (Maeshima *et al.*, 2000). Tumstatin inhibits angiogenesis by blocking the interactions of VEGF, a promoter of angiogenesis, with the αvβ3 integrin (Byzova *et al.*, 1998), on endothelial cells, causing inhibition of DNA synthesis and inducing apoptosis of proliferating endothelial cells (Maeshima *et al.*, 2001b). Tumstatin inhibits aberrant angiogenesis but is not involved in embryogenesis and wound healing. Hamano *et al.* also showed that the inhibitory effects of tumstatin were dependent on its binding to integrin αvβ3. They detected αvβ3 integrin on blood vessels and small

capillaries in proliferating tumours but did not detect this integrin in blood vessels of healing skin wounds or regenerating livers (Hamano *et al.*, 2003).

Integrins are cell surface receptors composed of transmembrane glycoproteins, with α and β subunits, which connect adhesive proteins in the extracellular matrix (ECM) to the cytoskeleton (Nisato *et al.*, 2005). They act as receptors for many proteins and growth factors, the αvβ3 integrin is a receptor for various proteins including fibronectin, fibrinogen, thrombospondin, tumstatin and the growth factor VEGF (Nisato *et al.*, 2005). A variety of cells express the αvβ3 integrin including endothelial cells and smooth muscle cells in postangioplasty restenosis, in healing arterial wounds and in osteoclasts (Byzova *et al.*, 1998). Integrins are able to mediate a number of biological events such as the migration of smooth muscle cells, adhesion of osteoclasts to bone matrix and angiogenesis (Kokubo *et al.*, 2007). Cell adhesion to the ECM mediated by integrins leads to bidirectional signalling events that regulate cell migration, survival and proliferation.

Goodpasture's disease results from autoantibodies against collagen IV α3 NC1. These antibodies are raised against cryptic epitopes in the collagen IV α3 NC1 domain in the alveolar and glomerular basement membrane (GBM). Collagen IV α3 chain undergoes a complex folding process that results in multiple conformers. The non-assembled conformers are structures which require specific activation by phosphorylation to enable its assembly into a

triple helical molecule (Raya *et al.*, 2000). The NC1 domain is responsible for determining the molecular organisation of the collagen IV α chains. The good pasture antigen-binding protein (GPBP) is a kinase that targets and phosphorylates the NC1 domain of the collagen IV α3 chain (Raya *et al.*, 2000). Alternative splicing of the GPBP generates two isoforms, GPBP and GPBPΔ26 (Raya *et al.*, 2000) of which GPBP is the most efficient at phosphorylating collagen IV α3 NC1 and has a higher binding affinity to the α3 chain. GPBP is associated with the GBM collagen organisation. Increased expression of GPBP is linked to the induction of the pro-autoimmune inflammatory response and a disorganisation of collagen IV in the GBM in Goodpastures disease (Granero *et al.*, 2005). This suggests the GPBP plays a role in the GBM collagen organisation. New Zealand white mice (NZW) naturally over express the GPBP, which results in glomerular abnormalities, including defective fusion of epithelial and endothelial components of the capillary GBM as well as defective fusion of the α3.α4.α5 and α1.α1.α2 networks in the capillary GBM (Revert *et al.*, 2007). The mechanism by which increased expression of the GPBP induces GBM disruption is unknown. Revert *et al.* suggested that the collagen IV α3 chain NC1 domain conformers produced by cells expressing high levels of GPBP, may be defective in their assembly of the α1.α1.α2 and α3.α4.α5 protomers, resulting in network disassociation and GBM disruption (Revert *et al.*, 2007). Increased levels of the GPBP will generate misfolding of the collagen IV α3 chain (Raya *et al.*, 2000). Levels of the GPBP in association with asthma have not been investigated.

It was hypothesised that tumstatin plays a crucial role as an angiogenic inhibitor in the airway. The specific aims of this study were:

1) to confirm the presence of angiogenesis in asthmatic and determine if it is present in LAM airway samples,

2) to examine the expression of the six collagen IV α chain NC1 domains in diseased (asthma and LAM) and non-diseased lungs,

3) to investigate the presence and level of expression of the GPBP in asthmatic and LAM airways,

4) to examine the expression of the αvβ3 integrin in asthmatic airways compared to non-asthmatic.

2.2 Materials and Methods

2.2.1 Confirmation of angiogenesis

2.2.1.1 Immunohistochemical detection of angiogenesis

Paraffin embedded airway sections from asthmatic, non-asthmatic and LAM individuals were stained for CD31 platelet/endothelial cell adhesion molecule -1 (PECAM-1) and von Willebrand factor (vWF). Sections were de-paraffinised, and re-hydrated through graded alcohol. Sections were blocked with a peroxidase blocking agent (DakoCytomation, Glostrup, CA) for 5 minutes and then washed with phosphate buffered saline (PBS) buffer. Primary antibodies, goat anti-human CD31 (PECAM-1) (Santa Cruz Biotechnology Inc, Santacruz, CA) [1µg/ml] and rabbit anti-human von Willebrand factor (vWF) (Santa Cruz Biotechnology Inc) [1µg/ml], were added and incubated at room temperature for one hour. Sections were washed in PBS and the secondary antibodies anti-mouse horseradish peroxidase (HRP) (DakoCytomation) and rabbit anti-goat HRP (DakoCytomation), were added and incubated at room temperature for one hour. After washing in PBS for 5 minutes, substrate chromogen, liquid 3,3'-diaminobenzidine (DAB) (DakoCytomation), was added to the sections and incubated for 5 minutes at room temperature. Distilled water was used to wash sections for 5 minutes and an aqueous mounting medium (Faramount, DakoCytomation) was used to mount sections prior to coverslipping.

Sections were imaged on an Olympus BX51 microscope and processed using Leica imaging software IM1000 (Leica, Heerbrugg St Gallen, Switzerland).

Detection of angiogenesis was also performed in other chronic respiratory diseases. Paraffin embedded tissue sections of bronchial rings from individuals with chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and bronchiectasis were stained for vWF as above, except for bronchiectasis sections where fast red, substrate chromogen (Dakocytomation) was used. COPD sections stained for vWF were also counterstained with Heamatoxylin for 1 minute prior to coverslipping.

2.2.2 Detection of collagen IV α1-α6 NC1 domains

Paraffin embedded tissue sections of bronchial rings from asthmatic, non-asthmatic and LAM individuals were stained for the six collagen IV α chain NC1 domains. Sections were deparaffinised and re-hydrated through graded alcohol. Blocking serum (10% non-immune horse serum) was then added to the sections for 20 minutes at room temperature. Without rinsing, either primary antibodies (collagen IV α1-6 NC1 a kind gift from Dr Sado at Shigei Medical Research Institute, Okayama, Japan [1ng/ml]) or isotype control antibody (Rat IgG, Jackson ImmunoResearch, West Grove PA [1ng/ml]) were added to the sections and incubated for 1 hour at room temperature. Sections were then washed with PBS and a goat anti-rat fluorescein isothiocyanate (FITC) (MP Biomedicals, Solon, OH, USA [1ng/ml]) or horse anti-mouse texas red (Vecta laboratories, Burlingame, CA, USA [1ng/ml]) conjugated secondary antibody was added, and incubated for 30 minutes at room temperature. Following a rinse with PBS, slides were mounted using vectashield mounting media (Vecta Laboratories). Images were taken on an

Olympus BX51 fluorescence microscope and captured using Leica imaging software IM1000 (Leica).

In addition, sections from fresh frozen biopsies from asthmatic and non-asthmatic individuals were also stained for collagen IV α1, 3 and 5 NC1 domains as above, except frozen sections were thawed and placed into PBS buffer for 5 minutes and the deparaffinising step was omitted.

Semi-quantitative analysis for the presence of collagen IV α1-6 chain NC1 domains was performed. Images from all asthmatic, non-asthmatic and LAM patients were scored by three independent observers, who were blinded to the diagnosis of the subject, for the presence and intensity of staining. Images were scored as follows;

0 = Absence of stain

1 = Thin and discontinuous staining

2 = Thin and continuous staining

3 = Strong and continuous staining

Scores from the three observers were averaged and the standard error of the mean was calculated.

2.2.3 Detection of collagen IV α3 and α5 NC1 domains in COPD, CF and bronchiectasis

Paraffin embedded tissue sections of bronchial rings from individuals with COPD, CF and bronchiectasis were stained for collagen IV α3 and α5 chain NC1 domains using the same protocol as in 2.2.2 with the exception of the primary and secondary antibodies used for the detection of the collagen IV α3 chain in CF and bronchiectatic airways, where mouse anti-human collagen

IV α3 NC1 (Wieslab, Lund, Sweden) at [1ng/ml] was used, followed by a horse anti-mouse texas red conjugated secondary antibody (Vecta Laboratories) at [1ng/ml]. Mouse IgG (Chemicon International, Temecula, CA) was used at [1ng/ml] as an isotype control.

2.2.4 Detection of collagen IV GPBP

Frozen sections from asthmatic and non-asthmatic individuals were thawed and placed in PBS for 5 minutes. Paraffin sections from asthmatic, non-asthmatic and LAM individuals were deparaffinised and placed in water for 5 minutes. Sections were blocked using a peroxidase block (DakoCytomation) for 5 minutes. Primary antibodies, mouse anti-human collagen IV α3 NC1 (Wieslab) at [1ng/ml], chicken anti-human collagen IV GPBP (GenWay Biotech, Inc, San Diego, CA) at [1ng/ml] or isotype control antibodies Mouse IgG_1 (R&D Systems) or Chicken IgY (Abcam, Cambridge, MA) respectively, were then added and incubated for 1 hour at room temperature. Sections were rinsed with PBS and a secondary peroxidase labelled antibody was added, anti-mouse HRP (DakoCytomation) against the α3 NC1 primary antibody, rabbit anti-chicken HRP labelled antibody (Abcam) against the GPBP antibody, and incubated for 1 hour at room temperature. Following a wash in PBS for 5 minutes, substrate chromogen solution (Liquid DAB+) (DakoCytomation) was added to the sections for 5 minutes. Distilled water was used to rinse the section prior to mounting in an aqueous mounting medium (Faramount aqueous mounting medium, DakoCytomation) and

coverslipping. Images were taken on an Olympus BX51 microscope and captured and analysed using Leica imaging software IM1000 (Leica).
The level of expression of the Collagen IV α3 chain NC1 domain and GPBP were quantified using grey scale image analysis, with QWIN image analysis software (Leica). From each patient within a single image which contained an airway and parenchyma, 10 random areas were selected for grey scale analysis. Final grey values were obtained by subtracting the corresponding grey values obtained from the respective isotype control. Statistical analysis was performed using one-way ANOVA with Dunnett's post test.

2.2.5 Detection of αv and β3 integrins

Frozen biopsy sections from asthmatic and non-asthmatic individuals were thawed and placed into PBS buffer for 5 minutes. Ten percent horse serum was used to block any non-specific binding in the sections for 20 minutes at room temperature. Sections were then blotted dry and primary antibodies added, mouse anti-human integrin αv (Chemicon International) at 1ng/ml or mouse anti-human integrin β3 (Chemicon International) at 1ng/ml or isotype control antibody (Mouse IgG_1 (R&D Systems, Minniapolas, Mn) and incubated at room temperature for 1 hour. Following a wash with PBS for 5 minutes the secondary antibody, horse anti-mouse texas red (Vecta Laboratories) at 1ng/ml was added and incubated at room temperature for 30 minutes. After a wash in PBS for 5 minutes the sections were mounted using vectashield mounting media (Vecta Laboratories). Images were taken on an

Olympus BX51 fluorescence microscope and captured using Leica imaging software IM1000 (Leica).

2.2.6 Co-localisation of αvβ3 integrin with VEGF and tumstatin expression

Paraffin sections from asthmatic, non-asthmatic and LAM individuals were deparaffinised and placed in water for 5 minutes. Ten percent horse serum was used to block sections for 20 minutes at room temperature. Primary antibodies, rat anti-human collagen IV α3 NC1 (Shigei Medical Research Institute, Okayama, Japan) at 1ng/ml, mouse anti-human integrin αvβ3 (Chemicon International) at 1ng/ml, rabbit anti-human $VEGF_{165}$ (Chemicon International) at 1ng/ml, were mixed and added to the sections. Isotype controls, rat IgG, (Jackson ImmunoResearch [1ng/ml]), mouse IgG (R&D Systems [1ng/ml]), rabbit IgG (R&D Systems [1ng/ml]) were added in the same manner as primary antibodies and incubated at room temperature for 1 hour. Following a wash in PBS for 5 minutes the secondary antibodies Alexa fluor 633 anti-rat [1ng/ml], Alexa fluor 405 anti-mouse [1ng/ml] and Alexa fluor 488 anti-chicken [1ng/ml] (Molecular Probes, Leiden, Netherlands) were added to sections and incubated for 30 minutes at room temperature. After a wash in PBS for 5 minutes the sections were mounted using DABCO anti-fade mounting medium (Sigma, St Louise, MO). Images were taken using the Zeiss LSM 510 Meta confocal microscope which utilises the Zeiss LSM 510 imaging software. Images were automatically processed by the Zeiss software to generate superimposed images of the three colour channels.

Three lasers were employed to excite the different fluorochromes used. For the Alexa 633 the red NeHe laser 633nm line was used, for the Alexa 405 the 404nm diode laser was used and for the Alexa 488 the Argon laser 488nm line was used. Z stack images were also obtained, whereby images of the section are taken at intervals throughout the whole depth of the tissue. The first point of focus of the tissue is set as the starting point and the last point of focus as the end point. Interval points are then automatically calculated for the three channels, ensuring a slight overlapping in each slice, from the start to the end point.

2.3 Results

2.3.1 Patient details

Asthmatic and non-asthmatic airway sections were obtained from post mortem tissue, lung resections, explanted lungs and endobronchial biopsies. COPD airway sections were obtained from lung biopsies, LAM airway sections were obtained from explanted lungs. CF and bronchiectasis airway sections were obtained from lung resections and explanted lungs. Complete patient details are found in table 2.1.

2.3.2 Confirmation of angiogenesis

Angiogenesis is a prominent feature in the airway remodelling seen in asthma and is also a feature reported in LAM, CF, COPD and bronchiectasis. The airway sections in this study had not previously been examined for the presence of angiogenesis.

Confirmation of increased angiogenesis in asthmatic (n=8) and LAM (n=8) tissue was observed as an increase in the number of CD31 and vWF positive blood vessels compared to non-asthmatic sections (n=8), figure 2.2. Also, the presence of angiogenesis in COPD (n=8), CF (n=4) and bronchiectasis (n=1) was observed as an increase in the number of vWF positive blood vessels compared to non-asthmatic sections. Images in figure 2.2 are representative of all individuals tested.

Table 2.1: Patient details

Patient #	Age	Sex	Disease	Type of sample	Derived from	Medication
1	17	Male	Asthmatic	Bronchial rings	Explanted lung	Salbutamol
2	48	Male	Asthmatic	Bronchial rings	Explanted lung	Prednisone 10mg/day
3	80	Male	Asthmatic	Bronchial rings	Explanted lung	Not known
4	15	Female	Asthmatic	Bronchial rings	Explanted lung	Not known
5	33	Female	Asthmatic	Bronchial rings	Explanted lung	Not known
6	15	Male	Asthmatic	Bronchial rings	Explanted lung	Not known
7	10	Female	Asthmatic	Bronchial rings	Explanted lung	Not known
8	17	Male	Asthmatic	Bronchial rings	Explanted lung	Not known
9	47	Male	Non-asthmatic	Bronchial rings	Explanted lung	Not known
10	62	Male	Non-asthmatic	Bronchial rings	Explanted lung	Not known
11	12	Male	Non-asthmatic	Bronchial rings	Explanted lung	Not known
12	17	Male	Non-asthmatic	Bronchial rings	Explanted lung	Not known
13	15	Female	Non-asthmatic	Bronchial rings	Explanted lung	Not known
14	57	Male	Carcinoma	Bronchial rings	Resection	Not known
15	84	Male	Carcinoma	Bronchial rings	Resection	Not known
16	56	Female	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
17	20	Male	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
18	42	Male	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week

Patient #	Age	Sex	Disease	Type of sample	Derived from	Medication
19	45	Male	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
20	60	Male	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
21	56	Female	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
22	20	Male	Asthmatic	Endobronchial biopsy	Bronchoscopy	Inhaled corticosteroids/day Bronchodilator/week
23	29	Male	Non-asthmatic	Endobronchial biopsy	Bronchoscopy	Not on medication
24	20	Male	Non-asthmatic	Endobronchial biopsy	Bronchoscopy	Not on medication
25	22	Male	Non-asthmatic	Endobronchial biopsy	Bronchoscopy	Not on medication
26	38	Female	LAM	Bronchial rings	Explanted lung	Not known
27	39	Female	LAM	Bronchial rings	Explanted lung	Not known
28	46	Female	LAM	Bronchial rings	Explanted lung	Not known
29	56	Female	LAM	Bronchial rings	Explanted lung	Not known
30	66	Female	LAM	Bronchial rings	Explanted lung	Not known
31	34	Female	LAM	Bronchial rings	Explanted lung	Not known
32	36	Female	LAM	Bronchial rings	Explanted lung	Not known
33	51	Female	LAM	Bronchial rings	Explanted lung	Not known

Patient #	Age	Sex	Disease	Type of sample	Derived from	Medication
34	57-75	Female	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
35	57-75	Female	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
36	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
37	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
38	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
39	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
40	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Not on medication
41	57-75	Female	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
42	57-75	Female	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
43	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
44	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
45	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
46	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day

Patient #	Age	Sex	Disease	Type of sample	Derived from	Medication
47	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
48	57-75	Male	COPD	Endobronchial biopsy	Bronchoscopy	Theophylline 200µg/day
49	19	Female	CF	Bronchial rings	Explanted lung	Not known
50	22	Female	CF	Bronchial rings	Explanted lung	Not known
51	25	Female	CF	Bronchial rings	Explanted lung	Not known
53	29	Male	CF	Bronchial rings	Explanted lung	Not known
54	46	Male	Bronchiectasis	Bronchial rings	Explanted lung	Not known

COPD patient age- only an age bracket was available.

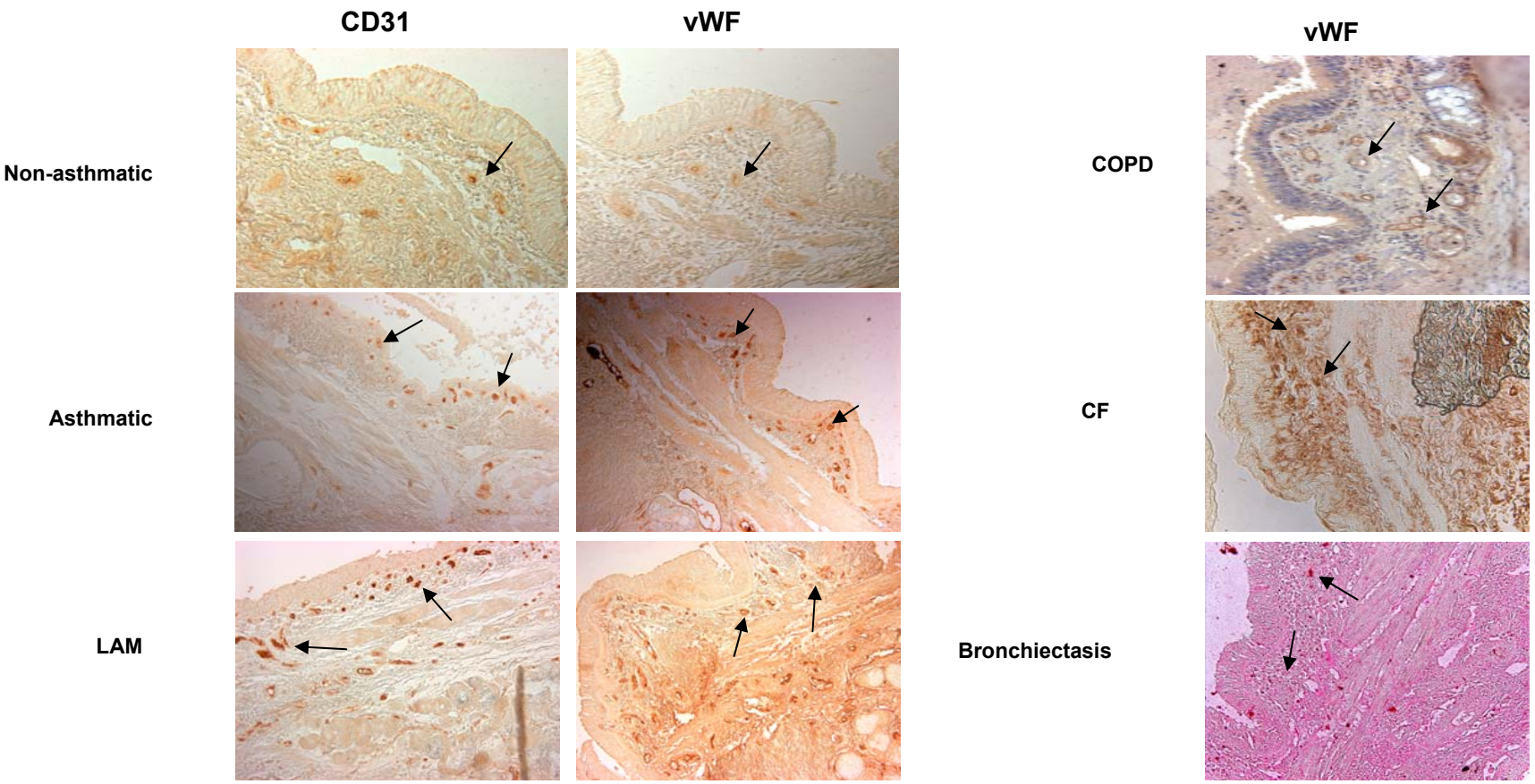


Figure 2.2: Angiogenesis in airway sections. Bronchial airway sections stained for CD31 and vWF from asthmatic, non-asthmatic, LAM, COPD and CF individuals showing blood vessel detection (arrows) using DAB (brown). Bronchial airway section from bronchiectasis showing blood vessels (arrows) detected using fast red (red). Images are representative of all subjects tested (X200).

2.3.3 Characterisation of collagen IV α1-α6 chain NC1 domains in asthmatic and non-asthmatic airways

Asthmatic airway sections (n=5) from post mortem tissue were negative for the collagen IV α3 chain NC1 domain (tumstatin) and positive for the remaining α chain NC1 domains as shown in figure 2.3. Non-asthmatic sections (n=4) were positive for all six collagen IV α chain NC1 domains (figure 2.4). Asthmatic and non-asthmatic sections were scored for the level of staining observed (table 2.2). All asthmatic sections scored 0 for the level of tumstatin observed. To confirm that this was not a feature of post mortem tissue, asthmatic (n=6) and non-asthmatic (n=5) biopsies taken from live volunteers were stained for collagen IV α3 and α5 NC1 domains. Collagen IV α5 was chosen as a comparative control as it showed consistent staining previously in all asthmatic and non-asthmatic sections. All asthmatic biopsy sections were negative for tumstatin and positive for collagen IV α5, non-asthmatic biopsy sections were positive for both tumstatin and collagen IV α5 as shown in figure 2.5. Isotype controls were negative.

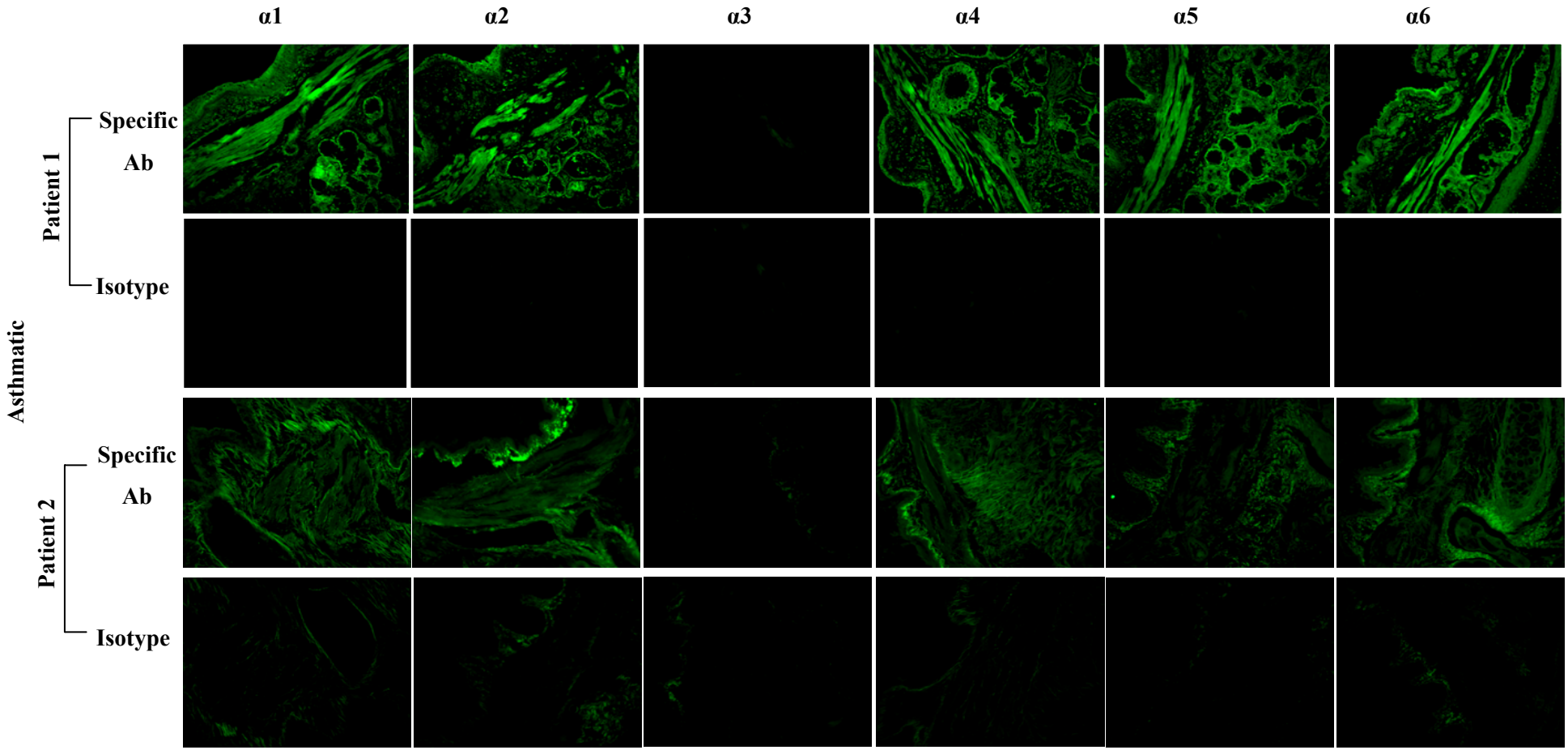


Figure 2.3: Detection of the six collagen IV α chain NC1 domains in paraffin embedded airway sections from 2 asthmatic individuals. Specific antibodies detected using FITC fluorochrome (green). IgG_1 isotype controls were negative for non-specific staining. Images are representative of results obtained from 5 asthmatic individuals (all images at X200)

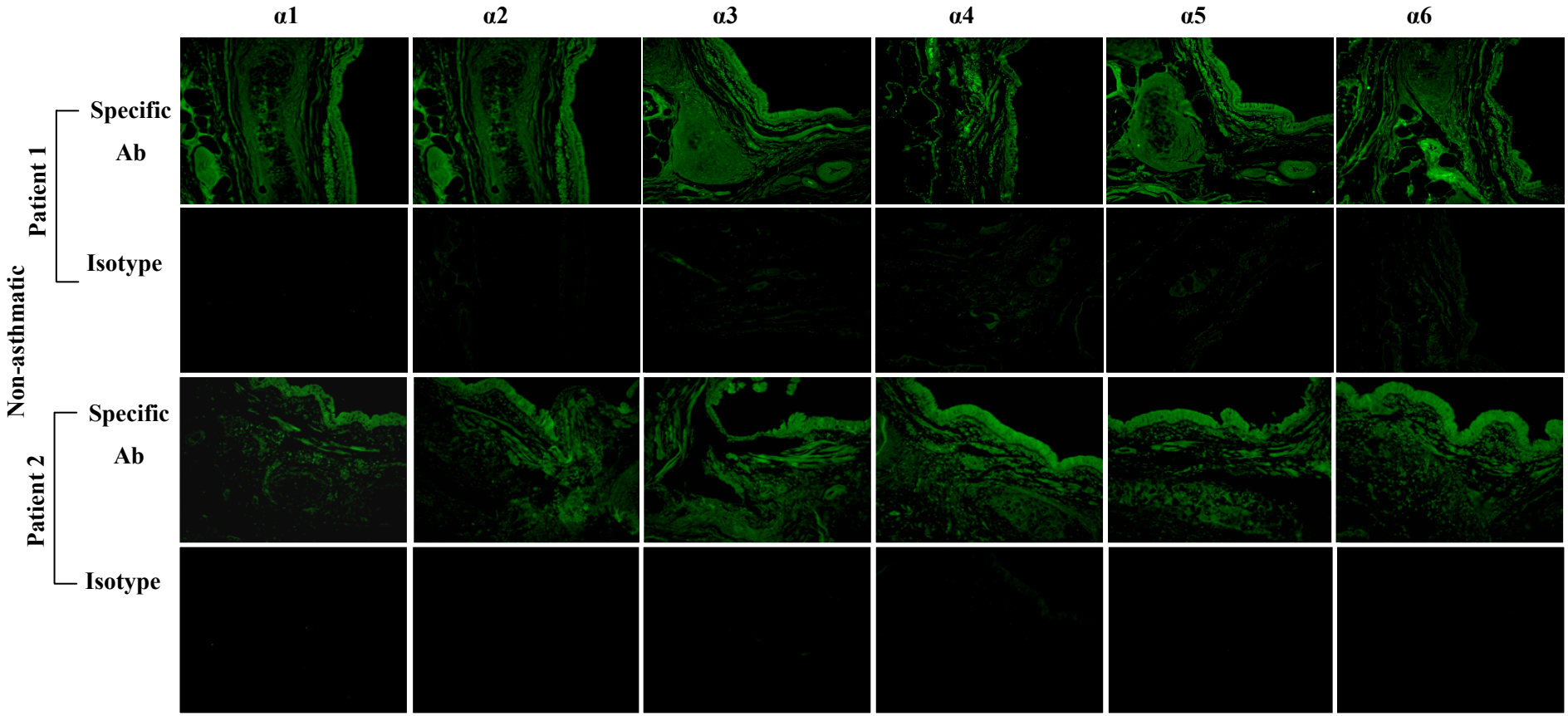


Figure 2.4: Detection of the six collagen IV α chain NC1 domains in paraffin embedded airway sections from 2 non-asthmatic individuals. Specific antibodies detected using FITC fluorochrome (green). IgG_1 isotype controls were negative for non-specific staining. Images are representative of results obtained from 4 non-asthmatic individuals (all images at X200).

	n=	α 1	α 2	α 3	α 4	α 5	α 6
Asthmatic (SEM)	5	2.1 (±0.3)	2.55 (±0.15)	0 (0)	1.95 (±0.3)	2.15 (±0.21)	2.38 (±0.21)
Non-asthmatic (SEM)	4	1.72 (±0.3)	2.21 (±0.17)	2.29 (±0.20)	2.72 (±0.20)	2.47 (±0.22)	2.5 (±0.20)

Table 2.2: Levels of the six collagen IV α chain NC1 domains observed in airway sections from asthmatic and non-asthmatic individuals. Scoring results obtained from 3 independent observers using the following scale: 0 = Absence of stain, 1 = Thin and discontinuous staining, 2 = Thin and continuous staining, 3 = Strong and continuous staining.

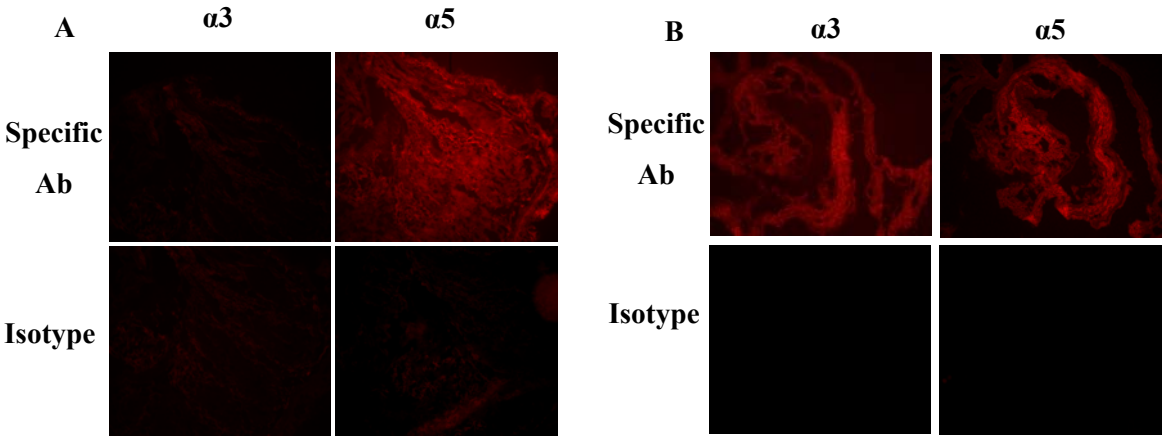


Figure 2.5: Airway biopsies stained for collagen IV α3 and α5 chain NC1 domains from asthmatic (n=6 (A)) and non-asthmatic (n=5 (B)) individuals. Specific staining was detected using a fluorochrome Texas red (red). Images are representative of all individuals tested (all images at X200).

2.3.4 Characterisation of collagen IV α1-α6 chain NC1 domains in LAM

Airway sections from LAM individuals (n=8) were negative for collagen IV α3 chain NC1 domain (tumstatin) and collagen IV α5 chain NC1 domain. The remaining collagen IV α chain NC1 domains were positive, figure 2.6. Images were scored as in 2.3.3, results are shown in table 2.3. All LAM sections scored 0.5 (± 0.16) for the level of tumstatin observed as well as for the α5 chain NC1 domain (0.56 ± 0.12).

	n=	α 1	α 2	α 3	α 4	α 5	α 6
LAM (SEM)	8	2.16 (±0.26)	2.28 (±0.18)	0.5 (±0.16)	2.63 (±0.17)	0.56 (±0.12)	2.33 (±0.12)

Table 2.3: Levels of the six collagen IV α chain NC1 domains observed in stained airway sections from LAM individuals. Scoring results obtained from 3 independent observers using the following scale: 0 = Absence of stain, 1 = Thin and discontinuous staining, 2 = Thin and continuous staining, 3 = Strong and continuous staining.

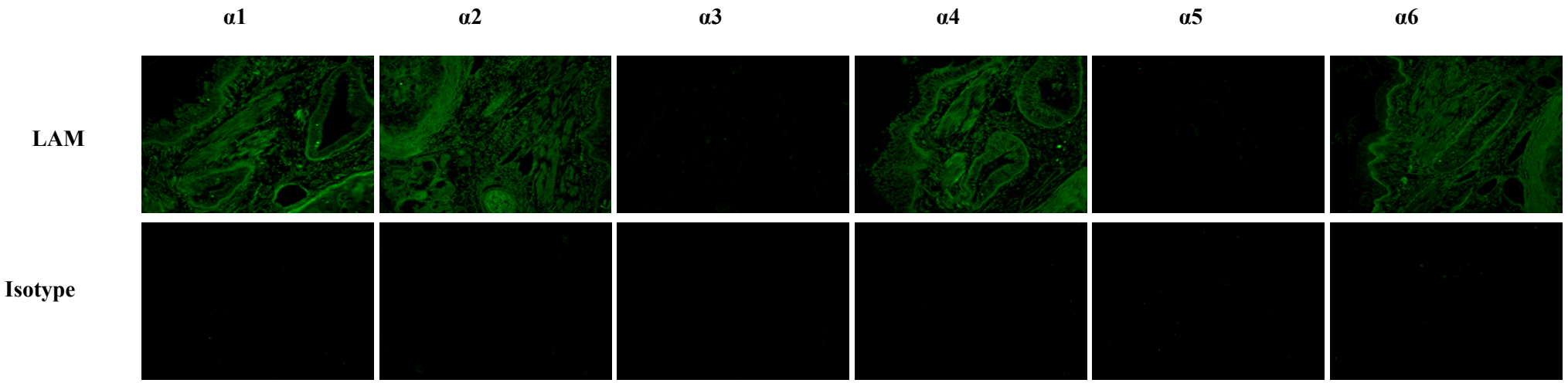


Figure 2.6: Detection of the six collagen IV α chain NC1 domains in paraffin embedded airway sections from LAM individuals. Specific antibodies detected using FITC fluorochrome (green). IgG_1 isotype controls were negative for non-specific staining. Images are representative of results obtained from 8 LAM individuals (all images at X200).

2.3.5 Characterisation of tumstatin in other chronic respiratory diseases

To test whether the absence of tumstatin was a feature of all chronic respiratory disorders, tissue sections from COPD (n=8), CF (n=4) and bronchiectasis (n=1) patients were stained for tumstatin and all were positive for tumstatin as shown in figure 2.7. CF and bronchiectasis sections were also positive for collagen IV α5 NC1 domain. Positive staining for collagen IV α5 NC1 domain in COPD sections was varied across the individuals tested with 4 out of 8 individuals examined being negative.

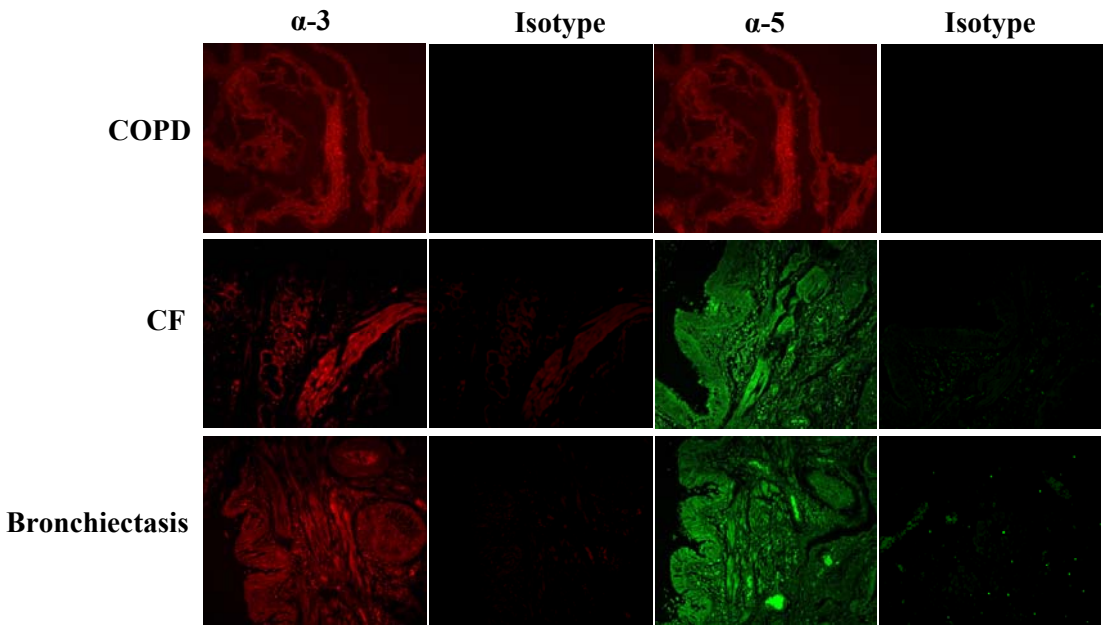


Figure 2.7: Bronchial airway sections stained for tumstatin and collagen IV α5 chain NC1 domain from COPD (n=8), CF (n=4) and bronchiectasis (n=1) individuals. Specific antibody detected using Texas red (red) or FITC (green) fluorochrome. Images are representative of all individuals tested. (All images at X200).

2.3.6 Detection of collagen IV GPBP

Collagen IV GPBP is a protein kinase that binds and phosphorylates the NC1 domain of the α3 chain (Raya *et al.*, 2000). Levels of expression of the GPBP can play an important role in the phosphorylation-dependent folding of the α3 chain. An increase in the GPBP in the asthmatic and LAM airway tissue may produce misfolded α3 chains which could result in the clearing of the tumstatin or make tumstatin undetectable by our antibody.

Colorimetric staining was used to enable quantitation by digital image analysis. We stained for both tumstatin and the GPBP in 6 asthmatic biopsies, 3 non-asthmatic airway sections, 2 non-asthmatic biopsies and 5 LAM airway sections. As in our earlier findings, the asthmatic biopsies and LAM sections were negative for tumstatin and the non-asthmatics were positive (figure 2.8). However, all samples were positive for the collagen IV GPBP (figure 2.8).

Digital image analysis revealed a significant difference in the level of tumstatin detected between asthmatics and non-asthmatics ($p<0.01$, students t-test), as well as a significant difference between LAM and non-asthmatics ($p<0.01$, students t-test). There was no significant difference in the levels of the collagen IV GPBP between asthmatic and non-asthmatic tissue sections ($p>0.05$, students t-test) or LAM and non-asthmatics ($p>0.05$, students t-test) as shown in figure 2.9.

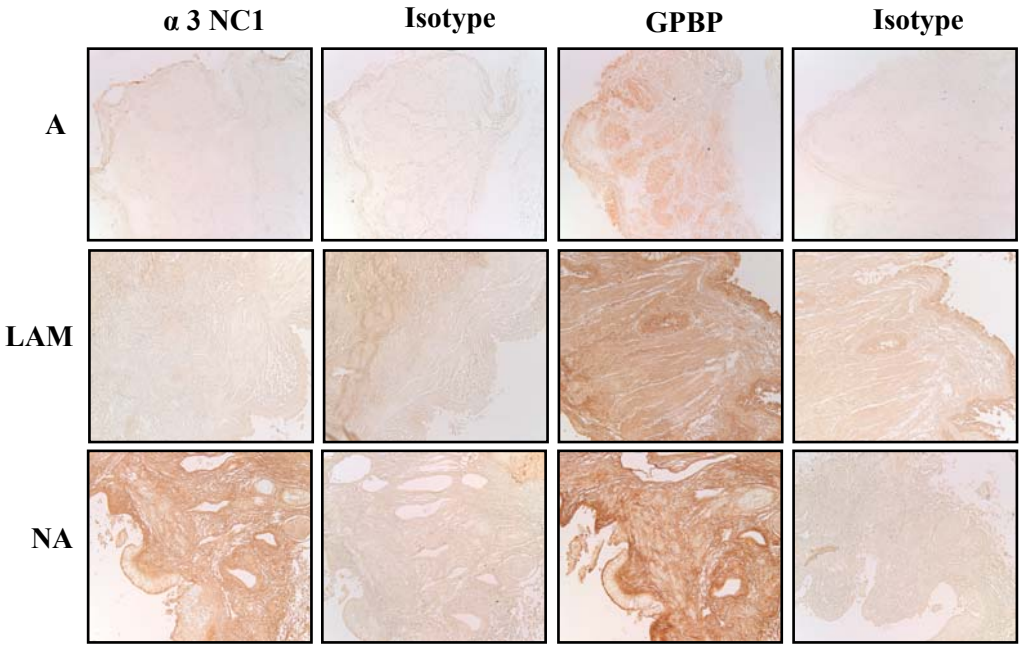


Figure 2.8: Bronchial biopsy sections stained for collagen IV α3 NC1 domain and collagen IV GPBP in asthmatic (n=6) and non-asthmatic (n=5) individuals and bronchial rings from LAM (n=5) individuals. Specific antibody detected using DAB (brown). Isotype controls were negative. Images are representative of all individuals tested (all images at X200).

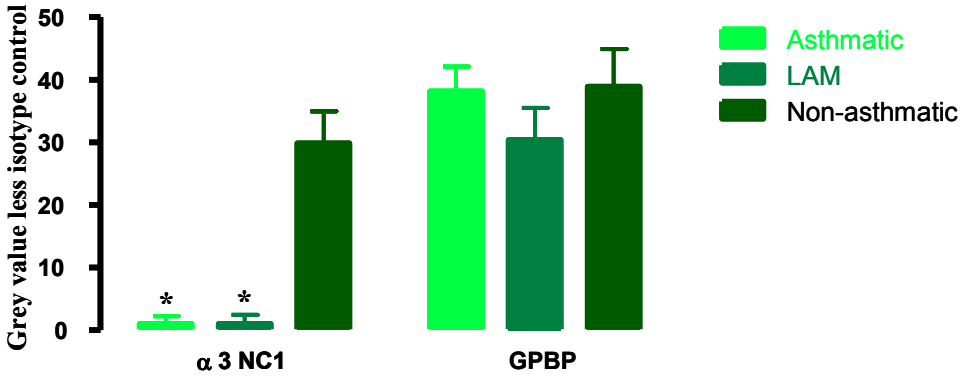


Figure 2.9: Level of collagen IV α3 NC1 domain and GPBP in asthmatic and non-asthmatic biopsies and from LAM airway sections. Levels of α3 and GPBP were quantified using grey scale digital image analysis. Final grey values were obtained by subtracting the corresponding grey values obtained from respective isotype controls. ((*p<0.01 compared to non-asthmatic) (one way ANOVA with Dunnett's post test)). Data is expressed as mean ± standard error of the mean.

2.3.7 αv and β3 integrins detected in airway sections

The anti-angiogenic activity of tumstatin is mediated via the interaction with the αvβ3 integrin on endothelial cells. It was important to determine whether both subunits of this integrin were present in the airway of asthmatics and non-asthmatics. Integrin αv and β3 subunits were both detected in asthmatic (n=5) and non-asthmatic (n=4) biopsies. Expression of both integrin subunits appears similar in terms of both level of expression and area of distribution in asthmatic and non-asthmatic airways (figure 2.10).

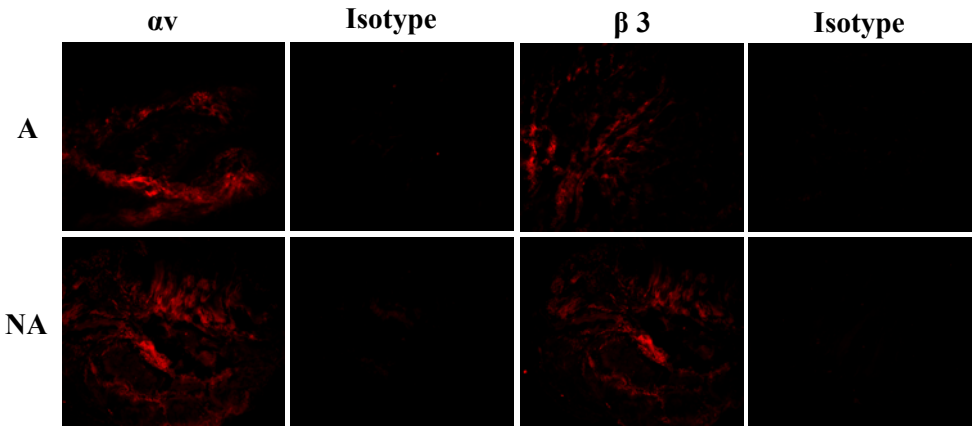


Figure 2.10: Airway sections and biopsies stained for integrin subunits αv and β3 in sections from an asthmatic biopsy and non-asthmatic biopsy. Isotype controls were negative. Specific staining was detected using Texas red (red) fluorochrome. Images are representative of all individuals tested ((n=5 asthmatics, n=4 non-asthmatics (all images at X200)).

2.3.8 Co-localisation of VEGF, tumstatin and integrin αvβ3

Tumstatin binds αvβ3 integrin to exert its anti-angiogenic properties, whereas VEGF binds αvβ3 integrin to promote angiogenesis. Co-localisation of both factors with the integrin αvβ3 in the airway has not been established. Tumstatin was absent in the asthmatic (n=4) sections as seen in previous results (2.3.3). The αvβ3 integrin was expressed broadly across the airway section from asthmatic individuals with a consistent amount of expression in the ASM bundle (figure 2.11). However, VEGF expression was confined, with specific localised staining in the ASM bundles. Co-localisation of VEGF and the αvβ3 integrin in the asthmatic sections was present on the ASM bundles but was minimal overall. This was also true for airway sections

from LAM (n=3) individuals. In contrast, co-localisation of tumstatin, VEGF and the αvβ3 integrin was clearly distinguishable in the non-asthmatic (n=3) sections (figure 2.11). Tumstatin was expressed throughout the airways of non-asthmatic individuals with a notable increase in expression in the basement membrane. VEGF and the integrin αvβ3 were also evenly expressed in the non-asthmatic airways. Although there was a clear co-localisation in the non-asthmatic airways, areas of some ASM bundles as well ECM within the airway expressed tumstatin, VEGF and the integrin αvβ3 separately. This feature is highlighted in the Z axis slices. As shown in figure 2.12 and 2.13, the Z slices from asthmatic and LAM individuals show the specific yet separate expression of VEGF and the αvβ3 integrin throughout the airway. The Z axis slices from non-asthmatic sections show the co-localisation of the three factors as well as their separate expression (figure 2.14).

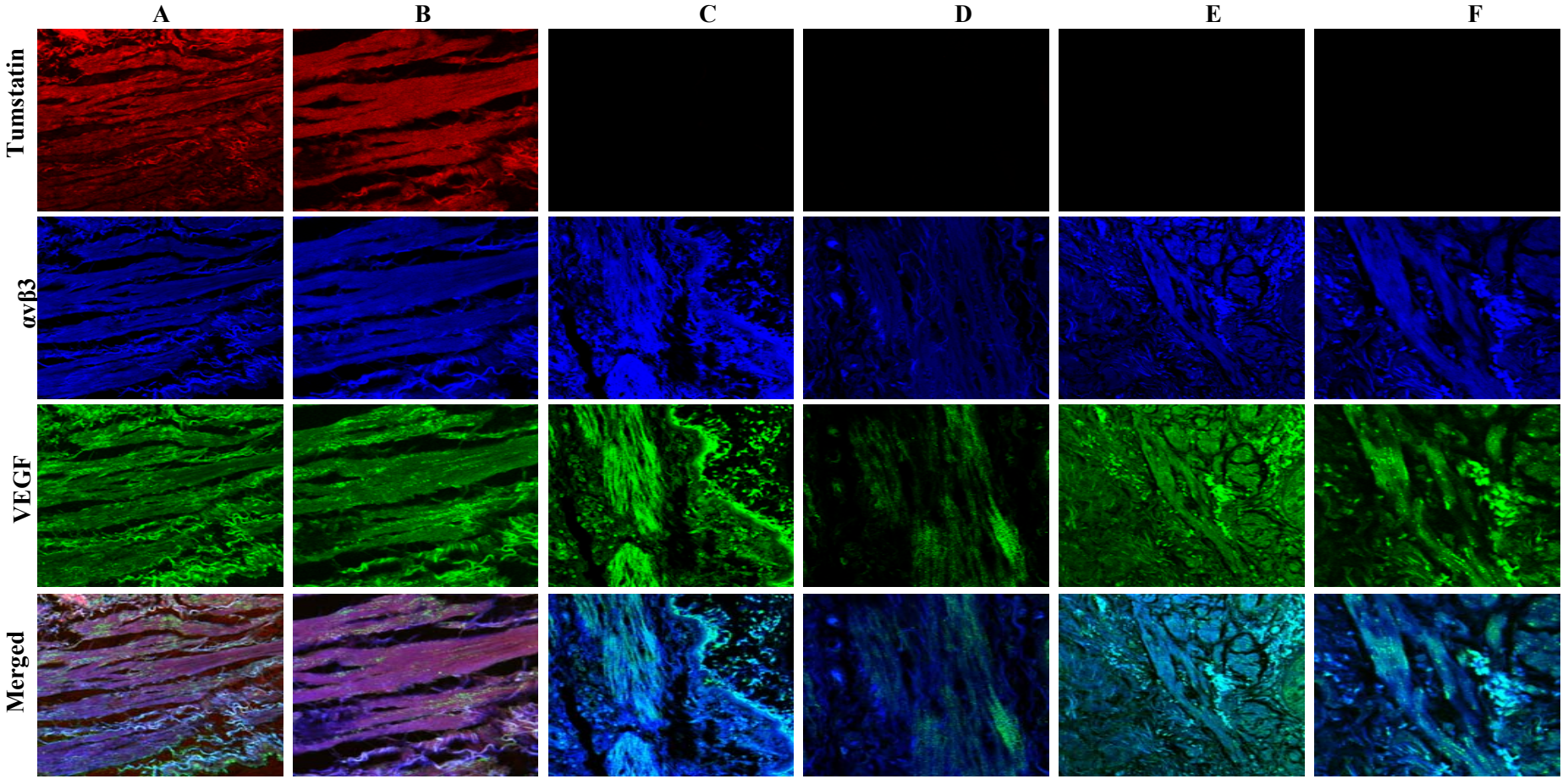


Figure 2.11: Bronchial airway sections from non-asthmatic (A and B), asthmatic (C and D) and LAM (E and F) individuals stained for tumstatin (red), αvβ3 integrin (blue) and VEGF (green). Images are representative of all individuals (non-asthmatic n=3, asthmatic n=4 and LAM n=3) tested. Images A, C and E are at X200 and B, D and F are at X400.

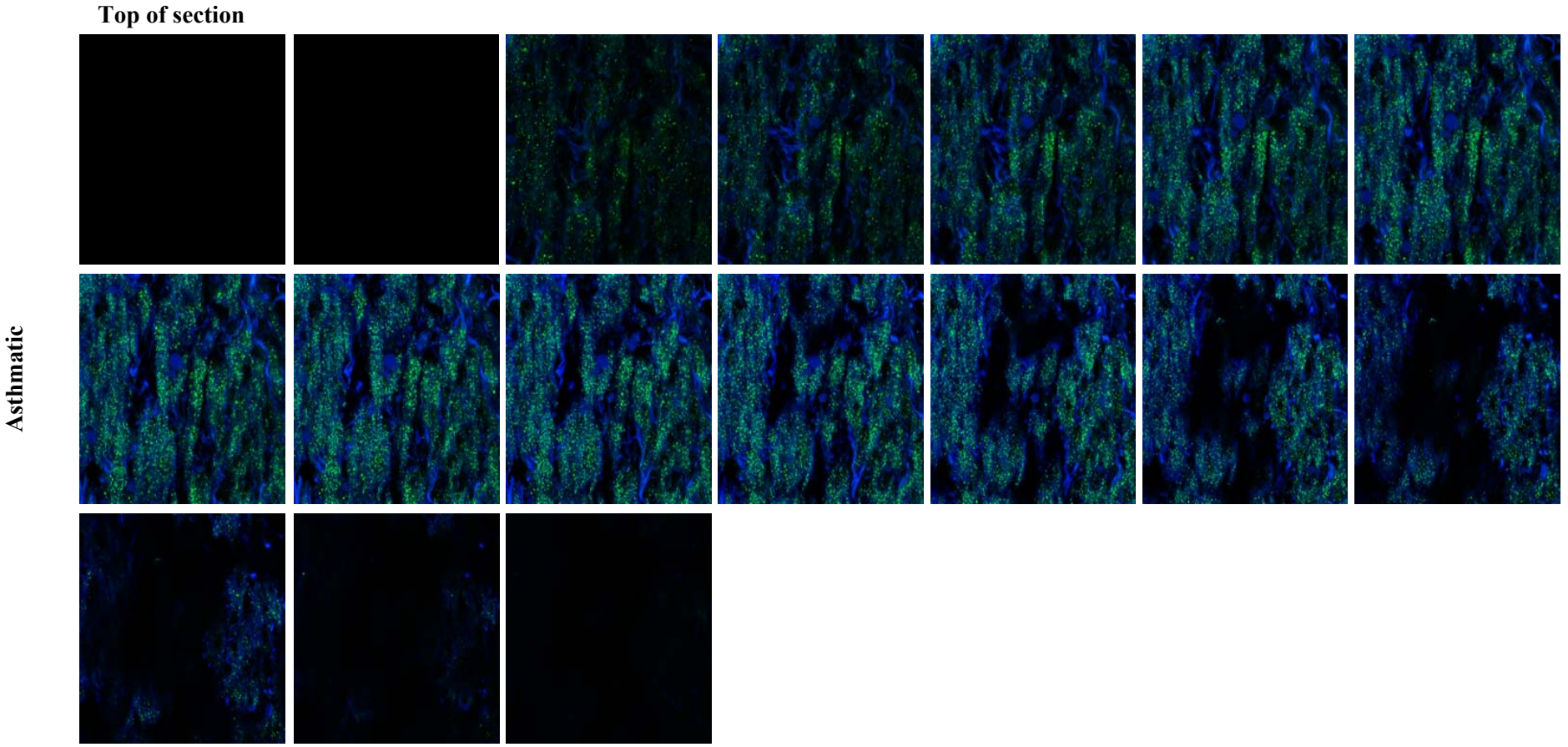


Figure 2.12: Consecutive Z-axis slices of bronchial airway section from an asthmatic individual stained for tumstatin (red), VEGF (green) and the integrin αvβ3 (blue). Images above represent the three (red, green and blue) merged channels. All images at X100. Images are representative of all asthmatic (n=4) individuals tested.

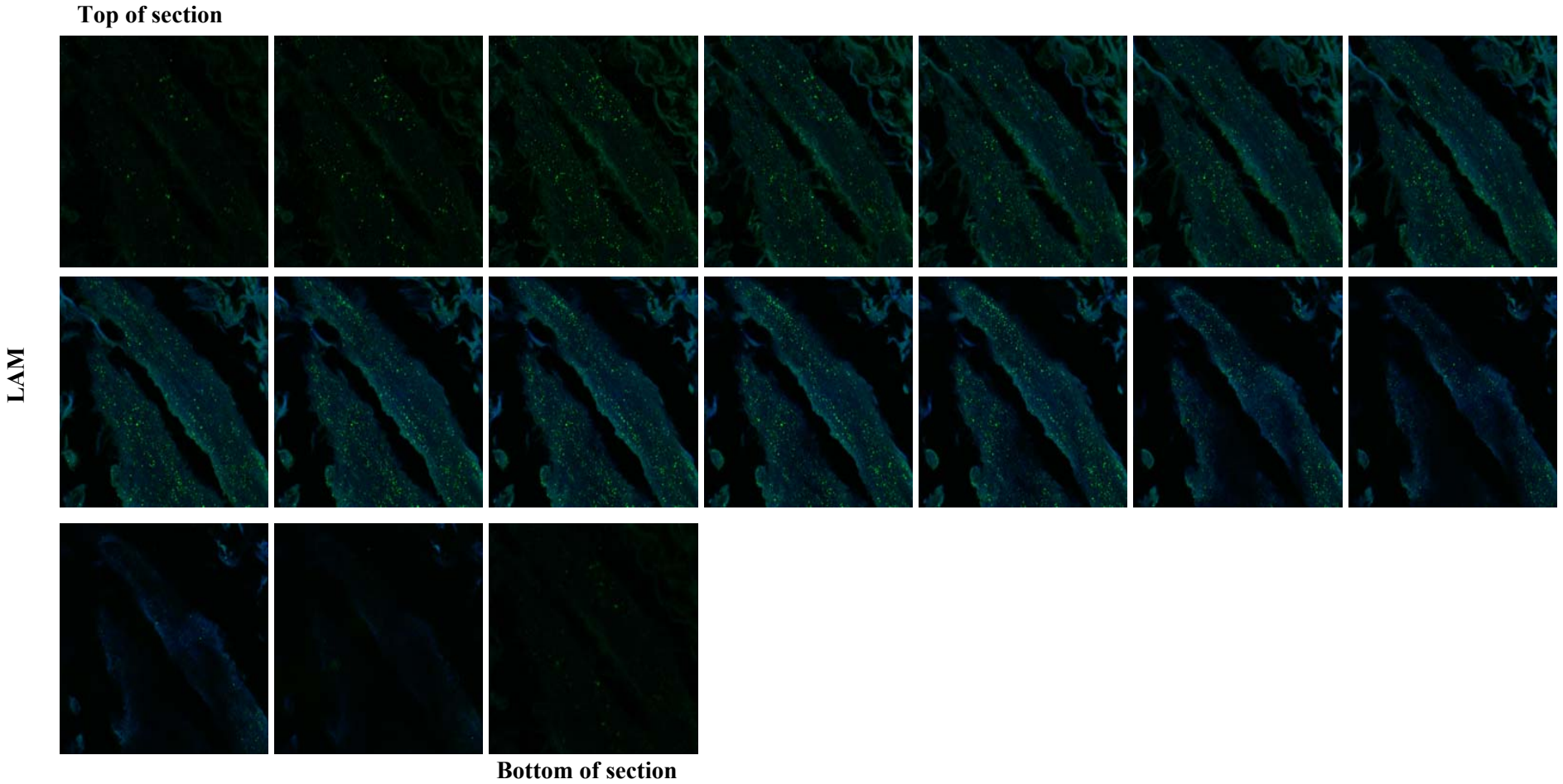


Figure 2.13: Consecutive Z-axis slices of bronchial airway section from a LAM individual stained for tumstatin (red), VEGF (green) and the integrin αvβ3 (blue). Images above represent the three (red, green and blue) merged channels. All images at X100. Images are representative of all LAM (n=3) individuals tested.

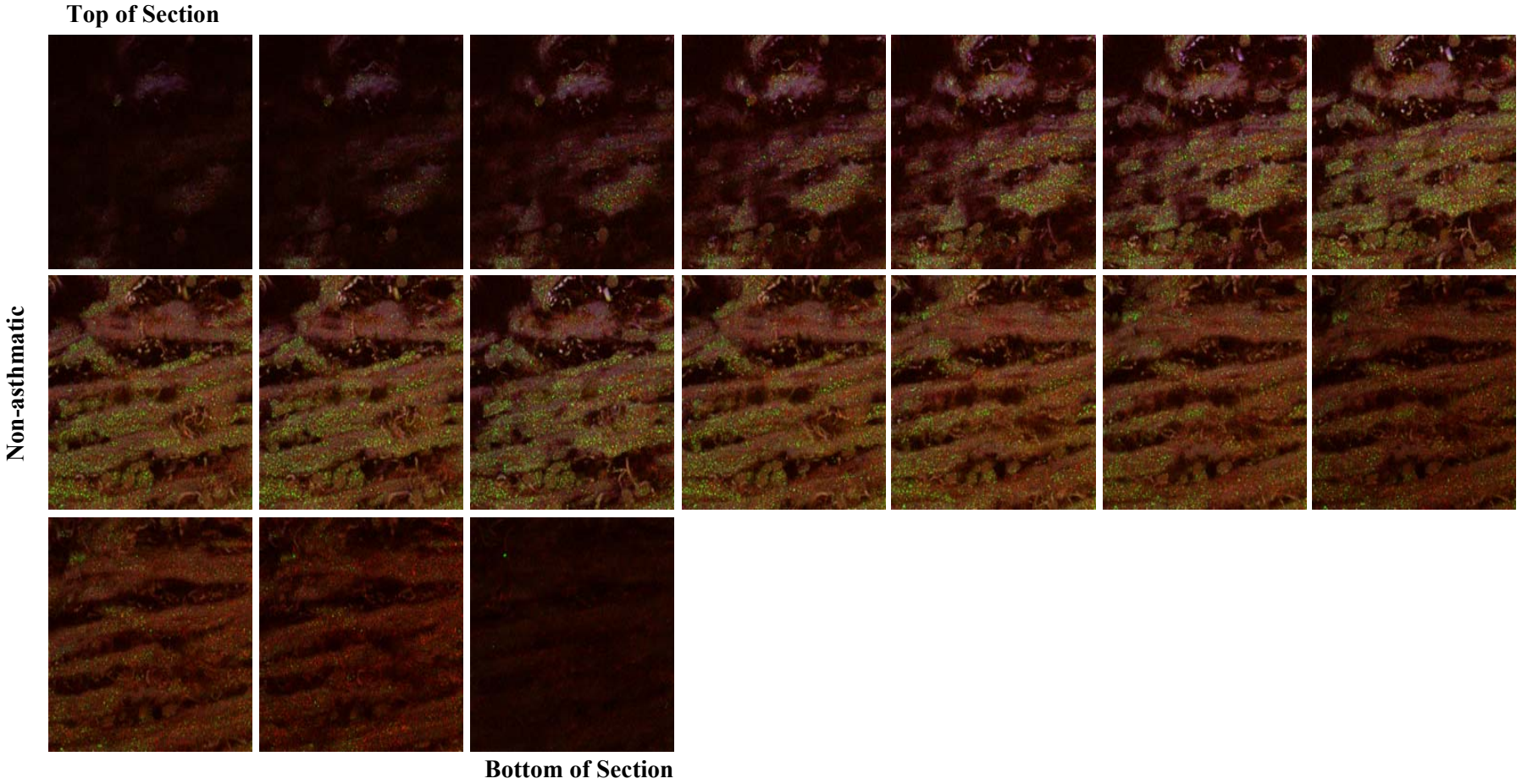


Figure 2.14: Consecutive Z-axis slices of bronchial airway section from a non-asthmatic individual stained for tumstatin (red), VEGF (green) and the integrin αvβ3 (blue). Images above represent the three (red, green and blue) merged channels. All images at X100. Images are representative of all non-asthmatic (n=3) individuals tested.

2.4 Discussion

Tumstatin is absent in the airways of asthmatic and LAM individuals and present in the airways of non-asthmatic, COPD, CF and bronchiectic individuals. Expression of the GPBP was not increased in asthmatic and LAM airways compared to non-asthmatics. The αv and β3 integrin subunits were present in both asthmatic and non-asthmatic airways. The αvβ3 integrin was present in asthmatic, LAM and non-asthmatic individuals, and the co-localisation between the VEGF and αvβ3 integrin was shown to be limited in the asthmatic and LAM airways compared to non-asthmatics.

Angiogenesis is one of the major features of airway remodelling in asthma (Hashimoto *et al.*, 2005; Li *et al.*, 1997), yet its role in the airway remains undetermined. In order to characterise angiogenic inhibitors in the airway of asthmatics and LAM individuals the presence of increased angiogenesis in the asthmatic and LAM bronchial airway sections used in this study was confirmed. CD31, a cell adhesion molecule expressed on platelets and at endothelial cell intercellular junctions as well as vWF, a complex produced by endothelial cells and megakaryocytes, were used to identify blood vessels in the airway. The presence of increased angiogenesis in the COPD, CF and bronchiectasis sections used in this study was also confirmed.

Tumstatin was not present in the airways of asthmatics but was present in the airways of non-asthmatic individuals. In contrast, both in asthmatics and non-

asthmatics the remaining five collagen IV α chain NC1 domains were present. It has previously been reported that collagen IV levels are decreased in the airways of asthmatics (Bousquet *et al.*, 1992), however it is not known if that reduction is due to a specific reduction of one or more α chains. Levels of collagen IV were also found to be decreased in asthmatic ASM cells compared to non-asthmatics *in vitro* (Johnson *et al.*, 2004). However, once again, it is not known which α chain the collagen IV antibody used in this study detected.

Asthma and LAM are both chronic respiratory diseases with reported increased angiogenesis. Both disease states were shown to be lacking tumstatin in the airway. To establish whether the absence of tumstatin is a feature of all chronic respiratory diseases shown to exhibit angiogenesis, airway sections from individuals with COPD, CF and bronchiectasis were examined for the presence of tumstatin. The presence of tumstatin was confirmed in all three chronic respiratory diseases. Therefore the absence of tumstatin is not a feature of chronic respiratory disease but is specific to asthma and LAM.

The absence of tumstatin could be implicated in the increase in angiogenesis in the asthmatic airway. McDonald reported that the development of angiogenesis is necessary to supply nutrients to accumulations of inflammatory cells in chronically inflamed tissue (McDonald, 2001). It can be hypothesised that the chronic inflammation and structural changes

observed in asthma, such as smooth muscle hyperplasia and hypertrophy, both result from the increased supply of nutrients brought into the local environment by angiogenesis. Therefore, the absence of the angiogenic inhibitor, tumstatin, in asthmatic individuals may result in increased angiogenesis in the airway.

Tumstatin, as well as the collagen IV α5 NC1 domain, was absent in the airways of LAM individuals. Smooth muscle in LAM airways is hyperproliferative (Black *et al.*, 2005; Merrilees *et al.*, 2004), therefore the absence of tumstatin in this disease may be further exaggerating this feature, again via the increase in the availability of nutrients. The collagen IV α5 chain NC1 domain to date has not been well characterised but preliminary findings suggest it may also have a role as an angiogenic inhibitor (Ortega *et al.*, 2002). The absence of the collagen IV α5 chain in the LAM tissues was not further investigated as it was beyond the scope of this thesis. However, this would potentially be of interest as the collagen IV α3 and α5 chains are associated in other diseases, such as Alports Syndrome where a mutation in the collagen IV α3 and α5 gene exists and results in kidney failure (Heidet *et al.*, 2001).

The GPBP is a kinase targeting the NC1 domain of the collagen IV α3 chain in order to determine its molecular organisation. The collagen IV α3 chain has a complex folding process that potentially gives rise to multiple conformers. Non-assembled conformers are specifically activated by

phosphorylation which is a signal the cell uses to determine the folding of the α3 NC1 domain (Raya *et al.*, 2000). The N-terminus of the α3 NC1 domain contains a phosphorylation site that the GPBP binds to and phosphorylates. Increased expression of GPBP in the airway may generate misfolded stuctures of the α3 chain. This misfolding may render the collagen IV α3 chain NC1 domain unrecognisable by the immune system and therefore lead to its destruction. There were no differences in the levels of expression of the GPBP between asthmatic, LAM and non-asthmatic individuals. This suggests that the lack of detection of tumstatin in the airway of asthmatic and LAM individuals is not due to an autoimmune response which leads to the clearing of tumstatin from the airways as a result of altered GPBP dependent phosphorylation. However, in this study the antibody used to detect the GPBP did not differentiate between its two isoforms, of which GPBP and not GPBPΔ26 is the more active isoform of the two. Therefore, even though there was no difference in the levels detected in the asthmatic, LAM and non-asthmatic airways, a difference in the ratio of GPBP to GPBPΔ26 could still exist.

The angiogenic inhibitory actions of tumstatin occur via its interaction with the integrin αvβ3 on endothelial cells, as described in 1.5.1. For tumstatin to have a role in the airways of asthmatics as an angiogenic inhibitor, integrin αvβ3 must be present in these airways. Both the αv and β3 integrin subunits were detected in the airways of asthmatic and non-asthmatic individuals. VEGF, a promoter of angiogenesis, also interacts with the αvβ3 integrin to

mediate its angiogenic activity. Co-localisation of tumstatin, VEGF and αvβ3 integrin was possible through the use of confocal microscopy. It was shown that in the asthmatic and LAM airways VEGF was localised to specific regions on the ASM bundles and elsewhere in the tissue section, whereas the expression of the αvβ3 integrin was more widely distributed throughout the airway. Some areas in both asthmatic and LAM sections showed co-localisation between VEGF and the αvβ3 integrin. These results suggest that some co-localisation of VEGF and the αvβ3 integrin is occurring in the airway of both asthmatic and LAM individuals. However, these two molecules were also located in the same regions in the airway but were not co-localised. This is made apparent by the lack of colour change in the merged images, as co-localisation usually results in a combined colour formation. The lack of co-localisation was further confirmed in the Z-stacking images obtained, where the expression of both VEGF and αvβ3 integrin were seen at different levels throughout the tissue. In comparison, the expression of tumstatin, VEGF and αvβ3 in the non-asthmatic individuals was co-localised throughout the airway. It is important to note that certain regions in the tissue were expressing tumstatin and VEGF side by side. This suggests that tumstatin and VEGF are naturally occurring within the same vicinity in the airway for the purpose of maintaining a balance in the angiogenic process. This further suggests that the absence of tumstatin seen in the asthmatic and LAM airways results in the overexpression of VEGF which potentially leads to an increase in angiogenesis due to the lack of availability of an ‘off’ switch.

The various staining techniques used in this chapter have all confirmed the absence of tumstatin in asthmatic and LAM airways tissue. The limiting factor in the use of fluorescence staining is the inability to accurately quantitate the level of expression. Nevertheless, it was possible to overcome this with the use of colormetric staining which allowed for quantitation and reconfirmation of the absence of tumstatin in the airway section. The use of two different antibodies against tumstatin as well as different detection techniques confirmed the specificity of the staining techniques. Homogeneity in the staining was observed within each patient group, although fluorescent images across each patient group varied slightly in the intensity of signal. This was expected as the intensity of the laser used to excite the fluorochrome fluctuates. The difference in the intensity of the staining was not significantly different within each patient group and furthermore was confirmed by the use of quantitated colourmetric images.

In summary, the absence of tumstatin in the airways of asthmatic and LAM individuals is a specific feature of both diseases and not a general feature of chronic respiratory disease. The lack of alteration in the expression of the GPBP in asthmatic and LAM airways compared to non-asthmatics, suggests that the absence of tumstatin was not the result of altered phosphorylation. The absence of this angiogenic inhibitor may be contributing to the increased angiogenesis seen in the asthmatic and LAM airways. The presence of the αvβ3 integrin in asthmatic and LAM airways, suggest that tumstatin activity in the airways is possible.

Chapter 3

Regulation of angiogenesis in the airway

3.1 Introduction

Collagen IV α3 chain contains the angiogenic inhibitor tumstatin. It is a 188kDa molecule, of which tumstatin (28kDa) is the bioactive non-collagenous domain (NC1). Tumstatin is active as an angiogenic inhibitor when it is cleaved from the whole collagen IV α3 chain (Sund *et al.*, 2005). Matrix metalloproteinase (MMP)-9 cleaves collagen IV to release tumstatin with the greatest efficiency. However, several other matrix proteases such as MMP-2, 3 and 13 are also able to cleave tumstatin but less efficiently (Hamano *et al.*, 2003). MMP-9 deficient mice exhibited an accelerated rate of tumour growth. Yet, upon restoration of physiological levels of tumstatin in these mice, tumour growth rate was retarded to that seen in wild-type mice (Hamano *et al.*, 2003). Circulating physiological levels of tumstatin in serum in mice models, were between 300-350ng/ml (Hamano *et al.*, 2003). Levels of tumstatin expression in human serum have not been measured.

Tumstatin blocks angiogenesis by inhibiting the proliferation of endothelial cells and inducing apoptosis of proliferating endothelial cells (Maeshima *et al.*, 2000). It suppresses tumour growth in renal and prostate carcinoma cells as well as inhibiting the proliferation of human, bovine and murine endothelial cells. Tumstatin is a 244 amino acid molecule, with the angiogenic activity localised to amino acids 54-132. The activity of this region is specific for inhibition of proliferating endothelial cells as it had no effect on prostate cancer tumour cells (Maeshima *et al.*, 2001a; Maeshima *et*

al., 2001b). This region has been further defined and peptides T3 (69-88 amino acid) and T7 (74-98 amino acid (figure 3.1)) were identified as having anti-angiogenic activity in that inhibition of proliferation and induction of apoptosis was seen in bovine pulmonary arterial endothelial cells, human umbilical vein endothelial cells (HUVEC's) and a human prostate adenocarcinoma cell line (Maeshima *et al.*, 2001b).

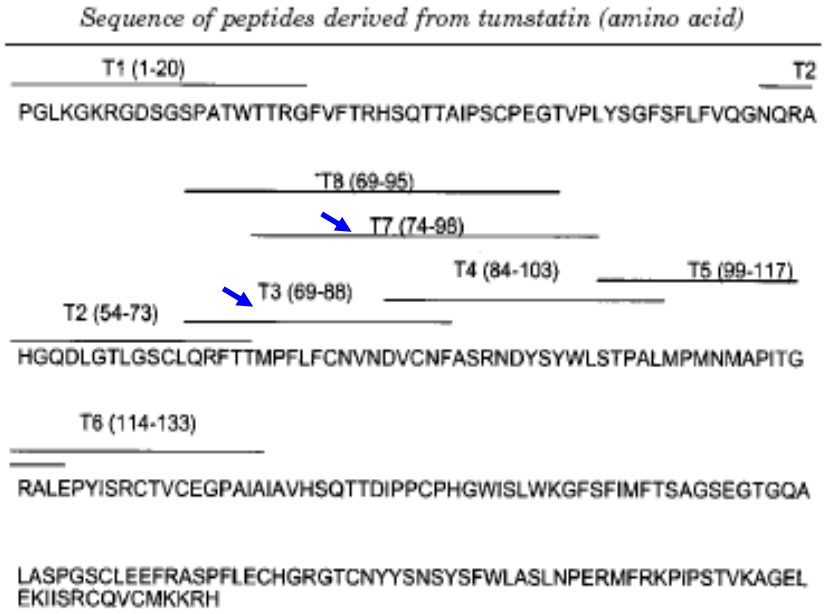


Figure 3.1: Sequence of peptides derived from tumstatin. Figure has been adapted from Maeshima *et al.* (2001). Blue arrows showing T3 and T7 peptides.

The angiogenic response consists of a balance between pro-angiogenic factors and anti-angiogenic factors. Aberrant angiogenesis occurs as a result of a disruption to this balance. Angiogenic stimulators such as vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) are reported to be increased in asthma (Hoshino *et al.*, 2001a; Lee *et al.*,

2001). These factors are secreted by inflammatory cells, pericytes, keratinocytes, tumour cells and airway smooth muscle cells (ASM) (Hoshino *et al.*, 2001b; Knox *et al.*, 2001). VEGF regulates both physiological and pathological angiogenesis. It exists in five isoforms, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, which are generated by alternative splicing of a single gene (Hutchings *et al.*, 2003). $VEGF_{165}$ is the most abundant of the isoforms and has a high binding affinity to heparin. Although this molecule is secreted by cells, a significant fraction remains bound to the cell surface and the extracellular matrix (ECM) (D'Andrea *et al.*, 2006). $VEGF_{165}$ adheres to endothelial cells, and this cell adhesion is mediated through the αvβ3 integrin, which facilitates the process of angiogenesis. However, tumstatin binds to the αvβ3 integrin and inhibits $VEGF_{165}$ binding of endothelial cell (Hutchings *et al.*, 2003). Although VEGF is responsible for initiating blood vessel growth, Angiopoietin-1 (Ang-1), an endothelial cell-specific growth factor, is responsible for the maturation of blood vessels from primitive tubes to network blood vessels (Carmeliet, 2004). Ang-1 is widely expressed in normal adult tissue, and is essential for the development of vasculature. Mice with an Ang-1 deficiency die due to the lack of endothelial cell tube maturation into blood vessels (McDonald, 2001). Ang-1 has also been reported to exhibit anti-angiogenic activity, in that it can suppress tumour angiogenesis in colon cancer (Ahmad *et al.*, 2001; Tian *et al.*, 2002). The effects of Ang-1 on blood vessels also serves to inhibit vascular leakage and this is due to its interaction with platelet/endothelial cell adhesion

molecule (PECAM). Inhibition of vascular leakage is an advantage in conditions of sepsis, lung injury and local inflammation.

The specific role of angiogenic inhibitors in the airway has not been elucidated. Endostatin (described in 1.4.1.1) is the only endogenous angiogenic inhibitor to be examined in asthma (Asai *et al.*, 2002). The levels of VEGF and endostatin in asthmatic sputum were measured and an increase in the VEGF/endostatin ratio in asthmatics compared to non-asthmatics was found. However, the increase in this ratio was due to an increase in the levels of VEGF rather than changes in endostatin (Asai *et al.*, 2002). To date there are no studies examining the levels of endogenous angiogenic inhibitors in tissue from asthmatic patients.

The inhibitory activity of tumstatin has been examined using bovine pulmonary arterial endothelial cells, HUVEC, human prostate adenocarcinoma cells and mouse endothelial cells (Maeshima *et al.*, 2000; Maeshima *et al.*, 2002). The effectiveness of tumstatin in human airways has not been directly investigated. Caudroy *et al.* showed, using human pulmonary carcinoma cells, an association between the expression of tumstatin and tumour vascularisation. Highly vascularised tumours expressed lower levels of tumstatin (Caudroy *et al.*, 2004). The ability of tumstatin to affect human primary pulmonary endothelial cells has yet to be examined.

The specific aims of this study were:

1) to determine and compare the levels of tumstatin in serum and bronchoalveolar lavage fluid (BAL-f) samples of asthmatic and non-asthmatic individuals.

2) to assess the action of tumstatin on human primary pulmonary endothelial cells.

3.2 Materials and Methods

3.2.1 Patient details

Serum and BAL-f were obtained from individuals with intermittent, mild persistent or moderate persistent atopic asthma, according to GINA guidelines (Global Initiative for Asthma, Global Strategy for Asthma Management and Prevention, NIH Publication, 2005). Patients were included if they had asthma symptoms in the preceding 12 months, a positive mannitol/methacoline bronchial provocation challenge test and fewer than 5 pack year history of smoking. Patients were excluded if their baseline forced expiratory volume in the first second (FEV_1) was less than 60% predicted. Non-asthmatic individuals had no history of asthma or other lung disease, had normal baseline spirometry (at least 80% predicted), negative mannitol/methacoline bronchial challenge test and no significant bronchodilator reversibility (12% and 200mL improvement in FEV_1). In this chapter these individuals will be referred to as healthy controls.

Mannitol/methacoline bronchial provocation challenge tests were performed by Dr Melissa Baraket, whereby volunteers were given increasing concentrations of mannitol/methacoline up to a final concentration of 6.1µmol or a fall in FEV_1 of >20% in which case the test was terminated. Bronchoalveolar lavage was performed by the instillation of 0.9% sodium chloride solution (4 successive aliquots of 60mL, total 240mL) warmed to 37˚C into the right middle lobe which was then aspirated for collection in a

sterile glass bottle (chilled to prevent cellular adhesion to the glass). Collection of samples was performed by Dr Melissa Baraket from volunteers participating in a clinical study.

ASM cells, fibroblasts and endothelial cells were isolated from explanted lung and resections. ASM cells were also isolated from biopsies. Complete patient details are outlined in table 3.1.

Table 3.1: Patient details

Patient #	Age	Sex	Disease	Type of sample	Derived from
1	26	Male	Healthy control	Serum, BAL-f	Study participant
2	22	Male	Healthy control	Serum, BAL-f	Study participant
3	33	Male	Healthy control	Serum, BAL-f	Study participant
4	25	Female	Healthy control	Serum, BAL-f	Study participant
5	26	Male	Asthmatic a	Serum, BAL-f	Study participant
6	30	Male	Asthmatic b	Serum, BAL-f	Study participant
7	21	Male	Asthmatic a	Serum, BAL-f	Study participant
8	21	Male	Asthmatic a	Serum, BAL-f	Study participant
9	20	Male	Asthmatic b	Serum, BAL-f	Study participant
10	22	Male	Asthmatic b	Serum, BAL-f	Study participant
11	27	Male	Asthmatic a	Serum, BAL-f	Study participant
12	21	Male	Asthmatic b	Serum, BAL-f	Study participant
13	23	Male	Asthmatic a	Serum, BAL-f	Study participant
14	64	Female	Emphysema	Fibroblast cells	Lung resection
15	55	Male	Emphysema	Fibroblast cells	Lung resection
16	44	Male	Pulmonary fibrosis	Fibroblast cells	Lung resection
17	74	Male	Carcinoma	ASM cells	Lung resection
18	75	Male	Carcinoma	ASM cells	Lung resection
19	20	Male	Healthy control	ASM cells	Endobronchial biopsy

a-100µg fluticasone twice daily, b- 500µg fluticasone twice daily

Patient #	Age	Sex	Disease	Type of sample	Derived from
20	56	Female	Emphysema	Pulmonary endothelial cells	Explanted lung
21	23	Male	Bronchiesctasis	Pulmonary endothelial cells	Explanted lung
22	53	Male	Bronchiectasis	Pulmonary endothelial cells	Explanted lung
23	55	Male	Emphysema	Pulmonary endothelial cells	Explanted lung
24	64	Female	Pulmonary fibrosis	Pulmonary endothelial cells	Explanted lung
25	56	Female	Asthmatic	Biopsy	Endobronchial biopsy
26	20	Male	Asthmatic	Biopsy	Endobronchial biopsy
27	42	Male	Asthmatic	Biopsy	Endobronchial biopsy
28	45	Male	Asthmatic	Biopsy	Endobronchial biopsy
29	60	Male	Asthmatic	Biopsy	Endobronchial biopsy
30	38	Female	LAM	ASM Cells	Explanted lung
31	39	Female	LAM	ASM Cells	Explanted lung
32	46	Female	LAM	ASM Cells	Explanted lung
33	40	Female	LAM	ASM Cells	Explanted lung
34	56	Female	LAM	ASM Cells	Explanted lung

a-100µg fluticasone twice daily, b- 500µg fluticasone twice daily

3.2.2 Airway smooth muscle cell isolation

ASM cells were isolated as previously described (Johnson *et al.*, 2001). Bronchial airways were dissected from the surrounding parenchyma and washed firstly in 70% alcohol followed by Hanks buffered salt solution (Trace Scientific, Melbourne, AUS) and cut longitudinally. The bronchus was then pinned down in a sterile petri dish with the epithelial surface facing upwards. With the use of a dissecting microscope, the epithelium was removed with fine forceps in order to expose the smooth muscle bundles. The smooth muscle bundles were dissected free from the surrounding tissue and placed in a sterile tube containing Hanks buffered salt solution (Trace Scientific). The isolated smooth muscle bundles were centrifuged at 150 x g for 5 minutes. Isolation medium was aspirated and isolated pieces of muscle were placed into 25cm^2 vented tissue culture flasks (Becton and Dickinson (BD) Franklin Lakes, UA, USA) containing 2.5mls Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS, JRH Biosciences, Melbourne, AUS), 20U/ml penicillin, 20g/ml streptomycin, and 2.5g/ml amphotericin and placed in a humidified CO_2 incubator (5% CO_2 in air) and maintained at 37°C. Following 2 weeks of culture at 37°C, 5% CO_2, the medium was aspirated and fresh medium was added. Cells were confluent in approximately 2-3 weeks, and were subcultured (following trypsinisation, see below). ASM cells used in this chapter were all between passage 5-7.

ASM cells were subcultured using the following procedure. Growth medium was aspirated from the culture flasks. Cells were washed in Hanks balanced salt solution (Trace Scientific), and incubated with 0.05% trypsin (w/v) (Sigma, St Louis, MO) supplemented with 1mM ethylenediaminetetraacetic acid solution salt (EDTA) (Biolab Australia, VIC, Australia) in DMEM for 3 minutes. Confirmation of detachment of cells was obtained using microscopy. Trypsinisation was stopped by the addition of 3 times the volume of growth medium. The cells were centrifuged at 150 x g for 5 minutes, and resuspended in 1ml of growth medium. Trypan blue exclusion was used for cell counting and viability using manual cell counting (haemocytometer).

Smooth muscle phenotype was confirmed by morphology, and positive immunofluorescent staining with a specific α-smooth muscle actin antibody and a calponin antibody.

3.2.3 Pulmonary endothelial cell isolation

Human pulmonary endothelial cells were isolated from explanted lungs. Blood vessels were dissected free from surrounding tissue, cut longitudinally and then cut into approximately $5mm^3$ segments. Blood vessel segments were then placed in digestive buffer (1mg (250U)/ml type 2 collagenase, 1U/ml dispase, 10mg/ml bovine serum albumin (BSA), Sigma) and incubated at 37°C for 10 minutes with agitation every 2 minutes. The supernatants were harvested and replaced with fresh digestive buffer every 10 minutes for a total of 50 minutes. To stop the reaction, 10% of the final volume of FBS

was added to the supernatants before centrifugation at 200g for 5 minutes. The cells were washed with nutrient mixture Ham's F-12 (F-12) supplemented with 10% FBS (JRH Biosciences), 10μg/ml endothelial cell growth supplement (BD), 20U/ml heparin (Sigma) and 20U/ml penicillin, 20g/ml streptomycin and 2.5g/ml amphotericin (Sigma) (culture medium). They were then re-centrifuged at 200g for 5 minutes before being placed in a $75cm^2$ vented tissue culture flask (BD) containing 10ml of culture medium and incubated for 1 hour at 37°C 5% CO_2. Unattached cells were harvested, centrifuged at 200g for 5 minutes and placed in a new $75cm^2$ vented tissue culture flask (BD) (pre-coated with 0.2% (w/v) gelatine (Sigma) containing 10ml of culture medium. The flask was then maintained at 37°C in a humidified CO_2 incubator (5% CO_2 in air). Cells were confluent in approximately 2-3 weeks and were subcultured (following trypsinisation, protocol same as in HASM, see above). Endothelial cells used in this chapter were all between passage 4-6.

Each endothelial cell line isolated was stained for CD105 (an endothelial cell specific marker) to confirm cell type.

3.2.4 Dot blot

Using specialised dot blotting equipment (Slot Blot manifold, PR 648, GE Healthcare Uppsala, Sweden) the amount of tumstatin and goodpasture antigen-binding protein (GPBP) in the serum and BAL-f of asthmatic individuals, pre and post steroid treatment (inhaled fluticasone propionate

either 100µg or 500µg twice daily) and healthy controls was compared. Nitrocellulose (NC) paper was cut to size (Hybond-ECL 0.45µm pore size, Amersham) and washed in tris buffered saline with 0.05% Tween (T-TBS) for 5 minutes before placing in the blot machine. Fifty µl of serum or fractionated BAL-f was loaded into each slot and the vacuum applied until samples were completely evacuated. Tumstatin (Recombinant peptide, NC1 domain from bovine collagen IV, Weislab, Lund, Sweden) was used as a positive control for the tumstatin blots and protein extracts from non-asthmatic lung tissue as the GPBP positive control (see below for method). The membrane was then washed twice with T-TBS, which was extracted with the aid of the vacuum, before being removed from the blotting apparatus and blocked in 5% BSA (w/v) in PBS for 1 hour at room temperature. Primary antibody, mouse anti-human collagen IV NC1 domain of the α3 chain (Wieslab) or chicken anti-human collagen IV α3 GPBP (GenWay Biotech, San Diego, CA, USA), was added at 1:1200 and 1:100 dilution in 0.1% BSA /T-TBS respectively and incubated for one hour at room temperature. T-TBS was used to wash the membrane (3 times) and the secondary antibody was added (rabbit anti-mouse HRP and rabbit anti-chicken HRP at a 1:2000 dilution in 0.1% BSA/T-TBS (DakoCytomation, Glostrup, Denmark, and Abcam, Cambridge, MA, USA) respectively) and incubated for 30 minutes at room temperature. The membrane was then washed with T-TBS for 15 minutes with a further 2 washes of 5 minutes each. The membrane was visualised, the image captured and densitometry calculated using a Kodak 4000MM image station, following the addition of

chemiluminescent substrate (West Dura Extended, Pierce, Rockford, IL, USA).

Protein from non-asthmatic airway tissue was extracted using the following method: airway tissue was dissected free from surrounding parenchyma and rinsed in PBS before being snap frozen in liquid nitrogen. Tissue was pulverised using a mortar and pestle and placed in ice cold buffer solution (PBS containing 0.5% sodium deoxycholate, 1% Sodium dodecyl sulphate (SDS), 10% protease inhibitor cocktail set III (Calbiochem, San Diego, CA)) for 15 minutes. The sample was then heated at 95°C for 5 minutes and centrifuged at 13000rpm for a further 5 minutes. Supernatant was collected and stored at -80°C until required.

3.2.4.1 Fractionation

BAL-f and serum samples were fractionated using centrifugal filter devices with a 100kDa pore size (Millipore Corporation, Temecula, CA). One ml of sample was loaded into the top tube of the device and capped. Tubes were then centrifuged at 4000rpm for 5 minutes. Samples from above and below the filter, referred to as top and bottom samples, were collected and analysed using the dot blot method described above.

3.2.5 Western blotting

A non-reducing western blot using antibodies against collagen IV α3 NC1 domain (tumstatin) was performed. Serum samples from asthmatic and health control individuals were used at a 1:500 dilution. Twenty µl of sample was

added to 20µl of loading buffer (20% glycerol, 1M Tris HCl pH 6.8, bromophenol blue) and loaded onto pre-cast gradient gels, 4%-20%, (Biorad, Hercules, CA, USA). The gels were run in running buffer (25mM tris, 192mM glycine, 1% w/v SDS) at 100 Volts for 1hour and 15 minutes.

Proteins were than transferred onto polyvinylidene difluoride (PVDF) membranes using transfer buffer (25nM tris, 192mM glycine) for 1 hour and 30 minutes at 30 Volts. Five percent BSA/TBS was used to block the membranes for 1 hour at room temperature and primary antibody, mouse anti-human collagen IV NC1 domain of the α3 chain (Wieslab) was added at a 1:2000 dilution and incubated overnight at 4°C with rocking. Membranes were then washed 3 times with T-TBS and incubated with rabbit anti-mouse HRP (DakoCytomation) at a 1:1000 dilution for 1 hour at room temperature. Membranes were visualised and images captured using the Kodak 4000MM image station, following the addition of chemiluminescent substrate (West Dura Extended, Pierce, QLD, AUS). A pre-stained SDS-PAGE standard broad range ladder was run to confirm band sizes.

Gels were checked for complete transfer using coomassie blue staining (50% methanol, 0.05% coomassie blue) for 2 hours at room temperature with rocking. Acetic acid was used to differentiate the staining and gels were de-stained for 20 minutes.

3.2.6 Inhibition of proliferation

ASM cells from asthmatic and non-asthmatic individuals and primary pulmonary endothelial cells from non-asthmatic individuals were seeded in 96 well plates at $1x10^4$cells /cm^2, ASM cells in DMEM containing 5% FBS and endothelial cells in F-12 10% FBS (growth medium), for a period of 24 hours. The cells were then washed three times using Hanks solution and placed in DMEM/F-12 containing 0.1% (v/v) insulin transferrin and selenium (ITS) (Invitrogen) for 24 hours. Following washing as before, growth medium was added to the cells. The synthetic tumstatin derived peptides T3 (Leu-Gln-Arg-Phe-Thr-Thr-Met-Pro-Phe-Leu-Phe-Cys-Asn-Val-Asn-Asp-Val-Cys-Asn-Phe) (Phoenix Pharmaceuticals, Burlingame, CA) dissolved in water or T7 (Thr-Met-Pro-Phe-Leu-Phe-Cys-Asn-Val-Asn-Asp-Val-Cys-Asn-Phe-Ala-Ser-Arg-Asn-Asp-Tyr-Ser-Tyr-Trp-Leu) (Phoenix pharmaceuticals) dissolved in 4% acetonitrile were added at 4.5µM to some wells. Four percent acetonitrile alone was used as a vehicle control. The peptides were replaced after the initial 24 hours and then after every 48 hours. Proliferation was assessed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay (Sigma) at day 3, 7 and 9. Ten µl of MTT was added to each well for 5 hours at 37°C followed by the addition of 100µl of filtered 10% SDS in 0.01M HCl overnight. The specific absorption of each well was measured using the Spectramax, MZ (Molecular Devices, Union City, CA) plate reader at 690nm (reference) and 570nm.

3.2.7 2D endothelial cell tube formation

Endothelial cell tube formation in the presence and absence of tumstatin was assessed with a commercial tube formation assay (BD BioCoat Angiogenesis system-endothelial cell tube formation, BD Biosciences). Primary pulmonary endothelial cells were seeded onto the 96 well plate at $4x10^5$ cells/ml in 10% FBS F-12 with endothelial cell growth supplement (ECG, BD Biosciences). Baseline tube formation was assessed and tumstatin (Recombinant peptide, NC1 domain from bovine collagen IV, Weislab) was added in duplicate wells in increasing concentrations from 8.4-2800 pg/ml. Following overnight incubation (37°C, 5% CO_2) tube formation (angiogenesis) was visualised using an inverted light microscope. Images were taken using a digital camera (Olympus Camedia C-4000 Zoom digital compact camera 4.O megapixel). Human umbilical vein endothelial cells (HUVECS) (American Type Culture Collection, Manassas, UA, USA) were used in this assay as a positive control for tube formation.

The number of tubes per well were counted and statistical analyses (one way ANOVA with Dunnett's post test) were performed.

3.2.8 3D endothelial cell tube formation

Twelve well plates (Falcon BD Labware) were coated with 400µl of Matrigel (BD Biosciences) and incubated at 37°C for 1 hour. Primary pulmonary

endothelial cells were seeded into each well of the 12 well plates at $4x10^4$ cells/ml in 10% FBS/F-12 with endothelial cell growth supplement in the presence of $VEGF_{165}$ 100ng/ml (R&D Systems), angiopoietin-1 (Ang-1) 100ng/ml (R&D Systems), ephrin-B2 (Eph-B2) 300ng/ml (R&D Systems) and 5% (w/v) fibrin (Sigma-Aldrich). Tumstatin (Recombinant peptide, NC1 domain from bovine collagen IV, Weislab) was added at 2800 pg/ml in duplicate wells. Plates were incubated at 37°C, 5% CO_2 for 7 days. Cells were restimulated every 48hrs. Images were taken using a digital camera (Olympus Camedia-4000 Zoom Digital Compact Camera 4.O megapixel) every day for 7 days. HUVECS (American Type Culture Collection) were used in this assay as a positive control for tube formation.

3.2.9 ASM cell supernatants

ASM cells isolated from asthmatic, non-asthmatic and LAM individuals were seeded onto six well plates at a density of $1x\ 10^4$ cells / cm^2 in 5% FBS /DMEM /1% Ab for 24hrs. Cells were then quiesced, media was aspirated and 0.1% BSA DMEM/1% Ab was added for 24hrs to quiesce cells. Media was aspirated and TGFβ (1ng/ml), in 625µl volume, was added to stimulate the cells for 8hrs. Supernatants were collected at time 0, 4 and 8hrs.

3.2.10 $VEGF_{165}$ and Ang-1 ELISA

Supernatants generated in 3.2.10 were measured for the level of $VEGF_{165}$ and Ang-1 using an ELISA assay.

Ninety six well plates (Falcon BD Labware) were coated with 50µl/well of capture antibody of either mouse anti-human VEGF at 4µg/ml (R&D systems) diluted in 0.1M Na_2HPO_4 buffer or mouse anti-human Ang-1 at 4µg/ml (R&D systems) diluted in PBS. Paraffin tape was used to seal plates and they were incubated overnight at 4°C. Plates were washed four times in PBS/0.05% Tween 20. One hundred µl of 1% BSA in PBS was used to block the plates which were incubated at room temperature for 1 hour on an orbital shaker before being washed three times using PBS/0.05% Tween 20. Samples and standards were added to appropriate wells (50µl/well) and the plate sealed with paraffin tape and incubated at 4°C overnight. Plates were washed four times in PBS/0.05% Tween 20. Detection antibody was added to wells, anti-mouse $VEGF_{165}$ antibody at 100ng/ml, anti-mouse Ang-1 antibody at 200ng/ml, diluted in 1% BSA/PBS/0.05%Tween 20, and incubated at room temperature on an orbital shaker for 1hr. PBS/0.05% Tween 20 was used to wash the plates six times. One hundred µl of streptavidin (conjugated with HRP) (R&D systems, MN, USA) diluted 1:200 with 1% (w/v) BSA/PBS-Tween was added to each well and incubated at room temperature on an orbital shaker for 30 minutes. Plates were washed eight times in PBS/0.05% Tween 20 and developed using 50 µL/well of 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate (ABTS)

(Sigma) on an orbital shaker in the dark until colour developed. The reaction was stopped using 1 mM phosphoric acid (Sigma) and quantified by reading the plate at 450 nm using the Spectramax MZ, plate reader.

Statistical analysis was performed using a one-way ANOVA to compare asthmatics versus non-asthmatic and LAM versus non-asthmatics. Comparison was also made between BSA and TGFβ-stimulated cells for each patient group using a paired students t-test.

3.3 Results

3.3.1 Detection of tumstatin in serum and BAL-f

The presence of tumstatin was assesed in serum samples from asthmatics pre and post steroid treatment and healthy control individuals to determine whether the absence of tumstatin detected in the airway sections in our asthmatic population was lung tissue specific. Serum and BAL-f samples were analysed using the dot blot method (figure 3.2). Densitometric evaluation showed no significant difference in the levels of tumstatin between asthmatics, pre (n=8) and post steroids (n=8) and healthy controls (n=6) in both serum and BAL-f samples as shown in figure 3.3. Fractionated samples of serum and BAL-f produced a top and bottom sample, whereby the top sample contained molecules greater than and the bottom sample molecules smaller than 100kDa. These samples were analysed by dot blot for the detection of tumstatin. Tumstatin was only detected in the top samples of both serum (figure 3.4) and BAL-f (figure 3.5) from asthmatic (n=10), pre and post steroids, and healthy controls (n=8).

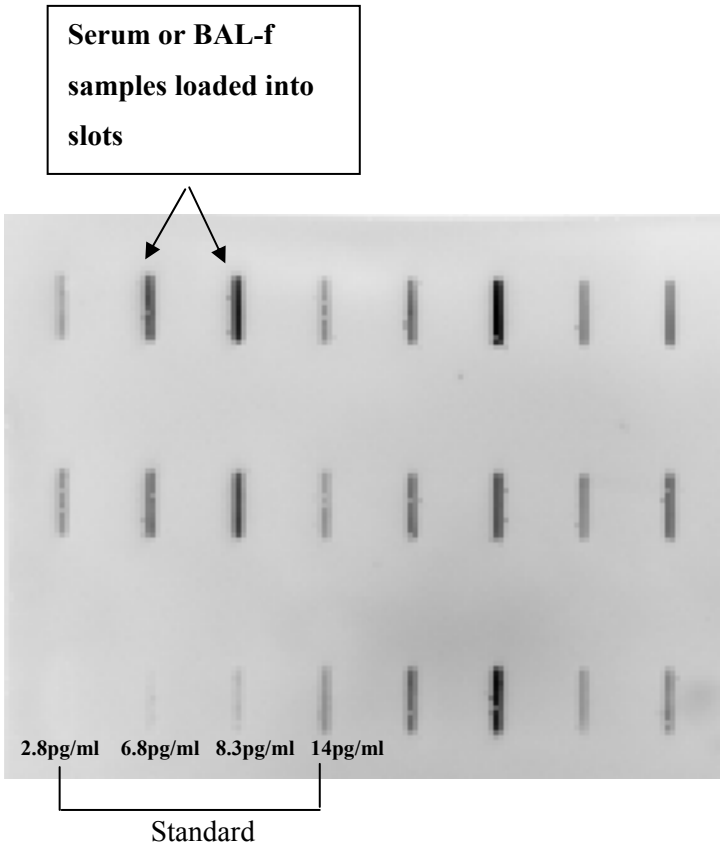


Figure 3.2: Nitrocellulose membrane showing sample detection using the dot blot method. Serum or BAL-f samples were loaded onto the membrane and visualised using chemiluminescent substrate. Dot blot membrane is representative of all membranes run. Intensity of each band was normalised to tumstatin or nonasthmatic protein standard.

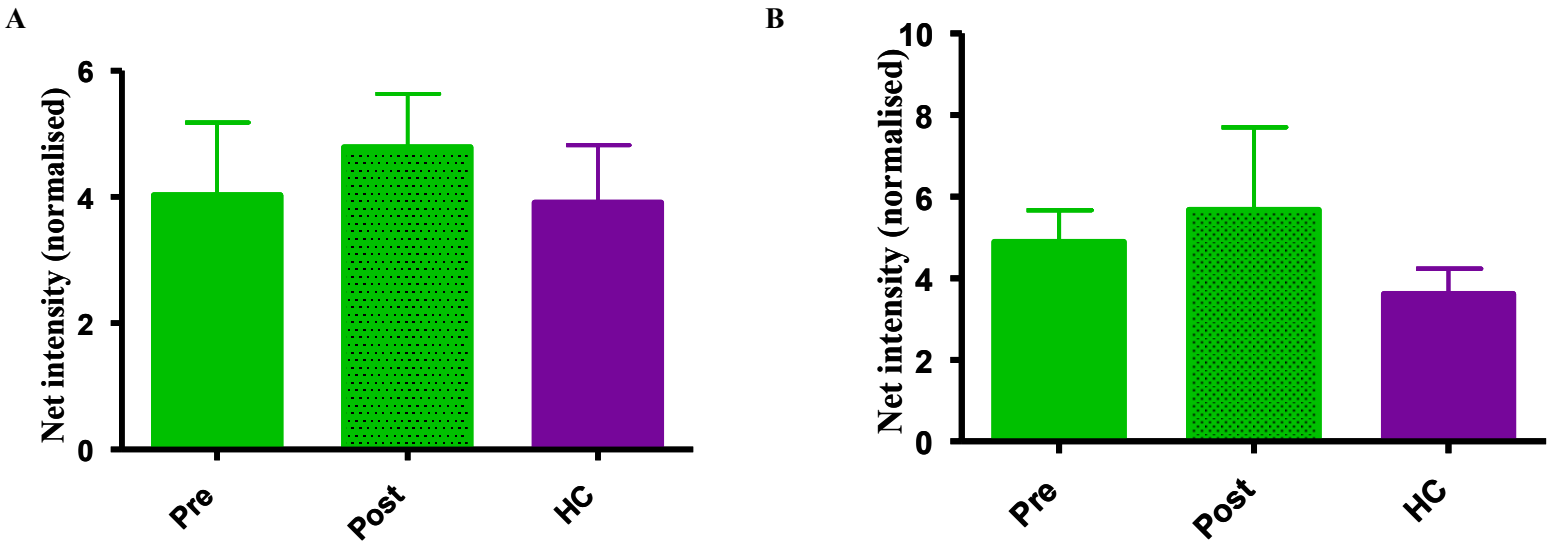


Figure 3.3: Levels of tumstatin in serum (A) and BAL-f (B) from asthmatics pre (n=8) and post (n=8) steroid treatment and from healthy control (HC, n=6) individuals were evaluated using a dot blot. The intensity of each band was normalised to an internal tumstatin control. No significant difference was seen in level of expression between any of the groups (One way ANOVA). Data are expressed as means ± standard error of the mean.

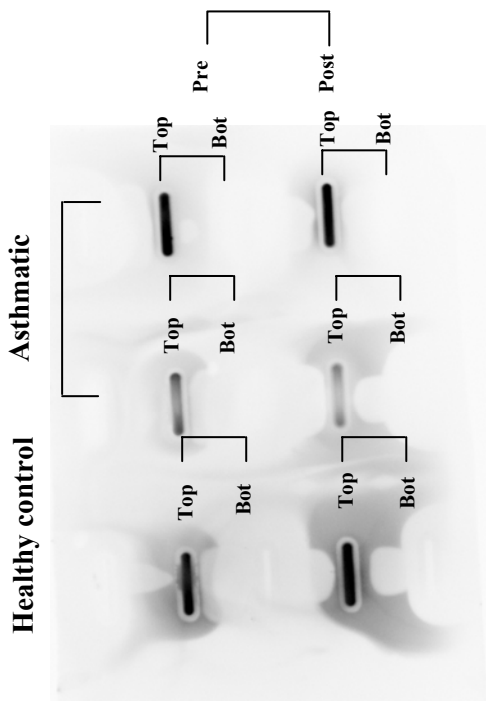


Figure 3.4: Representative nitrocellulose membrane showing the detection of tumstatin using the dot blot method. Fractionated serum top (Top) and bottom (Bot) samples from asthmatic (n=10) pre- (pre) and post (post)-steroids and healthy control (n=8) individuals. Membrane is representative of all membranes developed.

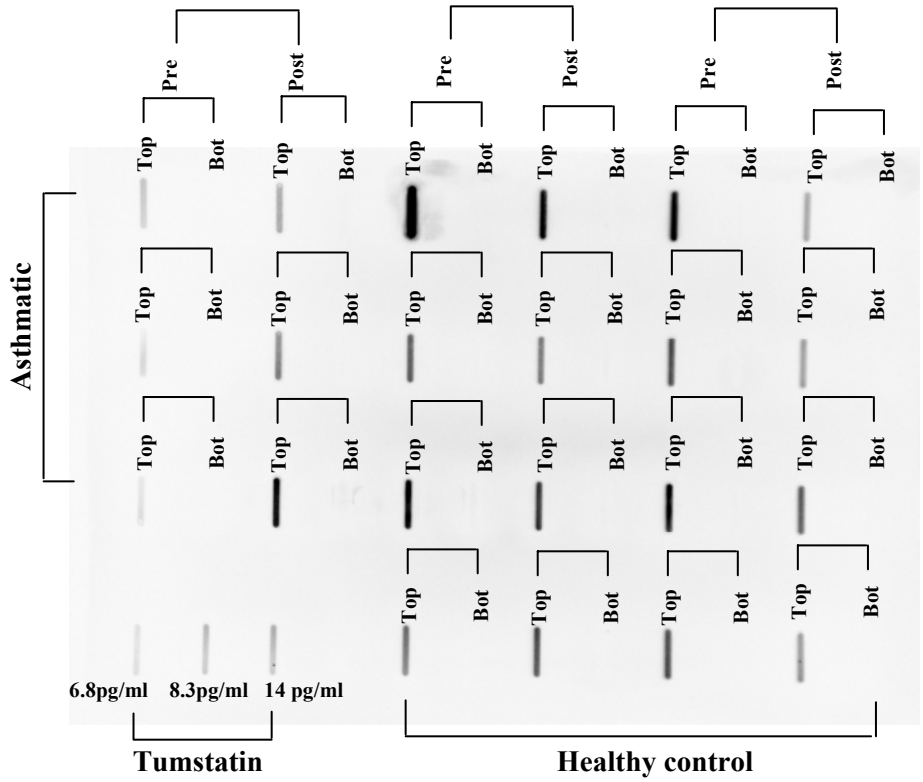


Figure 3.5: Representative nitrocellulose membrane showing the detection of tumstatin using the dot blot method. Fractionated BAL-f top (Top) and bottom (Bot) samples from asthmatic pre- (pre) and post (post)-steroids and healthy control individuals. Image is representative of results obtained from asthmatic (n=10) pre- and post-steroids and healthy control (n=10).

3.3.2 Detection of tumstatin 28kDa molecule

Tumstatin is only active as an angiogenic inhibitor when it is cleaved from the collagen IV α3 chain. Results from the fractionated samples of serum and BAL-f indicated that the tumstatin detected in serum and BAL-f was of a size greater than 100kDa indicating that tumstatin was not in its free 28kDa active form in these solutions. To confirm these results the serum samples were analysed using western blots. In both the asthmatic (n=10) and healthy control (n=8) serum we detected the whole collagen IVα3 chain at 188kDa rather than the active tumstatin at 28kDa (figure 3.6).

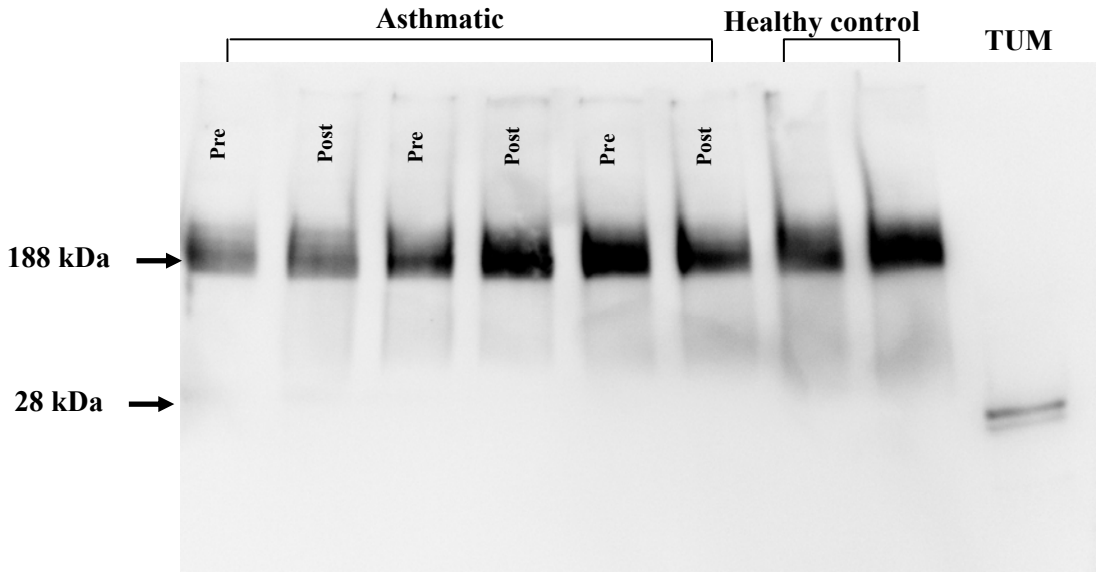


Figure 3.6: Western blot PVDF membrane showing detection of collagen IV α3 chain in serum samples from asthmatic and healthy control individuals. The band detected at 188kDa shows tumstatin detection as part of the whole collagen IV α3 chain. The positive control, recombinant tumstatin peptide (TUM) confirmed that the antibody was able to detect cleaved tumstatin at 28kDa. Image is representative of results obtained from asthmatic (n=10) pre- and post-steroids and healthy controls (n=8).

3.3.3 T3 and T7 peptides inhibit proliferation of primary pulmonary endothelial cells

Tumstatin inhibits the proliferation of endothelial cells (Maeshima *et al.*, 2000). The T3 and T7 peptides, regions within the tumstatin peptide identified to have anti-angiogenic properties, inhibited the proliferation of human prostate carcinoma cells and HUVEC's (Maeshima *et al.*, 2001a). However, the effect of both peptides on primary pulmonary endothelial cells was unknown.

The effect of the tumstatin derived peptides T3 and T7 on the proliferation of ASM cells was studied in cells from 4 asthmatic and 4 non-asthmatic individuals as well as 5 primary pulmonary endothelial cell lines. Tumstatin did not inhibit the proliferation of ASM cells from asthmatic (figure 3.7) or non-asthmatic (figure 3.8) individuals over a time course of 9 days, but was able to significantly inhibit primary pulmonary endothelial cell (n=5) proliferation over the same time period (figure 3.9).

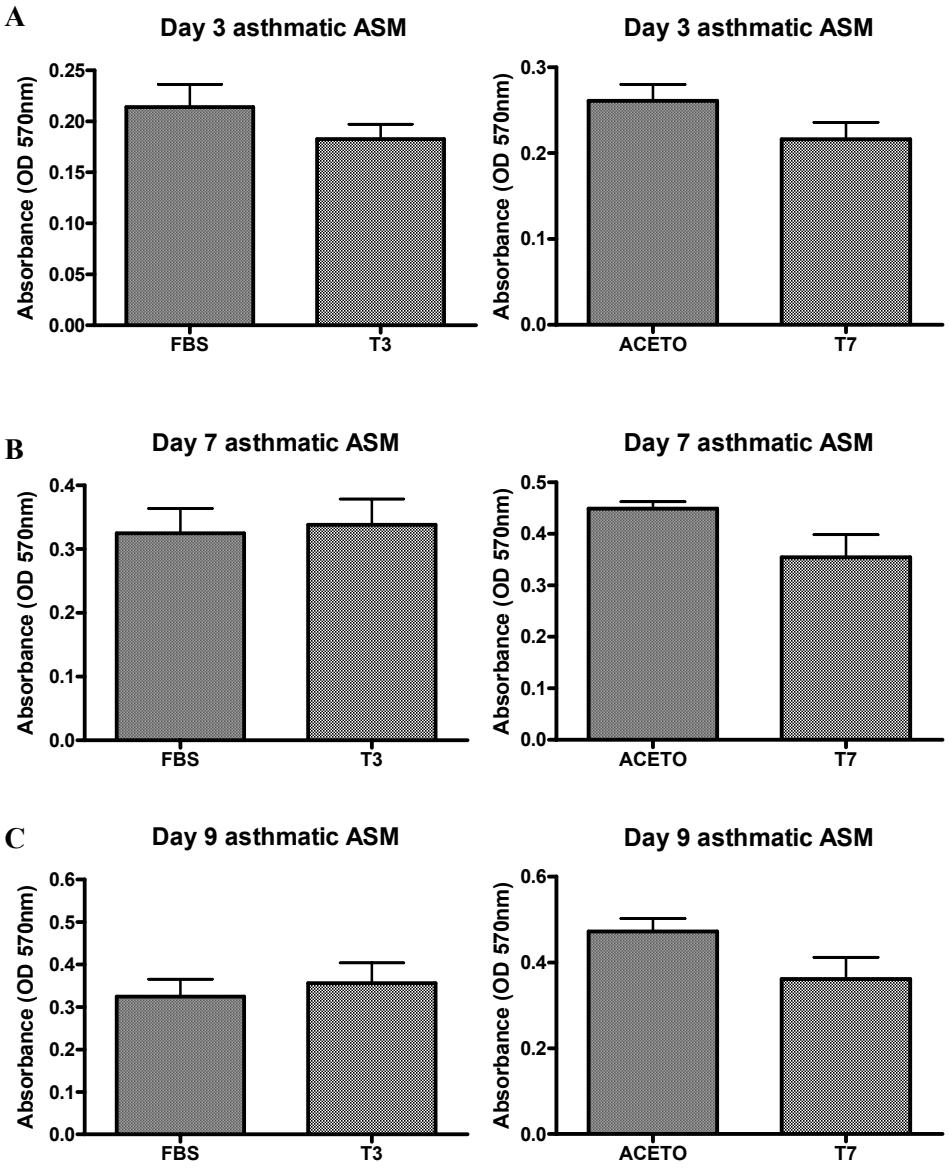


Figure 3.7: The proliferation of airway smooth muscle cells was not inhibited by the addition of T3 [4.5µM] or T7 [4.5µM] tumstatin derived peptides at A 3 days, B 7 days or C 9 days in asthmatic (n=4) individuals. Acetonitrile (ACETO) was added as vehicle control used to dissolve the T7 peptide. Data is expressed as mean ± standard error of the mean. Statistical analysis was performed using a paired students t-test. No significant difference were observed between peptides and controls.

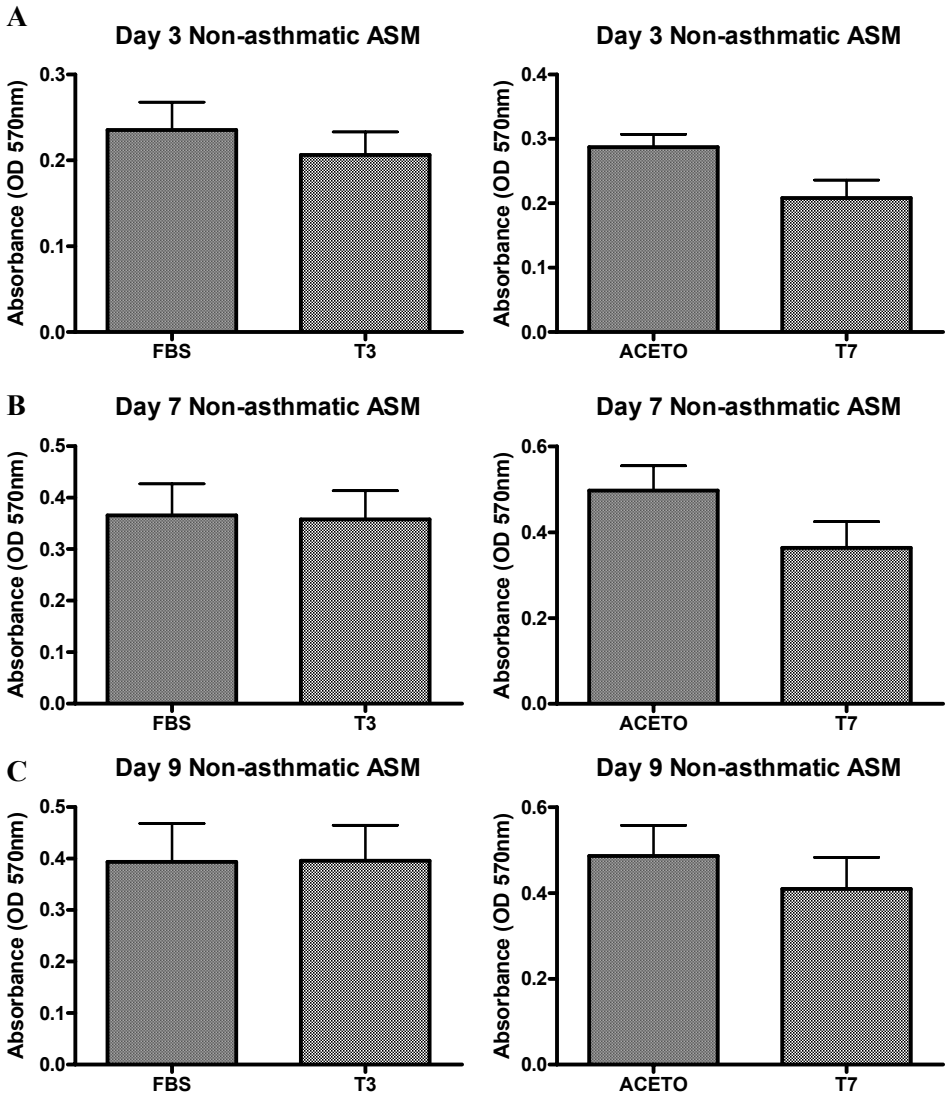


Figure 3.8: The proliferation of airway smooth muscle cells was not inhibited by the addition of T3 [4.5µM] or T7 [4.5µM] tumstatin derived peptides at A 3 day, B 7 days or C 9 days in non-asthmatic (n=4) individuals. Acetonitrile (ACETO) was added as vehicle control used to dissolve the T7 peptide. Data is expressed as mean ± standard error of the mean. Statistical analysis was performed using a paired students t-test. No significant differences were observed between peptides and controls.

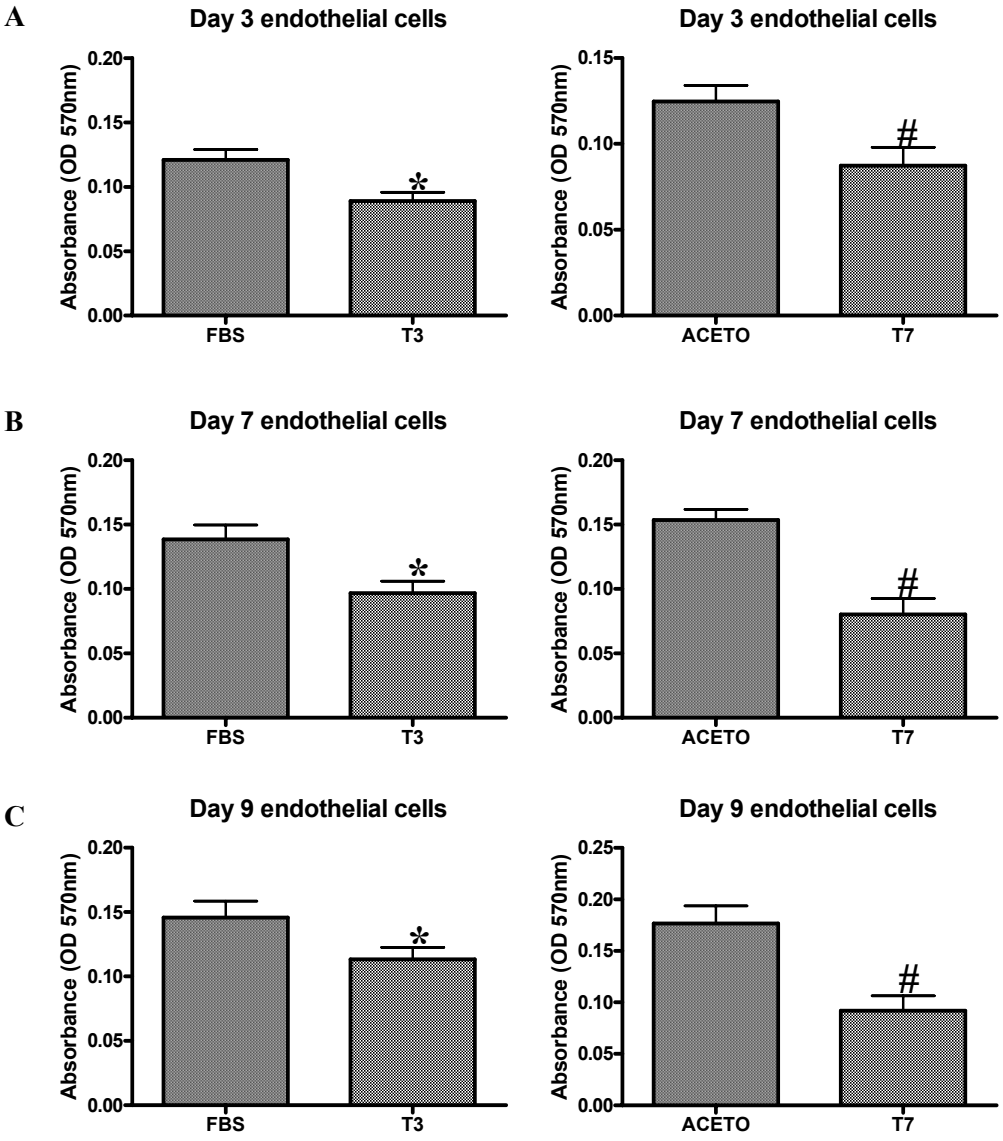


Figure 3.9: The proliferation of non-asthmatic primary pulmonary endothelial cells (n=5) was inhibited by the addition of T3 [4.5µM] or T7 [4.5µM] tumstatin derived peptides at day A 3 days, B 7 days and C 9 days. Acetonitrile was added as vehicle control used to dissolve the T7 peptide (ACETO). Data is expressed as mean ± standard error of the mean. Statistical analysis was performed using a paired students t-test * $p<0.05$ compared to FBS, # $p<0.05$ compared to ACETO.

3.3.4 Tumstatin inhibits 2D pulmonary endothelial cell tube formation

For the first time, the ability of tumstatin to inhibit primary pulmonary endothelial cell tube formation in a concentration related manner was demonstrated (figure 3.10). The number of tubes formed per well was quantified by manual counting. Tumstatin at concentrations of 28 and 84pg/ml resulted in a small, but statistically significant, reduction in the formation of tubes ($p<0.05$, n=5, repeated measures one way ANOVA with Dunnett's post test). However at a concentration of 280pg/ml approximately 40% of the tube formation was inhibited. This inhibition was further increased to 60% and 75% when cells were treated with 840 and 2800 pg/ml respectively (figure 3.11).

3.3.5 Tumstatin inhibits 3D pulmonary endothelial cell tube formation

The tube formation assay is an *in vitro* method for looking at angiogenesis. This assay is however limited as it is unable to demonstrate maturation of blood vessels. Therefore, a 3D matrigel experimental method was developed which was able to sustain tube formation long enough to observe sprouting (figure 3.12). Tumstatin was added to this assay at 2800pg/ml and complete inhibition of pulmonary endothelial cell tube formation and sprouting was observed (n=4) as shown in figure 3.13.

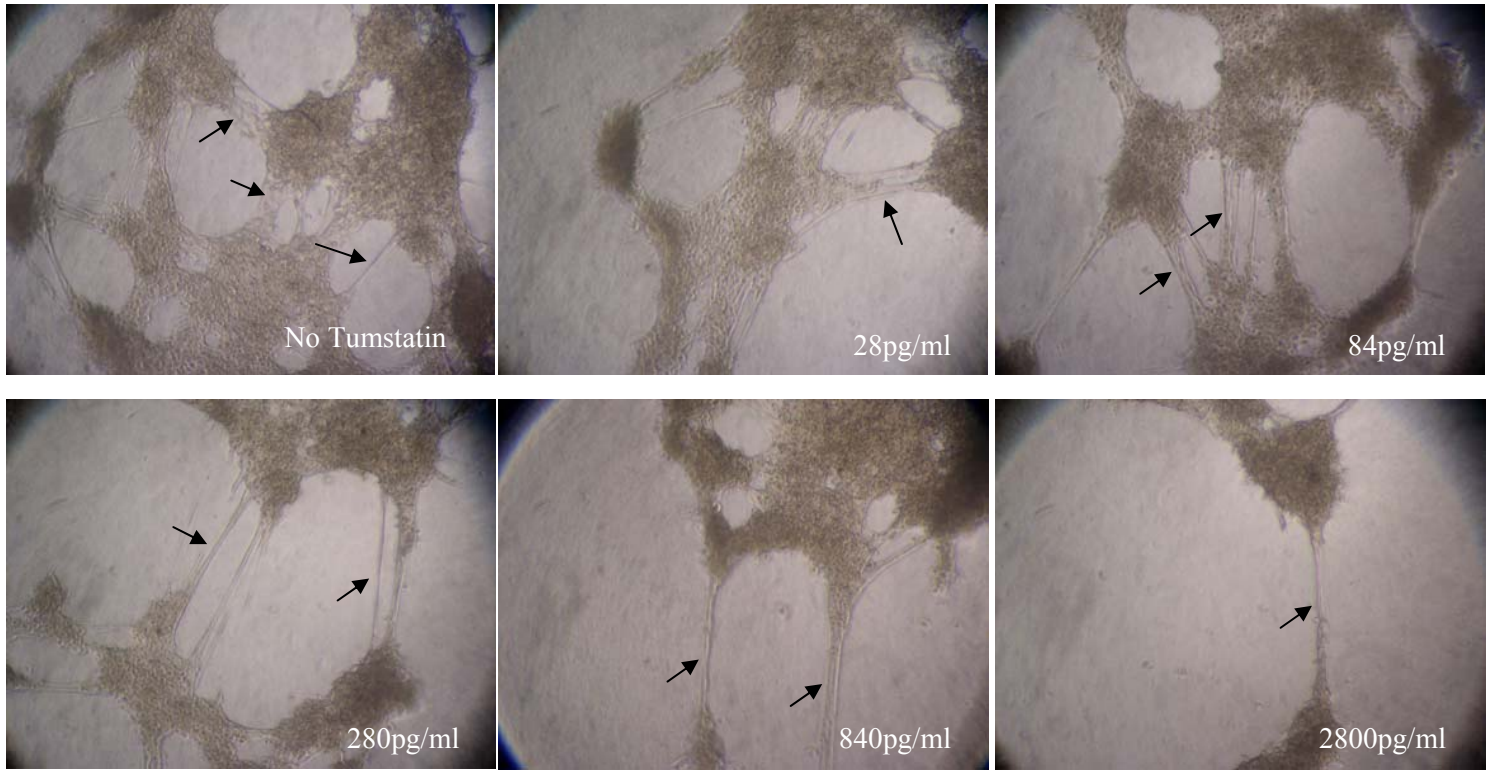


Figure 3.10: Tumstatin at the concentrations shown inhibits pulmonary endothelial cell tube formation in a concentration related manner. Images (X100) showing 2D pulmonary endothelial cell tube formation (arrows). Images are representative of all cell lines tested (n=5) non-asthmatic primary pulmonary endothelial cells.

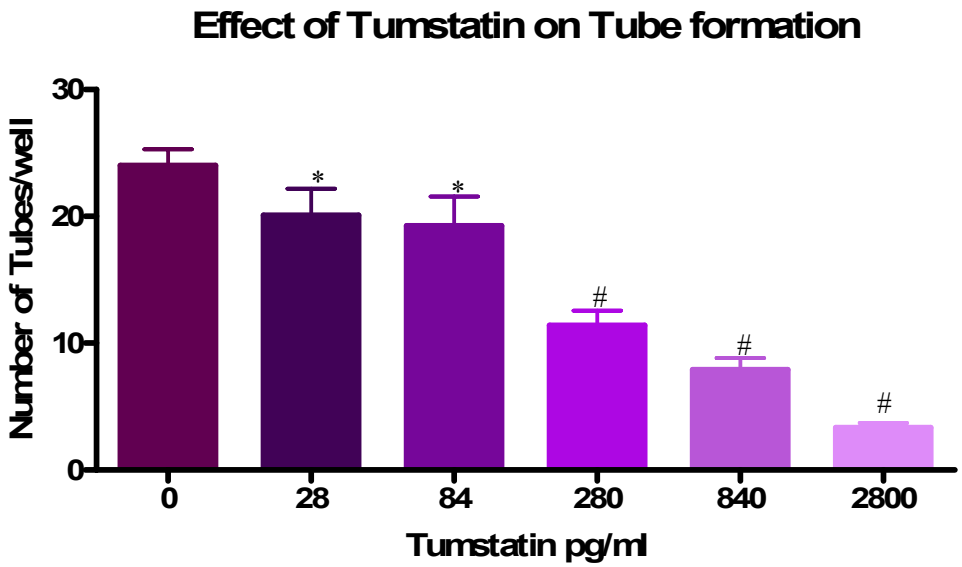


Figure 3.11: The effect of tumstatin on tube formation of primary pulmonary endothelial cells. Number of tubes counted per well from non-asthmatic (n=5) primary pulmonary endothelial cell lines.* $p<0.05$ versus 0 Tumstatin, # $p<0.01$ versus 0 Tumstatin (repeated measures one way ANOVA with Dunnett's post test). Data is expressed as mean ± standard error of the mean.

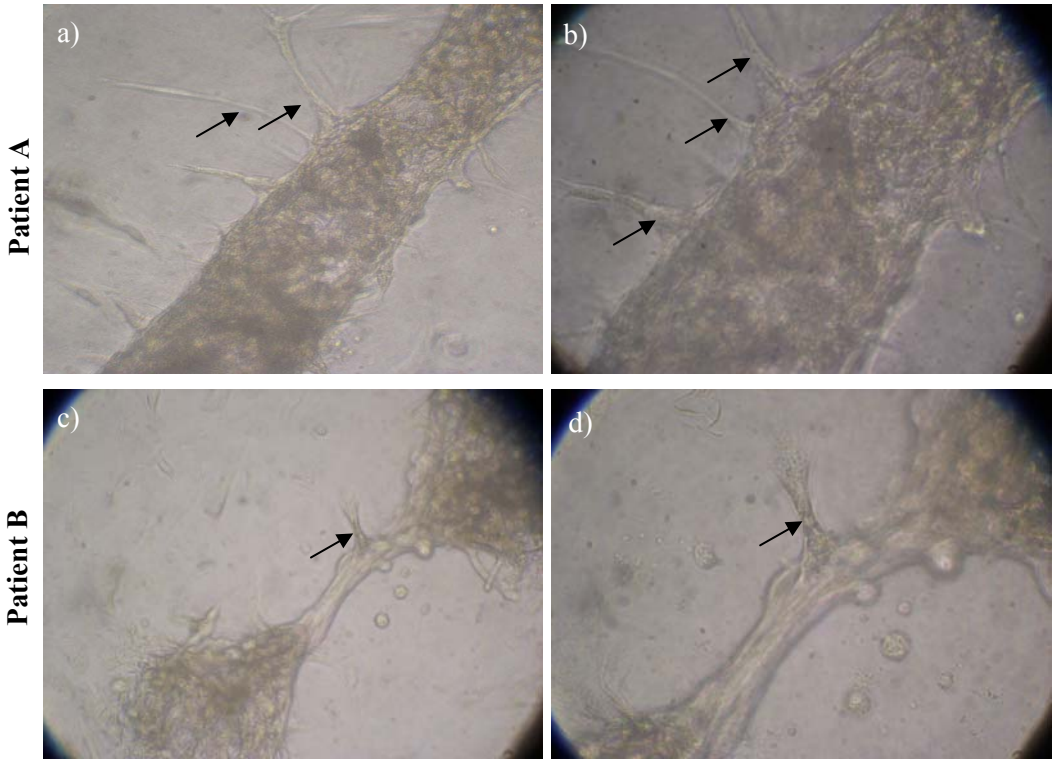


Figure 3.12: 3D endothelial cell tube formation showing sprouting (arrows). Images a) and c) are at X100 magnification, b) and d) are at X400 magnification. Image a) and b) are from patient A, images from c) and d) are from patient B. Images are representative of all primary pulmonary endothelial cell lines tested (n=4).

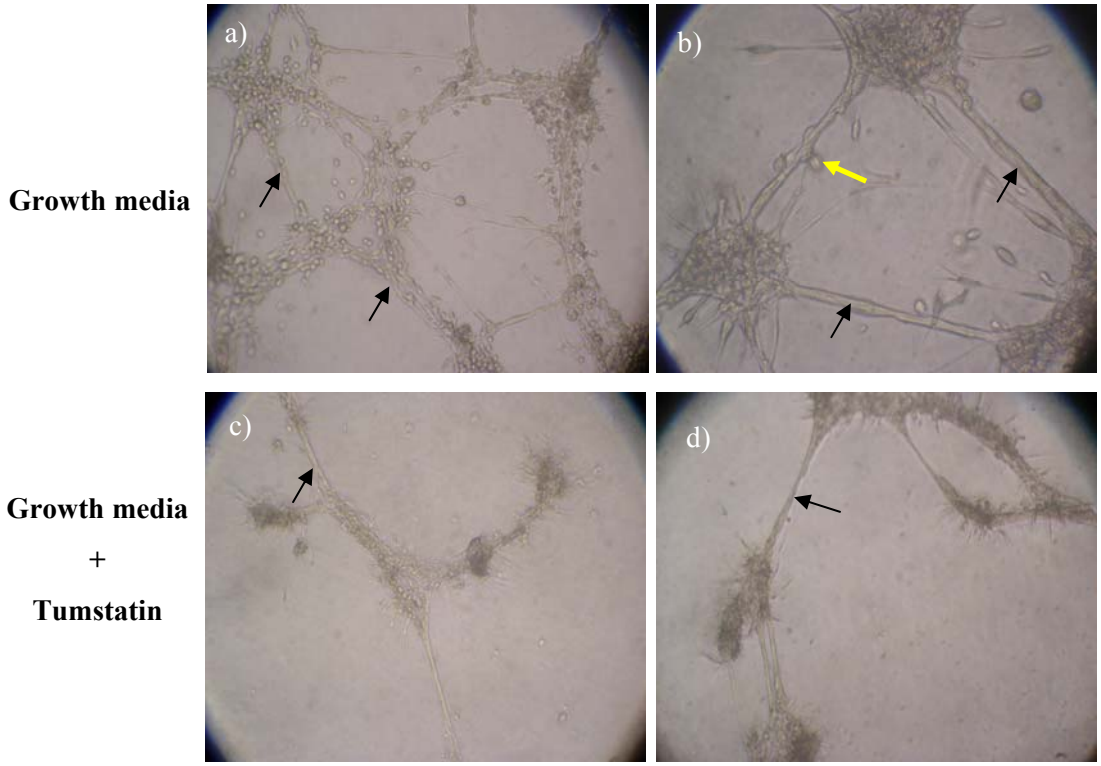


Figure 3.13: 3D tube formation assay showing primary pulmonary endothelial cell tube formation a) x100 and b) x200 and inhibition of tube formation by tumstatin at 2800pg/ml c) and d) (x100). Black arrows show tube formation, yellow arrow shows sprouting. Images a) and b) showing tube formation in growth medium alone. Images c) and d) showing tube formation in the presence of growth medium and tumstatin. Images are representative of all primary pulmonary endothelial cell lines tested (n=4).

3.3.6 Levels of VEGF and Ang-1 in TGFβ stimulated ASM cells

VEGF is a known potent angiogenic stimulator that requires Ang-1 to stabilise blood vessel formation. ASM cells from asthmatic, non-asthmatic and LAM individuals were stimulated with TGFβ (1ng/ml) and the level of VEGF and Ang-1 released was measured. VEGF release was significantly increased in TGFβ stimulated ASM cells from asthmatics (n=5 $p<0.02$ one-way ANOVA) and LAM (n=5 $p<0.05$ one-way ANOVA) individuals compared to non-asthmatics (n=5) (figure 3.14). Significant difference was also seen within each group when comparing TGFβ stimulated cells to their BSA control (asthmatic $p<0.01$, LAM $p<0.05$ and non-asthmatic $p<0.05$ (paired students t-test)). However, TGFβ did not stimulate the release of Ang-1 in asthmatic (n=3), LAM (n=3) or non-asthmatic (n=3) ASM cells (Fgure 3.15).

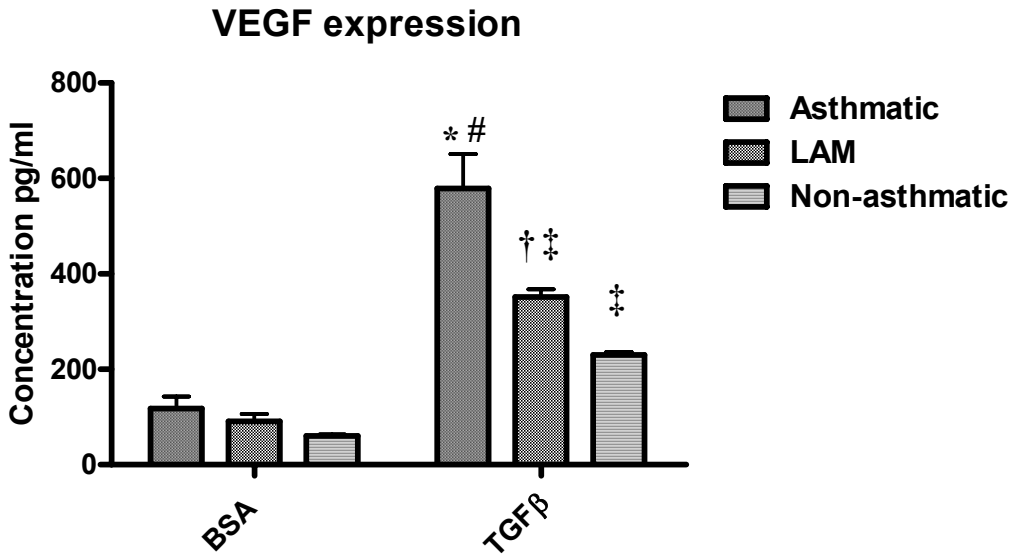


Figure 3.14: Levels of VEGF released into the supernatants by ASM cells in BSA and ASM cells stimulated with TGFβ from asthmatic (n=5), LAM (n=5) and non-asthmatic (n=5) individuals. (* $p<0.02$ compared to non-asthmatic, one-way ANOVA, † $p<0.05$ compared to non-asthmatic, one-way ANOVA, # $p<0.01$ compared to BSA paired students t-test, ‡ $p<0.05$ compared to BSA paired students t-test).

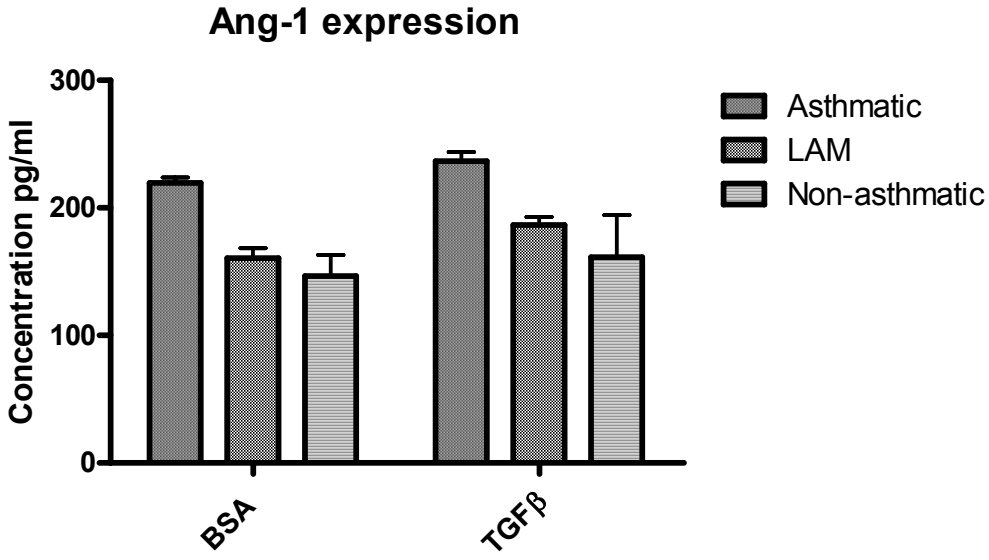


Figure 3.15: Levels of Ang-1 released into the supernatants by ASM cells in BSA and ASM cells stimulated with TGFβ from asthmatic (n=3), LAM (n=3) and non-asthmatic (n=3) individuals. Data are expressed as mean ± standard error of the mean.

3.4 Discussion

Tumstatin was detected in serum and BAL-f from asthmatic and healthy control individuals, with no significant difference in the levels of expression between the two groups. Detection of tumstatin was shown to be as part of the whole collagen IV α3 chain and not in its cleaved and therefore active form. Recombinant T3 and T7 peptides inhibited primary pulmonary endothelial cell proliferation but had no effect on ASM cells. Tumstatin inhibited primary pulmonary endothelial cell tube formation. Levels of VEGF released were shown to be increased in asthmatic and LAM ASM cells stimulated with TGFβ compared to non-asthmatics, whereas Ang-1 levels were the same in all groups.

Tumstatin was shown to be absent in the airways of asthmatic and LAM individuals (Chapter 2) but present in non-asthmatics. Therefore, it was necessary to determine if this absence was a systemic feature of asthma and LAM or if it was limited to the airway. We detected tumstatin in both serum and BAL-f from asthmatic individuals. However, the levels of tumstatin detected in serum from asthmatic and healthy control individuals' serum and BAL-f were not significantly different. Unfortunately it was not possible to investigate this in LAM individuals due to the lack of sample availability.

Detection of tumstatin in the serum suggests that tumstatin is being produced by asthmatic patients. This also indicates that the absence may be lung specific, but detection of tumstatin in the BAL-f samples indicates that tumstatin is available within the vicinity of the airways. While tumstatin is present in the airway, the question arises as to whether it is in an active state.

Detection of tumstatin in fractionated samples of serum and BAL-f, which rendered a top sample consisting of molecules of size greater than 100kDa and a bottom sample of molecules less than 100kDa, showed tumstatin to be detectable only in the top samples from both serum and BAL-f indicating it was part of the whole collagen IV molecule and not cleaved. Similarly, using western blotting, tumstatin was detected in the serum of asthmatics and healthy controls and was part of the whole collagen IV α3 chain and not cleaved tumstatin. Tumstatin is only active as an angiogenic inhibitor when it is cleaved from the remaining components of the collagen IV α3 chain. Hamano *et al*. showed that MMP-9 was most effective in releasing the NC1 domain, tumstatin, from the collagen IV α3 chain in the glomerular basement membrane. They showed that mice deficient in MMP-9 had significantly decreased levels of tumstatin in their serum (Hamano *et al.*, 2003). Levels of MMP-9 are increased in bronchial biopsy specimens, blood, induced sputum and BAL-f from patients with severe or uncontrolled asthma (Hoshino *et al.*, 1998a; Lemjabbar *et al.*, 1999). Other proteases, including MMP-2 have been reported to cleave tumstatin but with less efficiency (Hamano *et al.*, 2003).

Increased MMP-2 levels are associated with the increased cellular proliferation in LAM individuals (Matsui *et al.*, 2000). The levels of MMP-9 have not been examined in these individuals. The increase in MMP-2 levels in the airways of LAM individuals could potentially cleave tumstatin from the BM and possibly lead to its clearance from the airway. ASM cells from asthmatics on the other hand produce less pro and active MMP-2 compared to non-asthmatics, therefore eliminating MMP-2 as a candidate for the cleaving of tumstatin in the asthmatic airways. Whilst this study did not directly examine the relationship between MMPs and tumstatin in the airway it would be of interest to determine the association of these important factors in the future.

Our findings suggest that the collagen IV α3 chain is present in the airways of asthmatics and LAM individuals as reflected in the serum and BAL-f but is not recognised by the antibody in the airway sections. It can be postulated that the increase in the levels of MMP-9 in asthmatic airways (Cataldo *et al.*, 2002; Ko *et al.*, 2005) release tumstatin from the BM and potentially allow its clearance from the airway. The presence of another protease, yet to be identified, may be responsible for the cleavage and clearance of tumstatin in the asthmatic and LAM airways. It is also possible that tumstatin undergoes a structural change such that the antigenic site is no longer recognisable by the antibody.

Levels of VEGF in TGFβ-stimulated ASM cells were increased in asthmatics and LAM individuals compared to non-asthmatic individuals. However, levels of Ang-1 expression were the same in asthmatic, LAM and non-asthmatic individuals indicating that Ang-1 expression is not modulated by TGFβ. These results further confirm the inbalance between the pro- and anti-angiogenic factors in the asthmatic and LAM airways. The presence of Ang-1, despite it not being modulated, in this system confirms the potential for blood vessel stabilisation induced by the increased VEGF released by the ASM cells.

Proliferation of ASM cells was not inhibited by the addition of the tumstatin derived T3 and T7 peptides, whereas primary pulmonary endothelial cell proliferation was inhibited by both peptides. For the first time, the ability of tumstatin to inhibit primary pulmonary endothelial cell tube formation was demonstrated and that inhibition was shown to be concentration dependent. Pulmonary vessels were used to isolate the endothelial cells used in these experiments. Ideally it would have been desirable to be able to isolate bronchial vessels, however this was not possible due to the limitations of the currently available methods.

The ability of these peptides to only inhibit endothelial cell proliferation is consistent with previous findings by Maeshima *et al.* who showed the inhibition of proliferation of bovine pulmonary arterial endothelial cells, HUVECs and a human prostate adenocarcinoma cell line by T3 and T7

peptides (Maeshima *et al.*, 2001b). The inability of these peptides to inhibit the proliferation of ASM cells indicates the specificity of tumstatin's inhibitory action. ASM cells do express the αvβ3 integrin (chapter 2) which is required by tumstatin for the inhibition of angiogenesis. This would suggest that tumstatin should be able to interact with these cells. However, the effects of tumstatin on other funcions (such as cytokine release and extracellular matrix protein production) of ASM cells were not examined in this study. It would be of interest to characterise such effects in the future. The lack of effect of tumstatin in ASM cells despite the presence of the relevant integrin would indicate that the action of tumstatin in inhibiting proliferation is cell type specific.

Addition of tumstatin, in both the 2D and 3D tube formation assays, inhibited pulmonary endothelial cell tube formation in a concentration-related manner. This is the first report on the effect of tumstatin on primary pulmonary endothelial cells, and the effectiveness of tumstatin as an angiogenic inhibitor in the airways had not previously been examined. The results confirm the potential for tumstatin to act as an angiogenic inhibitor in the airways as it is now clear that tumstatin is able to exert its anti-angiogenic properties on pulmonary endothelial cells. The highest concentration of tumstatin administered in these experiments was approximately 300-fold lower than physiological levels reported in mouse models (Hamano *et al.*, 2003), yet it was able to inhibit almost all tube formation. It is expected that lower concentrations of tumstatin would be effective in a cell culture system as it is

less complex than in an animal model. However, the ability to inhibit tube formation at such a low concentration suggests that tumstatin may have a strong potency in the airways. Tumstatin causes tumour regression by inhibiting aberrant blood vessel formation resulting from the presence of the tumour. So, having demonstrated tumstatin's ability to inhibit blood vessel formation in the airways, the administration of tumstatin to asthmatic and LAM airways can potentially inhibit the angiogenesis seen in these airways and as a consequence decrease the amount of smooth muscle present. Decreasing the amount of smooth muscle present in the airways of asthmatics and LAM patients would potentially decrease airway hyperresponsiveness (AHR).

Improvement of AHR by reducing the ASM in the airways has been demonstrated with the use of thermoplasty (Cox *et al.*, 2007). This method uses controlled thermal energy which is delivered to the airway wall during a series of bronchoscopies (Pavord *et al.*, 2007). However, this is not only an invasive technique but is only applied to airways between three and 10mm in diameter. Administration of an alternate therapy using a less invasive method would be a more desirable way to decrease smooth muscle bulk.

Tumstatin has demonstratable effects in cell culture systems, however it is important to examine these effects in an *in vivo* system as it is a multicellular environment which could potentially influence the action of tumstatin. Chapter 4 in this thesis describes the effects of tumstatin in a virus-induced

mouse model of angiogenesis as well as a mouse model of ovalbumin-induced airway hyperresponsiveness.

Chapter 4

Effect of tumstatin in the airway '*in vivo*'

4.1 Introduction

Tumstatin is a potent angiogenic inhibitor. Its ability to inhibit tumour growth and endothelial cell proliferation as well as induce apoptosis of proliferating endothelial cells has been well documented (Maeshima *et al.*, 2000; Maeshima *et al.*, 2001a; Maeshima *et al.*, 2001b).

Knock-out mouse models have been used to further characterise the role of tumstatin. Hamano *et al*. using a collagen IV α3 chain knock-out mouse model (Andrews *et al.*, 2002) showed that tumours from lewis lung carcinoma cells placed on the backs of tumstatin deficient mice grew at a faster rate than those placed on wild-type mice and were eventually more than twice the size of wild-type tumors. Upon administration of tumstatin, at physiological concentrations (336±28ng/ml), the tumour growth rate decreased to that in wild-type mice (Hamano *et al.*, 2003). Characterisation of the role of tumstatin in an animal model of allergic airway disease has not previously been reported.

Animal models of asthma are now widely used to investigate both the immunological and physiological events occurring in this disease. The current chronic mouse model of asthma, which uses repetitive allergen exposure, is able to mimic important features seen in this human disease such as Th-2 dependent allergic inflammation, airway remodelling and airway

hyperreactivity (Braun *et al.*, 2006). However, this model is still missing some aspects of this human disease like acute 'asthma attacks'.

Respiratory syncitial virus (RSV) is one of the respiratory viruses implicated in asthma exacerbations (Dodge *et al.*, 1996). Infection with RSV occurs in annual epidemics infecting almost all children within the first two years of life (Weinberger, 2003) and is the most common cause of wheezing episodes in children. It has been reported that RSV infection causes vascular endothelial growth factor (VEGF)- a known angiogenic promoter- release in the airways (Lee *et al.*, 2000). Human bronchial epithelial cells were infected with RSV and levels of VEGF were increased only two hours post infection. Increased VEGF levels were sustained for up to 48 hours. Mouse models of RSV infection have been used to examine the pathogenesis of allergic responses to inactivated RSV virions and individual RSV components. This model is limited, as RSV is not a mouse pathogen and therefore produces only minimal symptoms and rapidly aborted primary infections (Byrd *et al.*, 1997). Bonville *et al.* established a mouse model of infection using a natural mouse pathogen pneumonia virus of mice (PVM) which resulted in an infection that replicated many of the symptoms of severe RSV infections in humans (Bonville *et al.*, 2003). RSV and PVM are both from the same family- *Paramyxoviridae*, subfamily *Pneumovirinae*. Chambers *et al.* isolated and characterised the nine distinct genes of PVM. They showed that the nucleoprotein and phosphoprotein in both PVM and RSV have conserved epitopes, suggesting that the immune response to these viruses would be

similar in their respective natural hosts (Chambers *et al.*, 1990). PVM mouse models have the potential to be used as a representative model of human respiratory infection.

A widely used mouse model of allergic airway disease is the chronic ovalbumin challenged BALB/c mice model. This model was shown to exhibit features of airway remodelling such as increased smooth muscle in the airways and angiogenesis in both the bronchus and surrounding parenchyma, as well as airway hyperresponsiveness (AHR) (Lee *et al.*, 2006). Mice chronically exposed to ovalbumin (OVA) developed increased angiogenesis (Lee *et al.*, 2006). Recently, Asosingh *et al.* also reported increased angiogenesis in airway sections from ovalbumin-challenged mice (Asosingh *et al.*, 2007). Furthermore, Suzaki *et al.* assessed AHR as an increase in PenH (enhanced respiratory pause) in response to increased doses of methacholine (Suzaki *et al.*, 2005). PenH is representative of the phase shift in thoracic and nasal flow curves; increased phase shift correlates with increased respiratory resistance. The authors showed that ovalbumanin-sensitised mice had an increased PenH value compared to control mice (Suzaki *et al.*, 2005). However, assessment of AHR using the the whole-body plethysmograph allows for measaurements of parameters such as resistance and compliance (Braun *et al.*, 2006), which are thought to provide better correlates to human asthma. In addition, this method is suitable for longitudinal studies and allows large throughput of animals for screening purposes which made it more suitable for this study. Therefore, the

assessment of AHR in this chapter was performed using the whole-body plethysmograph.

Endostatin is an endogenous angiogenic inhibitor that has been studied in a mouse model of asthma. Suzaki *et al.* showed, using ovalbumin-sensitised mice, that administration of endostatin/Fc inhibited AHR and pulmonary allergic inflammation. They also examined the expression of CD31 (an endothelial cell marker) and found it was reduced in the endostatin treated mice (Suzaki *et al.*, 2005).

Angiogenesis is a critical point in the development of most human tumours and angiogenic inhibitors have been trialled in mouse models to assess their ability to cause tumour regression. TNP-470, a synthetic analogue of fumagillin known to block a broad spectrum of angiogenic regulators, was shown to inhibit the growth of both B16 melanoma and AKR lymphoma tumours in mice (Kaptzan *et al.*, 2006). The ability of TNP-40 to inhibit angiogenesis in the airways was not examined.

The specific aims of this study were:

1) to identify the time required for angiogenesis to develop in the airways using a viral mouse model

2) to identify the efficacy of tumstatin in inhibiting AHR and angiogenesis in the airway *in vivo* using a mouse model of allergic airway disease.

4.2 Materials and Methods

Mouse models were generated in collaboration with: Prof Paul S. Foster, A/Prof Philip M. Hansbro and Dr Nicole G. Hansbro, Priority Research Centre for Asthma and Respiratory Disease, School of Biomedical Sciences, Faculty of Health, The University of Newcastle and Vaccines, Immunity, Viruses and Asthma Group, Hunter Medical Research Institute, Newcastle, Australia; and the Division of BioSciences, John Curtin School of Medical Research, Australian National University, Canberra, Australia.

All measurements in the live mice were carried out by the collaborators in their laboratories in Newcastle. All experiments were approved by The University of Newcastle Animal Care and Ethics Committee (approval # 1001).

4.2.1 Mouse model of respiratory infection with pneumonia virus of mice (PVM)

Newborn BALB/c mice (<24 hrs old) were infected intranasally (IN) with a low dose (2.5 plaque forming units) of PVM strain J3666 in 5ul Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal bovine serum (FBS). Tumstatin (Recombinant peptide, NC1 domain from bovine collagen IV, Weislab, Lund, Sweden) reconstituted in phosphate buffered saline

(PBS) was given to the mice IN at 300ng per 20g body weight. Tumstatin was administered daily for the first 10 days, and then at days 21, 24 and 27 post infection (figure 4.1). As per ethical guidelines, mice were lightly anaesthetised once they were 10 days old with isofluorane prior to the administration of tumstatin. Mice were sacrificed and the left lung removed, and fixed in 4% formaldehyde solution and paraffin embedded prior to immunohistochemical analysis.

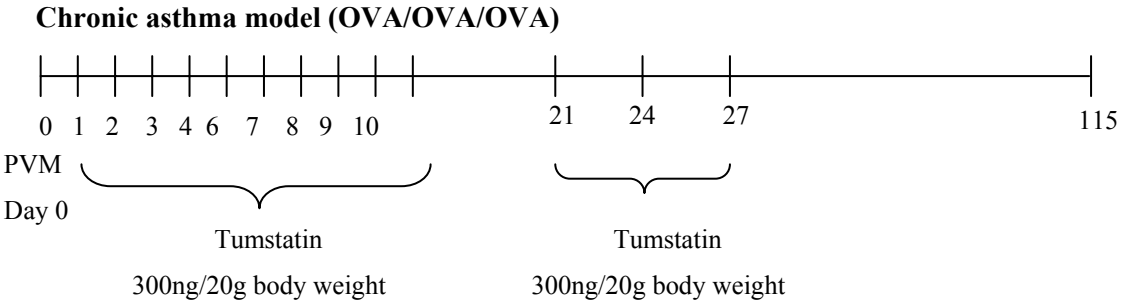
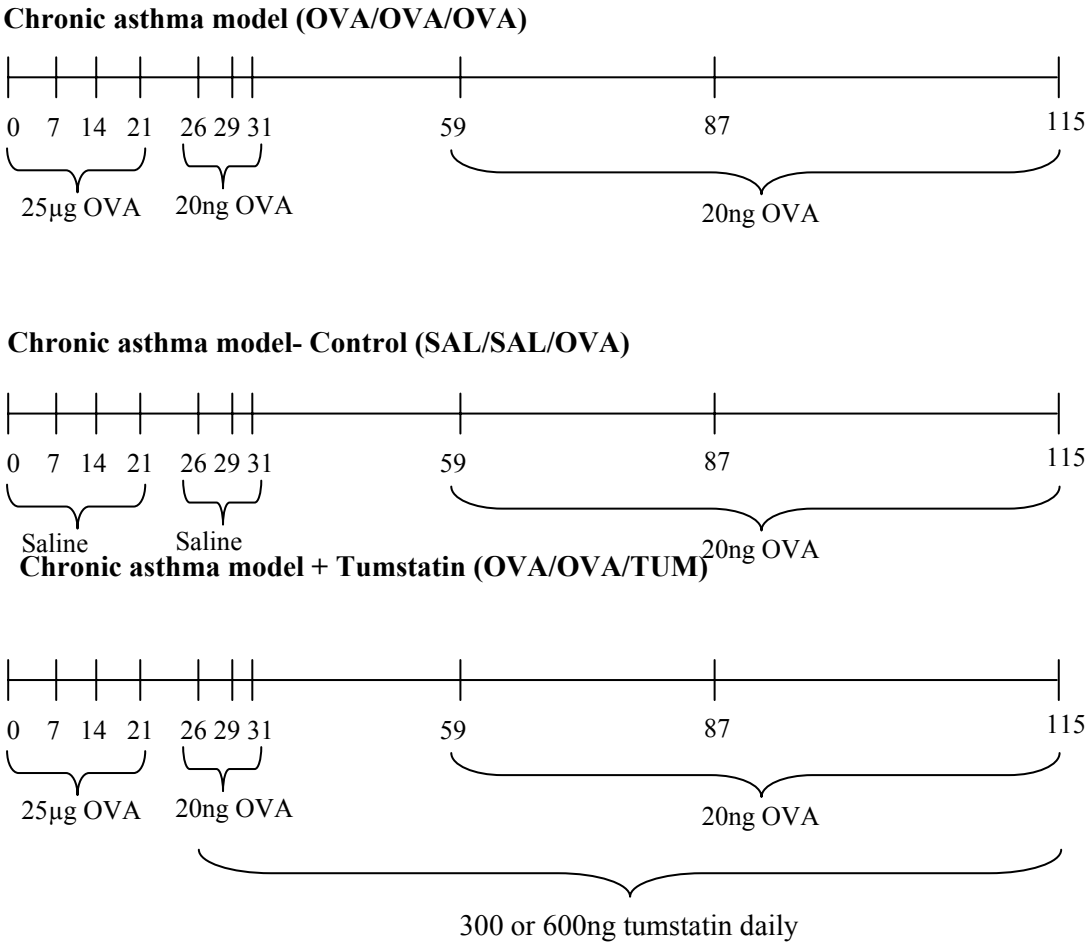


Figure 4.1: Pneumonia virus of mice (PVM) mouse model regime.

4.2.2 Mouse model of ovalbumin-induced airway hyperresponsiveness

Adult BALB/c mice (6-8 weeks old, ARC, Perth, Australia) were administered ovalbumin (OVA) on days 0, 7, 14 and 21: 25 μg OVA and 1 mg aluminium hydroxide in 200 μl sterile PBS were given subcutaneously (SC). On days 26, 29 and 31, 20 ng OVA in 50 μl sterile PBS was administered intranasally (IN), and then twice weekly from days 32 – 115. Some of the mice were administered tumstatin (reconstituted in PBS) IN at either 600ng or 300ng per 20g body weight once a day from days 25-115. OVA was omitted from sham-treated mice (figure 4.2).

Figure 4.2: Chronic asthma mouse model regime.



The airway resistance to inhaled methacholine (10 mg/ml) was calculated on day 115 (see following for method), at this time point the animals were sacrificed and the left lung removed and inflated with 30% formalin/PBS for 10 minutes. Lungs were then fixed in 4% formaldehyde solution and paraffin embedded prior to use in immunohistochemical analysis.

AHR was assessed to inhaled methacholine *in vivo* by measuring changes in transpulmonary resistance (RL) and dynamic compliance (Cdyn) using a supine whole-body plethysmograph (Buxco Electronics Inc, USA) attached to a computer. Mice were anaesthetised with an intraperitoneal injection of ketamine/xylazine and cannulated via the trachea with an 18G metal tube. Mice were mechanically ventilated by a minivent mouse ventilator at a rate of 120 breaths per minute and a tidal volume of 8ml/kg. Changes in lung volume were detected by a calibrated differential transducer connected to the plethysmograph chamber lumen which measured volume changes due to thoracic expansion with ventilation. A pressure transducer with a port near the tracheal tube measured alterations in forced ventilation tracheal pressure as a function of airway calibre. Aerosolised acetyl-β-methacholine (10mg/ml) in PBS generated by an ultrasonic nebuliser was delivered directly to the lungs via the inspiratory line for 5 minutes. The peak response to methacholine was compared to the response to saline alone.

4.2.3 Statistical analysis

For the analysis of AHR comparison between control (sham mice) and OVA exposed mice a one way analysis of variance (ANOVA) with Dunett's Multiple Comparison test was used. For comparison between OVA and tumstatin administered mice a two way ANOVA with Bonferroni post test was used. For the analysis of airway conductance a two way ANOVA with Bonferroni post test was used.

4.2.4 Detection of angiogenesis in PVM model

Paraffin embedded lung tissue sections were placed in xylene for 30 mins for de-paraffinisation, and re-hydrated through graded alcohol. Sections were blocked with a pre-made peroxidase blocking agent (DakoCytomation, Glostrup, Denmark) for 5 minutes. Sections were then washed with PBS and primary antibodies added, goat anti-mouse CD31 (PECAM-1) (Santa Cruz Biotechnology Inc, Santacruz, CA, USA) at 1ng/ml and rabbit anti-mouse von Willebrand factor (vWF) (Santa Cruz Biotechnology Inc) at 1ng/ml, and incubated at room temperature for one hour. Both CD31 and vWF are blood vessel markers. Sections were washed in PBS and the secondary antibodies added, pre-made anti-rabbit horseradish peroxidase (HRP) (DakoCytomation) and rabbit anti-goat HRP (DakoCytomation) respectively, and incubated at room temperature for one hour. Sections were washed in PBS for 5 minutes and a pre-made substrate chromogen, liquid 3,3'-

diaminobenzidine (DAB) (DakoCytomation), was added to the sections and incubated for 5 minutes at room temperature. Sections were then washed in distilled water for 5 minutes and mounted using an aqueous mounting medium (Faramount, DakoCytomation) and coverslipped. Images were taken on an Olympus fluorescence microscope BX51 and captured using Leica imaging software IM2000 (Leica, Heerbrugg St Gallen, Switzerland).
Four independent observers counted the number of blood vessels in four random fields from each of the six mice at days 14, 28 and 35, in comparison to images of the appropriate isotype controls, for each factor (CD31 or vWF) investigated.

4.2.5 Detection of angiogenesis in ovalbumin model

Paraffin embedded lung tissue sections were placed in xylene for 30mins, for de-paraffinisation and then re-hydrated through graded alcohol. Four slides per lung were selected at random for staining. Sections were blocked with peroxidase blocking agent (DakoCytomation) for 5 minutes before washing with PBS and the addition of primary antibody rabbit anti-mouse vWF (Santa Cruz Biotechnology Inc.) at 1ng/ml, for one hour at room temperature. Sections were washed in PBS and the secondary antibody added, anti-rabbit HRP (DakoCytomation) and incubated at room temperature for one hour. After washing in PBS for 5 minutes the substrate chromogen, liquid DAB (DakoCytomation) was added to the sections and incubated for 5 minutes at room temperature. A final wash in distilled water for 5 minutes was carried

out before sections were mounted using an aqueous mounting medium (Faramount, DakoCytomation) and coverslipped. Images were taken on an Olympus BX51 fluorescence microscope and captured using Leica imaging software IM 1000 (Leica).

4.2.6 Statistical analysis

Four mice from each group, SAL/SAL/OVA, OVA/OVA/OVA, OVA/OVA/TUM 300, OVA/OVA/TUM 600, were analysed. For each mouse, four slides per lung were selected at random and the entire section imaged. The total area of each field was measured and the number of blood vessels and the area of each vessel calculated. The mean blood vessel area, per field, was determined by dividing the total blood vessel area by the number of blood vessels in that field normalised to the total area of the section.

4.3 Results

4.3.1 Detection of angiogenesis in PVM model

We assessed the lungs of the mice (six per timepoint), which received treatment with PVM alone, for the presence of angiogenesis. There was a clear increase in blood vessel number at day 28, compared to the earlier time points (days 7 and 14) which subsequently decreased at the latter time points (days 35 and 49) (figure 4.3).

The mean number of blood vessels per image (combined CD31 and vWF) at day 14 was 0.63 ± 0.31 (SEM), which dramatically increased at day 28 to 35.6 ± 3, and subsequently reduced to 0.34 ± 0.34 by day 35.

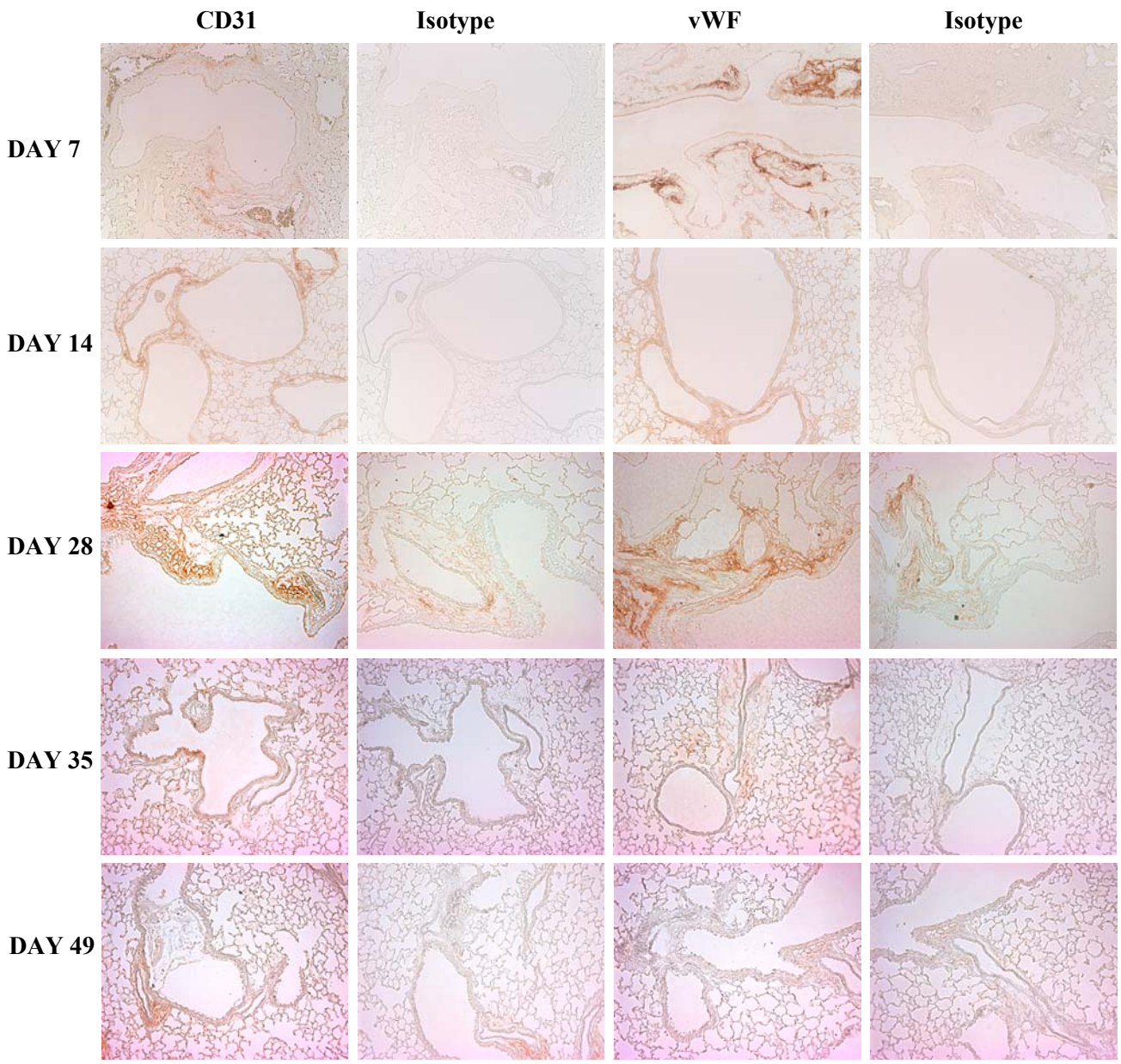


Figure 4.3: Detection of angiogenesis, in PVM infected mice, at days 7, 14, 28, 35 and 49 using CD31 and vWF staining. Positivity for the antibodies is indicated by the brown staining (DAB) compared to the isotype controls. These immunohistochemical images are derived from 5 different mice (one mouse per timepoint) and are representative of all six mice tested at each time point.

4.3.2 Inhibition of angiogenesis by tumstatin in PVM mouse model

The results indicated angiogenesis was occurring at day 28, therefore we investigated whether the addition of tumstatin to this system could inhibit the development of pulmonary angiogenesis. As shown in figure 4.4, tumstatin at 300ng/20g body weight was able to inhibit pulmonary angiogenesis seen in this model.

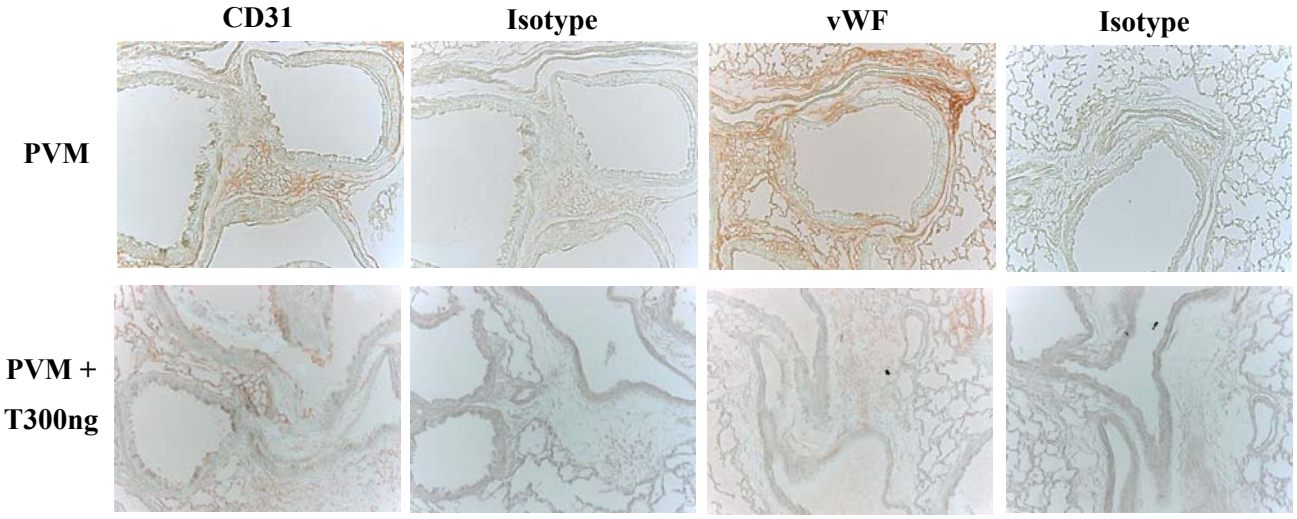


Figure 4.4: Detection of angiogenesis, in PVM infected mice, at day 28 with and without 300ng/20g body weight tumstatin (T300) using CD31 and vWF staining. Positivity for the antibodies is indicated by the brown staining (DAB) compared to the isotype controls. These immunohistochemical images are derived from 2 different mice (one mouse per treatment group) and are representative of all six mice which had PVM infection alone and the 4 mice which received tumstatin treatment in addition to PVM infection.

4.3.3 Inhibition of angiogenesis by tumstatin in ovalbumin mouse model

An increase in the number of blood vessels was observed in airway sections taken from OVA (OVA/OVA/OVA)-challenged mice when compared to the control mice (SAL/SAL/OVA) as shown in figure 4.5. Mice that were challenged with ovalbumin and administered with 300 or 600 ng/20g body weight of tumstatin (OVA/OVA/TUM) showed significant inhibition of angiogenesis compared to the untreated group (OVA/OVA/OVA).

The OVA/OVA/OVA mice had significantly more blood vessels and greater blood vessel area than the SAL/SAL/OVA, OVA/OVA/TUM 300 and OVA/OVA/TUM 600 mice. There was no significant difference between the SAL/SAL/OVA, OVA/OVA/TUM 300 and OVA/OAV/TUM 600 mice (figure 4.6).

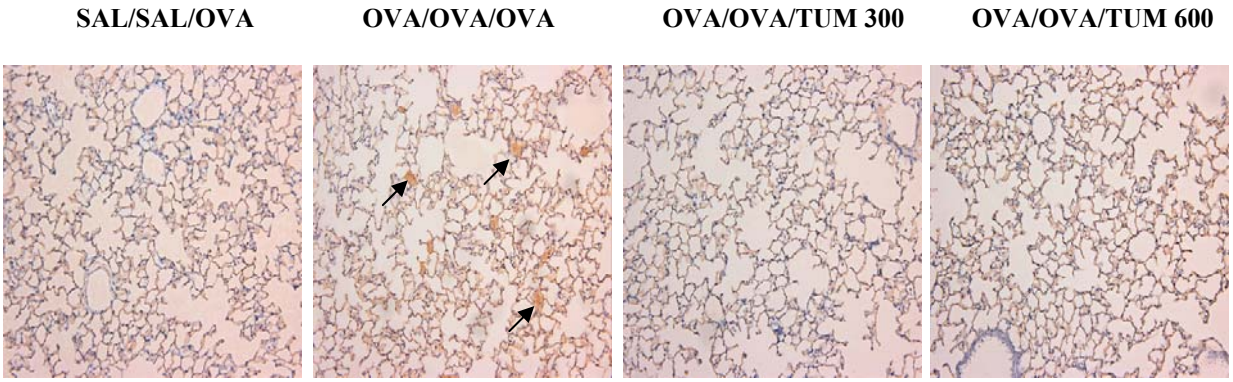


Figure 4.5: Detection of Angiogenesis in a mouse model of airway hyperresponsiveness. Angiogenesis in airway sections from control (SAL/SAL/OVA), ovalbumin-challenged (OVA/OVA/OVA), ovalbumin and tumstatin 300ng/20g body weight (OVA/OVA/TUM 300) and ovalbumin and tumstatin 600ng/20g body weight (Ova/Tum 600) mice was detected using vWF staining. Blood vessels (indicated by arrows) are stained brown (DAB). Images are representative of results obtained from 4 mice in each group.

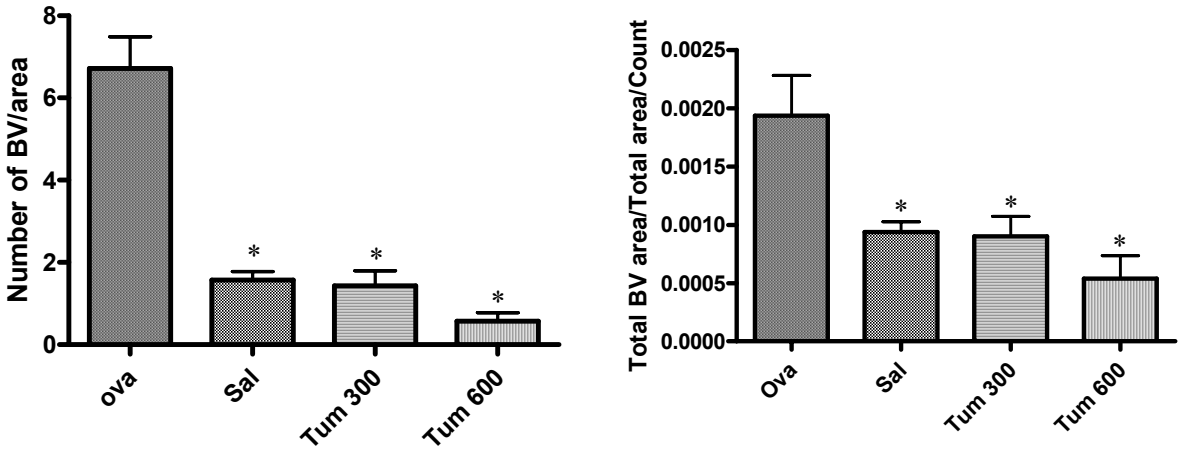


Figure 4.6: Tumstatin inhibits blood vessel formation in the airway of OVA treated mice. The number of blood vessels counted per area and the total blood vessel area divided by the total field area divided by the blood vessel count in airway sections from ovalbumin-challenged mice (OVA), control mice (SAL), ovalbumin and tumstatin 300ng/20g body weight (Tum 300) and ovalbumin and tumstatin 600ng/20g body weight (Tum 600). Results were obtained from 4 mice from each group. Data are expressed as mean ± standard error of the mean. (* $p < 0.02$ compared to OVA, one way ANOVA).

4.3.4 Effect of tumstatin on AHR in ovalbumin mouse model

The mice which received treatment with OVA alone demonstrated an increase in airway resistance to methacholine at a concentration of 10mg/ml in comparison to control animals demonstrating that a murine model of AHR had been successfully established ($p<0.01$, n=4, one way ANOVA with Dunnett's Multiple Comparison Test, figure 4.7). The mice which received concurrent treatment of OVA with tumstatin at 300 ng and OVA had reduced airway resistance to methacholine (10 mg/ml) in comparison to OVA alone ($p<0.01$, n=4, two way ANOVA with Bonferroni post tests, figure 4.8). Moreover, the mice which received concurrent treatment of OVA with tumstatin at 600 ng also had reduced airway resistance to methacholine (10 mg/ml) in comparison to OVA alone ($p<0.01$, n=4, two way ANOVA with Bonferroni post tests, figure 4.7). Similarily, mice which were sensitised and subsequently chronically exposed to OVA had decreased airway conductance in comparison to sham-treated mice ($p<0.05$, n=4, two way ANOVA with Bonferroni post tests). However concurrent administration of tumstatin and OVA significantly increased airway conductance in comparison to the OVA sensitised and exposed group only in the mice administered 600 ng tumstatin ($p<0.05$, n=4, two way ANOVA with Bonferroni post tests figure 4.8).

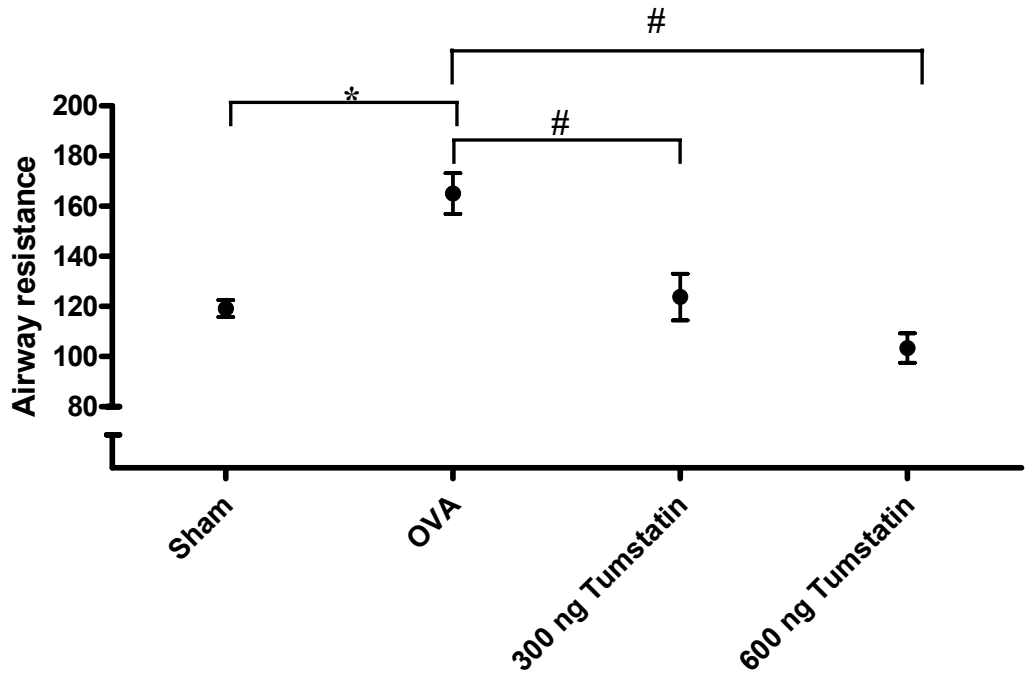


Figure 4.7: AHR induced by inhalation of methacholine (10 mg/ml) in mice chronically exposed to ovalbumin (OVA), ovalbumin plus 300ng of tumstatin (300ng Tumstatin), ovalbumin plus 600ng of tumstatin (600ng Tumstatin) and naïve mice (Sham). * $p<0.01$ compared to Sham, # $p<0.01$ compared to OVA (two way ANOVA with Bonferroni post tests n=4). Data are expressed as mean ± standard error of the mean.

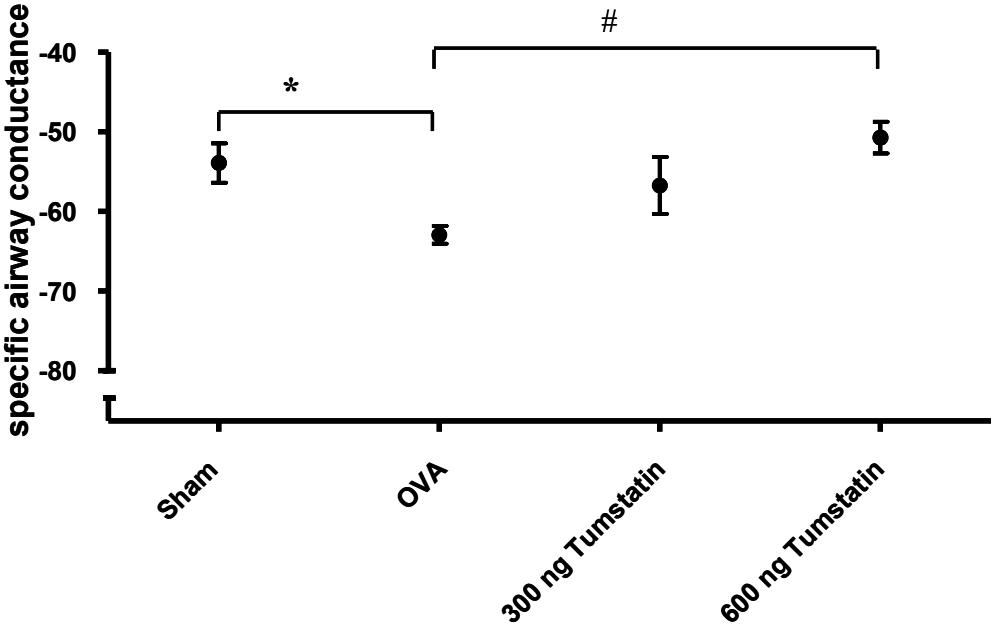


Figure 4.8: Specific airway conductance in mice chronically exposed to ovalbumin (OVA), ovalbumin plus 300ng of tumstatin (300ng Tumstatin), ovalbumin plus 600ng of tumstatin (600ng Tumstatin) and naïve mice (Sham). * $p<0.05$ compared to Sham, # $p<0.05$ compared to OVA (two way ANOVA with Bonferroni post tests, n=4). Data are expressed as mean ± standard error of the mean.

4.4 Discussion

This study examined the efficacy of tumstatin in the regulation of angiogenesis during allergic airway inflammation. Firstly, the ability of tumstatin to act as an angiogenic inhibitor in the lung was determined, using a virus mouse model (PVM model). In this model, tumstatin was able to inhibit pulmonary angiogenesis. Further, a well established mouse model of allergic airway disease, which exhibits features similar to those seen in human allergic asthma (Asosingh *et al.*, 2007; Lee *et al.*, 2006; Suzaki *et al.*, 2005), was employed to directly examine tumstatin's effectiveness in inhibiting angiogenesis and its effect on lung function (AHR). Tumstatin was able to inhibit angiogenesis but also improve AHR in this model.

Viral respiratory infections (VRI) in early childhood have been linked to the development of asthma (Weinberger, 2003). RSV infections trigger an inflammatory response in the airways which leads to epithelial damage as well as hypersecretion of mucus and oedema (Holt *et al.*, 2002). Tissue repair in the airways in humans is apparent within days post infection (Hall *et al.*, 1978). Therefore, the potential for remodelling, which includes angiogenesis, in the airway of PVM-infected mice was very likely and would occur in a shorter period of time than in a chronic mouse model.

The PVM model was used in this study as an initial step to evaluate the development of angiogenesis, which was identified in the airways at 28 days

post infection, and then to investigate whether tumstatin could prevent this angiogenesis with the knowledge that angiogenesis was detectable and that the effect of tumstatin was demonstrable at a defined time point. The PVM model made it possible to then investigate the effects of tumstatin on angiogenesis in the airways in an animal model. Tumstatin was able to inhibit angiogenesis in the airway, demonstrating for the first time the potential for tumstatin to be an effective angiogenic inhibitor in the airways.

VEGF is a potent angiogenic promoter, that is reported to increase vascular permeability (Lee *et al.*, 2004). Tumstatin has been shown to block VEGF-induced neovascularisation in C57BL/6 using a matrigel assay (Maeshima *et al.*, 2001a; Maeshima *et al.*, 2001b). Yamamoto *et al*. showed the inhibitory effect of tumstatin on monocyte/macrophage recruitment in diabetic mice (C57BL/6 mice infected with streptozotocin) which led them to speculate that this inhibition was due to the inhibition of VEGF activity (Yamamoto *et al.*, 2004). VEGF levels are elevated in asthma and inflammatory cell influx is increased in the airways of asthmatics in the vicinity of the airway smooth muscle (ASM) (Belda *et al.*, 2005). This cell influx, consequently supports the increased ASM proliferation and hence the remodelling in the airways. The improvement in AHR observed in mice administered tumstatin in concert with OVA in the present study may be due to the inhibition of inflammatory cells, which could in part be due to the decreased blood vessels, as well as a decrease in the permeability of the existing blood vessels. The decrease in permeability may be in part due to the inhibition of

VEGF binding by tumstatin. Yamamoto *et al.* showed, using a mouse model of diabetes, that treatment with tumstatin inhibited VEGF but had no effect on Ang-1 levels. In contrast, levels of Ang-2, an endogenous antagonist of Ang-1 involved in the induction of sprouting angiogenesis, was decreased upon administration of tumstatin (Yamamoto *et al.*, 2004). Levels of VEGF, Ang-1 and Ang- 2 were not measured in the asthma mouse model used in this study. It would be of interest in the future to determine the level of expression of these three factors and the effect of tumstatin on their level of expression in the airways and the consequences on AHR.

The assessment of lung function in a mouse model is not simple. The comparison between mouse and human can be difficult as, for example, the tidal volume of a mouse is approximately 0.2ml compared to 500ml in human. This problem has been overcome with the use of orotracheally intubated mice and the ability therefore to measure parameters such as resistance and compliance repetitively, as was used in this study. The improvement in AHR was seen at 10mg/ml of inhaled methacoline. These results show the ability of tumstatin to reverse bronchoconstriction, however, a direct relationship to humans cannot be drawn from concentrations used in this animal model. Further investigation would be needed to examine the ability of tumstatin to improve AHR in humans.

The ECM plays a big role in the remodelling of the airways. There is a complex interaction between the ECM and lung cells in the airway. For

instance, the response to different ECM components, such as collagen, may alter the proliferation, survival and inflammatory mediator release of ASM cells (Hirst *et al.*, 2000; Peng *et al.*, 2005). Tumstatin is able to affect ECM protein deposition, as was shown in diabetic mice whereby the administration of tumstatin inhibited the glomerular accumulation of collagen IV (Yamamoto *et al.*, 2004). Levels of collagen IV were not examined in this study, however it would be interesting to see whether any alterations have occurred in the levels of collagen IV or other ECM proteins as a result of the inhibition of angiogenesis by tumstatin.

Physiological angiogenesis, associated with tissue repair, is unaffected by tumstatin. Closure and repair of skin wounds in tumstatin deficient mice progressed at the same rate as in wild-type mice. In addition rates of regeneration of liver after partial hepatectomy were the same for both groups (Hamano *et al.*, 2003). The mouse model of asthma used in this study did not exhibit any growth related problems due to the administration of tumstatin as mice treated with tumstatin developed at the same rate as control mice. Therefore, the angiogenesis observed in this model was a feature of the remodelling caused by the chronic exposure to ovalbumin, which in turn was inhibited by tumstatin. Although limitations in this model exist, such as the inability of mice to exhibit allergen independent chronification or acute asthma attacks, it still exhibits a majority of the symptoms seen in asthma. The ability of tumstatin to inhibit the hallmark features of asthma suggests that tumstatin deficiency in asthma may be critically important to

remodelling and to alterations in lung function. Indeed, in a recent case report, a patient with concomitant severe persistent asthma was treated for rheumatoid arthritis with an angiogenic inhibitor (MEDI-522). It was noted that during the trial the patient had significant and substantial improvement in lung function (Saadeh *et al.*, 2007). Tumstatin could play a significant role in the inhibition of neovascularisation and inflammation in asthmatic airways, as well as decreasing AHR.

Chapter 5

Summary and conclusion

5.1 General introduction

It is well recognised that airway remodelling is a characteristic feature of chronic persistent asthma. One of the prominent features of this remodelling is angiogenesis. Angiogenesis is controlled by both pro- and anti-angiogenic factors which under homeostatic conditions are in a state of equilibrium (Carmeliet, 2004). Levels of pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), are reported to be increased in the airways of asthmatics (Chetta *et al.*, 2005; Feltis *et al.*, 2006; Hoshino *et al.*, 2001b; Lee *et al.*, 2001). However, the levels of angiogenic inhibitors have not been closely examined in asthma. Evidence to date suggests an imbalance between the pro-angiogenic factors and the anti-angiogenic factors, with the scale appearing to be tipped towards the pro-angiogenic factors.

Experiments described in this thesis, have for the first time described the expression of the endogenous angiogenic inhibitor tumstatin in the airways of asthmatics and its functional role in the airway. The absence of tumstatin in the airways of asthmatics was demonstrated, and tumstatin was shown to have a functional role as an inhibitor of angiogenesis in the airways, in addition to improving AHR in a mouse model of allergic airways hyperresponsiveness.

5.2 Characterisation of tumstatin in chronic respiratory disease

As shown in chapter two of this thesis, tumstatin is absent from the airways of individuals with asthma and lymphangioleiomyomatosis (LAM). However, in asthmatic individuals, the remaining collagen IV α chain non-collagenous (NC1) domains are present. The collagen IV α5 chain NC1 domain is also absent from LAM airways, however this was not further investigated in this thesis. It would be of interest to follow up this observation in LAM, since the absence of both the collagen IV α3 and α5 chains are reported in other disease states such as Alports Syndrome. The absence of both chains in LAM individuals may have similar consequences to those seen in the other disease states such as deficiencies of the kidney. By examining airway sections from individuals with chronic obstructive pulmonary disease (COPD), bronchiectasis and cystic fibrosis (CF) it was confirmed the absence of tumstatin was not a general feature associated with chronic respiratory diseases. Tumstatin was present in these three disease states, suggesting the absence seen in asthmatic and LAM individuals is a specific defect in those diseases. Levels of collagen IV are reported to be decreased in the airways of asthmatics, however it is not known if this decrease is a result of a specific decrease in one or more of the α chains of collagen IV. The absence of tumstatin may be a contributing factor to the decrease seen in the asthmatic airway.

The collagen IV α3 chain has a complex folding process that results in a number of different conformers, some of which require specific activation by phosphorylation to determine their folding. The good pasture antigen-binding protein (GPBP) is responsible for this phosphorylation and an increase in the GPBP can result in an increase in misfolding of the collagen IV α3 chain. Misfolding of the collagen IV α3 chain may have resulted in a conformational change which could result in failure of the antibody used in this study to recognise the altered form. However, levels of GPBP were examined in asthmatic and LAM individuals and no significant difference was found compared to the non-asthmatic individuals. The lack of difference in the expression of the GPBP between asthmatic, LAM and non-asthmatics suggests this is not the cause of the difference in the level of detection of tumstatin that is reported in this study.

Tumstatin exhibits its anti-angiogenic properties through binding to the αvβ3 integrin. Both the αv and β3 subunit were shown to be present and equally expressed in asthmatic and non-asthmatic individuals. The limiting factor in this technique was the inability to perform digital analysis in order to quantify the levels of expression in each tissue section, however, upon visual examination no difference was apparent. The presence of both the αv and β3 subunits in both asthmatic and non-asthmatic individuals indicates that tumstatin, when present, is able to be active as an angiogenic inhibitor. Further, the co-localisation of tumstatin, VEGF and the αvβ3 integrin showed that, as in the previous finding, tumstatin was absent in the asthmatic

and LAM airways and that VEGF and, in addition, the αvβ3 integrin co-localisation was minimal in the airways of asthmatic and LAM individuals. Co-localisation between the three molecules in the non-asthmatic airway was clearly seen. This confirms that, under normal conditions, both VEGF and tumstatin are within reach of the αvβ3 integrin to act as the "on" (VEGF) and "off" (tumstatin) switch for angiogenesis when necessary.

5.3 Functional role of tumstatin *in vitro*

To determine whether the absence of tumstatin is a general feature of asthma, or if it is specific to the airway, serum and BAL-f samples were examined from asthmatic individuals. It was not possible to further investigate the absence of tumstatin in LAM individuals due to the lack of availability of suitable samples. As shown in chapter 3, tumstatin was detected in both the serum and the BAL-f of asthmatic individuals. The presence of tumstatin in the asthmatic BAL-f samples suggests that asthmatic individuals have the ability to produce tumstatin in the airway but are unable to sequester it in the matrix/basement membrane. Matrix metalloproteinase (MMP) -9 activity is increased in bronchial biopsy specimens, blood, induced sputum and BAL-f from patients with severe or uncontrolled asthma (Hoshino *et al.*, 1998a; Lemjabbar *et al.*, 1999). MMP-9 is the most efficient protease for cleaving tumstatin. It can be postulated that the increase in the levels of MMP-9 in asthmatic airways are causing excess amounts of tumstatin to be released and potentially cleared from the airway. Alternatively, an as yet unidentified

protease may be responsible for the cleavage and clearing of tumstatin in the airways.

Using primary pulmonary endothelial cells, this thesis shows, for the first time, the ability of tumstatin to inhibit pulmonary endothelial cell tube formation in a dose-related manner, as well as to inhibit the proliferation of pulmonary endothelial cells. To date, reports on tumstatin have described its activity in tumour cells, prostate carcinoma cells and human umbilical vein endothelial cells (HUVECs), whereas its potential activity in the airway had not been investigated. The findings from chapter three suggest the presence of tumstatin in the airway has the potential to play a functional role in the inhibition of angiogenesis.

5.4 Functional role of tumstatin *in vivo*

As shown in chapter four, the ability of tumstatin to act as an angiogenic inhibitor *in vivo* in the airway was determined using a viral-induced mouse model of angiogenesis. Results showed that administration of tumstatin in this model was successful in inhibiting the angiogeneis caused by the viral infection in the lungs of mice. This is consistent with previous reports in the literature on the effectiveness of tumstatin administration in inhibiting angiogenesis. Hamano et al. showed, using tumstatin knock-out mice, that when tumstatin was administered to the mice, tumour growth was reduced to that of the wild-type (Hamano *et al.*, 2003). The use of the viral mouse model established the fact that tumstatin is able to inhibit angiogenesis in the lung.

Further, a chronic model of allergic airway disease that exhibits similar features to those seen in asthma, was employed to directly examine tumstatin's effectiveness in inhibiting angiogenesis in asthma and its effects on AHR. Tumstatin inhibited the angiogenesis associated with remodelling, decreased airway resistance and increased conductance in the mice. The decrease in AHR seen in this model as a result of tumstatin administration could be due to the reduction of inflammatory cell influx due to the reduction in the number of blood vessels and blood vessel permeability. VEGF is known to increase vascular permeability, therefore in turn increases the influx of inflammatory cells into the airway. However, tumstatin has been shown to block VEGF activity, thereby reducing cellular influx and potentially decreasing AHR. Saadeh *et al.* described a clinical trial in which a patient was being treated for rheumatoid arthritis with an angiogenic inhibitor (MEDI-522), the patient was also diagnosed with moderately severe persistent asthma. It was noted that, during the trial, the patient had significant and substantial improvement in lung function (Saadeh *et al.*, 2007). With this in mind and the results from the *in vivo* study, tumstatin has potential as a therapeutic intervention in asthma.

Figure 5.1 summarises the major findings of the preceding chapters, in addition to previous relevant reports in the literature.

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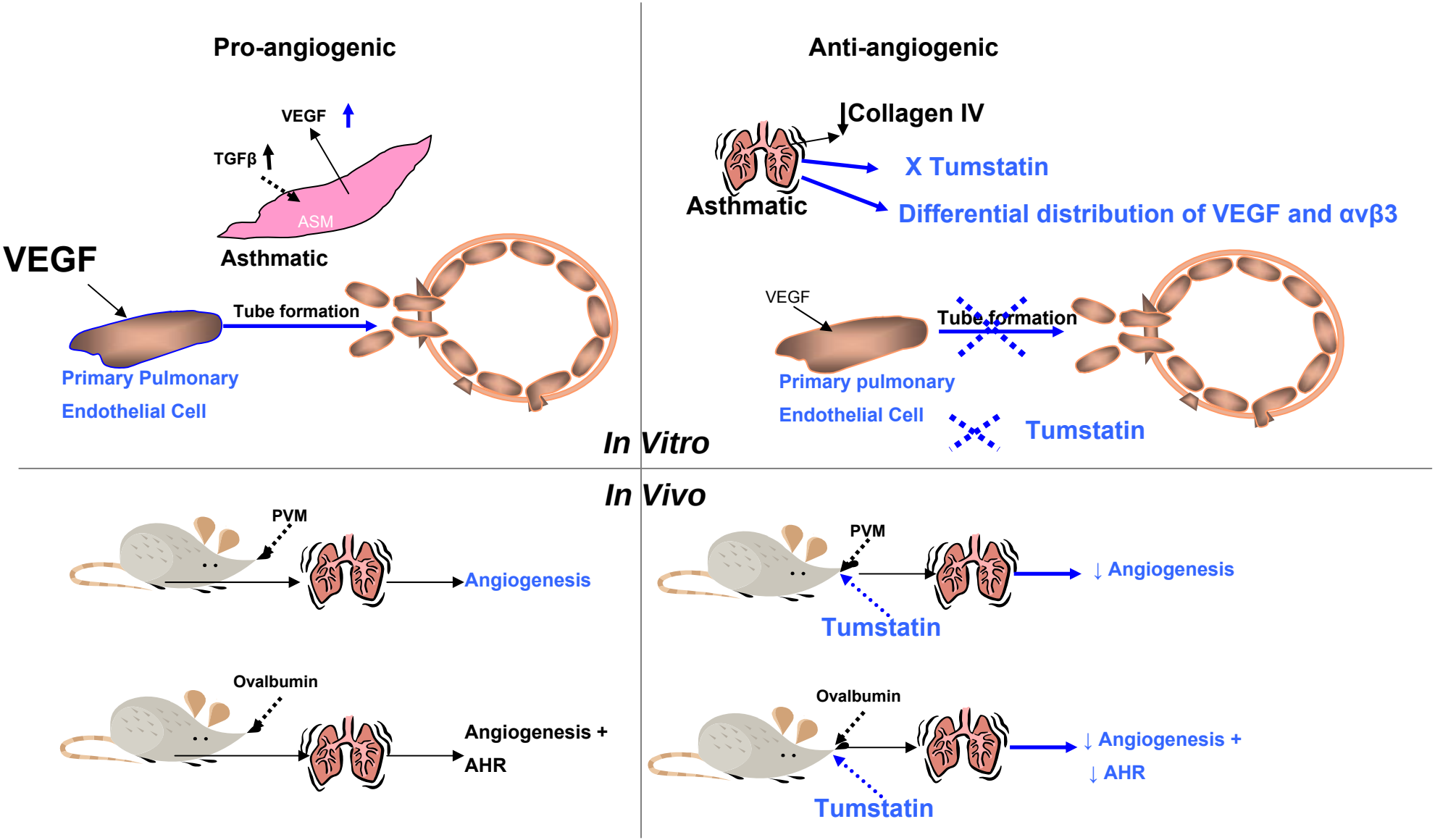

Future directions

The absence of tumstatin in the asthmatic airway, as shown in chapter two and its presence in asthmatic serum and BAL-f as shown in chapter three suggests that there is an underlying mechanism responsible for clearing tumstatin out of the airway in asthmatic individuals. It would be interesting and clinically beneficial to further investigate and identify all the agents which could cleave tumstatin and determine their roles in the airway, and further classify whether any changes in these agents exist in the asthmatic airway compared to the non-asthmatic. The identification of the mechanism responsible for clearing tumstatin in the airways and whether this process is altered in the asthmatic airway would be of great importance in any attempt to restore physiological tumstatin levels in the asthmatic airways.

The absence of tumstatin and the collagen IV α5 chain NC1 domain in LAM individuals, as shown in chapter two, raises the question of the role of collagen IV α5 chain in the airways and whether the absence of tumstatin and the α5 chain are related to each other in this disease. Alports Syndrome is a disease which features both collagen IV α3 and α5 chain mutations. This suggests the simultaneous absence of both molecules may play an aetiological role in the disease. Further characterisation of the role of the collagen IV α5 chain may possibly add some clarity to the relevance of its absence in LAM. In this thesis samples of blood and BAL-f could not be obtained from LAM individuals. If this problem can be overcome, it would

be ideal to further characterise tumstatin and the collagen IV α5 chain NC1 domain in serum and BAL-f samples of LAM individuals.

It is unknown if there is any genetic involvement in the absence of tumstatin in the asthmatic and LAM airways. The characterisation of the collagen IV α3 gene in asthmatic and LAM individuals may shed some light as to whether any mutations in this gene exist and are potentially the cause of the absence of tumstatin seen in both asthmatic and LAM airways.

Tumstatin decreased AHR *in vivo* in the mouse model of AHR used in this study, as shown in chapter four. However, the question of how tumstatin was able to decrease AHR remains unanswered. This thesis postulated that this mechanism may be due to the inhibition of VEGF, which in turn is inhibiting inflammation and decreasing AHR. Further investigation into the role of VEGF and tumstatin in this model may help to identify the mechanism of action involved. Levels of collagen IV in this model were not measured, thus it may be interesting to quantify the levels of collagen IV in these mice and identify whether tumstatin is having any effects on ECM protein deposition in the airways, as occurs in the kidney in diabetic mice.

Tumstatin can inhibit angiogenesis associated with tissue remodelling. In the airways in mice, it is capable of inhibiting aberrant angiogenesis as well as

decreasing AHR. Evidence from this thesis strongly suggests that tumstatin or a derivative has therapeutic potential in asthma.

Chapter 6

References

AHMAD, S.A., LIU, W., JUNG, Y.D., FAN, F., WILSON, M., REINMUTH, N., SHAHEEN, R.M., BUCANA, C.D. & ELLIS, L.M. (2001). The effects of angiopoietin-1 and -2 on tumor growth and angiogenesis in human colon cancer. *Cancer Res*, **61,** 1255-9.

ANDREWS, K.L., MUDD, J.L., LI, C. & MINER, J.H. (2002). Quantitative trait loci influence renal disease progression in a mouse model of Alport syndrome. *Am J Pathol*, **160,** 721-30.

ASAI, K., KANAZAWA, H., OTANI, K., SHIRAISHI, S., HIRATA, K. & YOSHIKAWA, J. (2002). Imbalance between vascular endothelial growth factor and endostatin levels in induced sputum from asthmatic subjects. *J Allergy Clin Immunol*, **110,** 571-5.

ASOSINGH, K., SWAIDANI, S., ARONICA, M. & ERZURUM, S.C. (2007). Th1- and Th2-dependent endothelial progenitor cell recruitment and angiogenic switch in asthma. *J Immunol*, **178,** 6482-94.

BARKER, A.F. (2002). Bronchiectasis. *N Engl J Med*, **346,** 1383-93.

BELDA, J., MARGARIT, G., MARTINEZ, C., CASAN, P., RODRIGUEZ-JEREZ, F., BRUFAL, M., TORREJON, M., GRANEL, C. & SANCHIS, J. (2005). [Bronchial exudate of serum proteins during asthma attack]. *Arch Bronconeumol*, **41,** 328-33.

BLACK, J.L., GE, Q., BOUSTANY, S., JOHNSON, P.R., PONIRIS, M.H., GLANVILLE, A.R., OLIVER, B.G., MOIR, L.M. & BURGESS, J.K. (2005). In vitro studies of lymphangioleiomyomatosis. *Eur Respir J*, **26,** 569-76.

BOEHM, T., PIRIE-SHEPHERD, S., TRINH, L.B., SHILOACH, J. & FOLKMAN, J. (1999). Disruption of the KEX1 gene in Pichia pastoris allows expression of full-length murine and human endostatin. *Yeast*, **15,** 563-72.

BONVILLE, C.A., EASTON, A.J., ROSENBERG, H.F. & DOMACHOWSKE, J.B. (2003). Altered pathogenesis of severe pneumovirus infection in response to combined antiviral and specific immunomodulatory agents. *J Virol*, **77,** 1237-44.

BOUSQUET, J., CHANEZ, P., LACOSTE, J.Y., WHITE, R., VIC, P., GODARD, P. & MICHEL, F.B. (1992). Asthma: a disease remodeling the airways. *Allergy*, **47,** 3-11.

BOUSQUET, J., JEFFERY, P.K., BUSSE, W.W., JOHNSON, M. & VIGNOLA, A.M. (2000). Asthma. From bronchoconstriction to airways inflammation and remodeling. *Am J Respir Crit Care Med*, **161,** 1720-45.

BOUSQUET, J., LACOSTE, J.Y., CHANEZ, P., VIC, P., GODARD, P. & MICHEL, F.B. (1996). Bronchial elastic fibers in normal subjects and asthmatic patients. *Am J Respir Crit Care Med*, **153,** 1648-54.

BOUTAUD, A., BORZA, D.B., BONDAR, O., GUNWAR, S., NETZER, K.O., SINGH, N., NINOMIYA, Y., SADO, Y., NOELKEN, M.E. & HUDSON, B.G. (2000). Type IV collagen of the glomerular basement membrane. Evidence that the chain specificity of network assembly is encoded by the noncollagenous NC1 domains. *J Biol Chem*, **275,** 30716-24.

BRAMAN, S.S. (2006). The global burden of asthma. *Chest*, **130,** 4S-12S.

BRAUN, A. & TSCHERNIG, T. (2006). Animal models of asthma: innovative methods of lung research and new pharmacological targets. *Exp Toxicol Pathol*, **57 Suppl 2,** 3-4.

BREWSTER, C.E., HOWARTH, P.H., DJUKANOVIC, R., WILSON, J., HOLGATE, S.T. & ROCHE, W.R. (1990). Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol Biol*, **3,** 507-11.

BUIST, A.S., MCBURNIE, M.A., VOLLMER, W.M., GILLESPIE, S., BURNEY, P., MANNINO, D.M., MENEZES, A.M., SULLIVAN, S.D., LEE, T.A., WEISS, K.B., JENSEN, R.L., MARKS, G.B., GULSVIK, A. & NIZANKOWSKA-MOGILNICKA, E. (2007). International variation in the prevalence of COPD (the BOLD Study): a population-based prevalence study. *Lancet*, **370,** 741-50.

BURGESS, J.K., GE, Q., PONIRIS, M.H., BOUSTANY, S., TWIGG, S.M., BLACK, J.L. & JOHNSON, P.R. (2006). Connective tissue growth factor and vascular endothelial growth factor from airway smooth muscle interact with the extracellular matrix. *Am J Physiol Lung Cell Mol Physiol*, **290,** L153-61.

BYRD, L.G. & PRINCE, G.A. (1997). Animal models of respiratory syncytial virus infection. *Clin Infect Dis*, **25,** 1363-8.

BYZOVA, T.V., RABBANI, R., D'SOUZA, S.E. & PLOW, E.F. (1998). Role of integrin alpha(v)beta3 in vascular biology. *Thromb Haemost*, **80,** 726-34.

CARMELIET, P. (2004). Manipulating angiogenesis in medicine. *J Intern Med*, **255,** 538-61.

CATALDO, D.D., BETTIOL, J., NOEL, A., BARTSCH, P., FOIDART, J.M. & LOUIS, R. (2002). Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. *Chest*, **122,** 1553-9.

CAUDROY, S., CUCHEROUSSET, J., LORENZATO, M., ZAHM, J.M., MARTINELLA-CATUSSE, C., POLETTE, M. & BIREMBAUT, P. (2004). Implication of tumstatin in tumor progression of human bronchopulmonary carcinomas. *Hum Pathol*, **35,** 1218-22.

CHAMBERS, P., BARR, J., PRINGLE, C.R. & EASTON, A.J. (1990). Molecular cloning of pneumonia virus of mice. *J Virol*, **64,** 1869-72.

CHETTA, A., FORESI, A., DEL DONNO, M., BERTORELLI, G., PESCI, A. & OLIVIERI, D. (1997). Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest*, **111,** 852-7.

CHETTA, A., ZANINI, A., FORESI, A., D'IPPOLITO, R., TIPA, A., CASTAGNARO, A., BARALDO, S., NERI, M., SAETTA, M. & OLIVIERI, D. (2005). Vascular endothelial growth factor up-regulation and bronchial wall remodelling in asthma. *Clin Exp Allergy*, **35,** 1437-42.

CHIAPPARA, G., GAGLIARDO, R., SIENA, A., BONSIGNORE, M.R., BOUSQUET, J., BONSIGNORE, G. & VIGNOLA, A.M. (2001). Airway remodelling in the pathogenesis of asthma. *Curr Opin Allergy Clin Immunol*, **1,** 85-93.

CHOE, M.M., SPORN, P.H. & SWARTZ, M.A. (2006). Extracellular matrix remodeling by dynamic strain in a three-dimensional tissue-engineered human airway wall model. *Am J Respir Cell Mol Biol*, **35,** 306-13.

COLORADO, P.C., TORRE, A., KAMPHAUS, G., MAESHIMA, Y., HOPFER, H., TAKAHASHI, K., VOLK, R., ZAMBORSKY, E.D., HERMAN, S., SARKAR, P.K., ERICKSEN, M.B., DHANABAL, M., SIMONS, M., POST, M., KUFE, D.W., WEICHSELBAUM, R.R., SUKHATME, V.P. & KALLURI, R. (2000). Anti-angiogenic cues from vascular basement membrane collagen. *Cancer Res*, **60,** 2520-6.

COX, G., THOMSON, N.C., RUBIN, A.S., NIVEN, R.M., CORRIS, P.A., SIERSTED, H.C., OLIVENSTEIN, R., PAVORD, I.D., MCCORMACK, D., CHAUDHURI, R., MILLER, J.D. & LAVIOLETTE, M. (2007). Asthma control during the year after bronchial thermoplasty. *N Engl J Med*, **356,** 1327-37.

D'AMORE, P.A. (1992). Mechanisms of endothelial growth control. *Am J Respir Cell Mol Biol*, **6,** 1-8.

D'ANDREA, L.D., DEL GATTO, A., PEDONE, C. & BENEDETTI, E. (2006). Peptide-based molecules in angiogenesis. *Chem Biol Drug Des*, **67,** 115-26.

DERRY, C.J. & PUSEY, C.D. (1994). Tissue-specific distribution of the Goodpasture antigen demonstrated by 2-D electrophoresis and western blotting. *Nephrol Dial Transplant*, **9,** 355-61.

DODGE, R., MARTINEZ, F.D., CLINE, M.G., LEBOWITZ, M.D. & BURROWS, B. (1996). Early childhood respiratory symptoms and the subsequent diagnosis of asthma. *J Allergy Clin Immunol*, **98,** 48-54.

EBINA, M., TAKAHASHI, T., CHIBA, T. & MOTOMIYA, M. (1993). Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am Rev Respir Dis*, **148,** 720-6.

EL-HASHEMITE, N. & KWIATKOWSKI, D.J. (2005). Interferon-gamma-Jak-Stat signaling in pulmonary lymphangioleiomyomatosis and renal angiomyolipoma: a potential therapeutic target. *Am J Respir Cell Mol Biol*, **33,** 227-30.

FELTIS, B.N., WIGNARAJAH, D., ZHENG, L., WARD, C., REID, D., HARDING, R. & WALTERS, E.H. (2006). Increased vascular endothelial growth factor and receptors: relationship to angiogenesis in asthma. *Am J Respir Crit Care Med*, **173,** 1201-7.

FOLKMAN, J. (2004a). Angiogenesis and c-Jun. *J Natl Cancer Inst*, **96,** 644.

FOLKMAN, J. (2004b). Endogenous angiogenesis inhibitors. *Apmis*, **112,** 496-507.

GRANERO, F., REVERT, F., REVERT-ROS, F., LAINEZ, S., MARTINEZ-MARTINEZ, P. & SAUS, J. (2005). A human-specific TNF-responsive promoter for Goodpasture antigen-binding protein. *Febs J*, **272,** 5291-305.

GRANT, M.A. & KALLURI, R. (2005). Structural basis for the functions of endogenous angiogenesis inhibitors. *Cold Spring Harb Symp Quant Biol*, **70,** 399-410.

HALL, W.J., HALL, C.B. & SPEERS, D.M. (1978). Respiratory syncytial virus infection in adults: clinical, virologic, and serial pulmonary function studies. *Ann Intern Med*, **88,** 203-5.

HAMANO, Y., ZEISBERG, M., SUGIMOTO, H., LIVELY, J.C., MAESHIMA, Y., YANG, C., HYNES, R.O., WERB, Z., SUDHAKAR, A. & KALLURI, R. (2003). Physiological levels of tumstatin, a fragment of collagen IV alpha3 chain, are generated by MMP-9 proteolysis and suppress angiogenesis via alphaV beta3 integrin. *Cancer Cell*, **3,** 589-601.

HASHIMOTO, M., TANAKA, H. & ABE, S. (2005). Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest*, **127,** 965-72.

HEIDET, L., ARRONDEL, C., FORESTIER, L., COHEN-SOLAL, L., MOLLET, G., GUTIERREZ, B., STAVROU, C., GUBLER, M.C. & ANTIGNAC, C. (2001). Structure of the human type IV collagen gene COL4A3 and mutations in autosomal Alport syndrome. *J Am Soc Nephrol*, **12,** 97-106.

HIRST, S.J., MARTIN, J.G., BONACCI, J.V., CHAN, V., FIXMAN, E.D., HAMID, Q.A., HERSZBERG, B., LAVOIE, J.P., MCVICKER, C.G., MOIR, L.M., NGUYEN, T.T., PENG, Q., RAMOS-BARBON, D. & STEWART, A.G. (2004). Proliferative aspects of airway smooth muscle. *J Allergy Clin Immunol*, **114,** S2-17.

HIRST, S.J., TWORT, C.H. & LEE, T.H. (2000). Differential effects of extracellular matrix proteins on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol*, **23,** 335-44.

HOLT, P.G. & SLY, P.D. (2002). Interactions between RSV infection, asthma, and atopy: unraveling the complexities. *J Exp Med*, **196,** 1271-5.

HOSHINO, M., NAKAMURA, Y. & HAMID, Q.A. (2001a). Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J Allergy Clin Immunol*, **107,** 1034-8.

HOSHINO, M., NAKAMURA, Y., SIM, J., SHIMOJO, J. & ISOGAI, S. (1998a). Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin Immunol*, **102,** 783-8.

HOSHINO, M., NAKAMURA, Y. & SIM, J.J. (1998b). Expression of growth factors and remodelling of the airway wall in bronchial asthma. *Thorax*, **53,** 21-7.

HOSHINO, M., TAKAHASHI, M. & AOIKE, N. (2001b). Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol*, **107,** 295-301.

HOSTIKKA, S.L., EDDY, R.L., BYERS, M.G., HOYHTYA, M., SHOWS, T.B. & TRYGGVASON, K. (1990). Identification of a distinct type IV collagen alpha chain with restricted kidney distribution and assignment of its gene to the locus of X chromosome-linked Alport syndrome. *Proc Natl Acad Sci U S A*, **87,** 1606-10.

HUTCHINGS, H., ORTEGA, N. & PLOUET, J. (2003). Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. *Faseb J*, **17,** 1520-2.

HYNES, R.O. (2002). A reevaluation of integrins as regulators of angiogenesis. *Nat Med*, **8,** 918-21.

JAMES, A. & CARROLL, N. (2000). Airway smooth muscle in health and disease; methods of measurement and relation to function. *Eur Respir J*, **15,** 782-9.

JEFFERY, P. (2001). Inflammation and remodeling in the adult and child with asthma. *Pediatr Pulmonol Suppl*, **21,** 3-16.

JEFFERY, P.K. (1998). Structural and inflammatory changes in COPD: a comparison with asthma. *Thorax*, **53,** 129-36.

JOHNSON, P.R., ROTH, M., TAMM, M., HUGHES, M., GE, Q., KING, G., BURGESS, J.K. & BLACK, J.L. (2001). Airway smooth muscle cell proliferation is increased in asthma. *Am J Respir Crit Care Med*, **164,** 474-7.

JOHNSON, P.R.A., BURGESS, J.K., UNDERWOOD, P.A., AU, W., PONIRIS, M.H., TAMM, M., GE, Q., ROTH, M. & BLACK, J.L. (2004). Extracellular matrix proteins modulate asthmatic airway smooth muscle cell proliferation via an autocrine mechanism. *J Allergy Clin Immunol***,** 690-96.

KALLURI, R. (2003). Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer*, **3,** 422-33.

KAMPHAUS, G.D., COLORADO, P.C., PANKA, D.J., HOPFER, H., RAMCHANDRAN, R., TORRE, A., MAESHIMA, Y., MIER, J.W., SUKHATME, V.P. & KALLURI, R. (2000). Canstatin, a novel matrix-derived inhibitor of angiogenesis and tumor growth. *J Biol Chem*, **275,** 1209-15.

KANAZAWA, H., NOMURA, S. & YOSHIKAWA, J. (2004). Role of microvascular permeability on physiologic differences in asthma and eosinophilic bronchitis. *Am J Respir Crit Care Med*, **169,** 1125-30.

KAPTZAN, T., SKUTELSKY, E., ITZHAKI, O., SINAI, J., HUSZAR, M., SIEGAL, A., BEN-ZVI, R., JOSSIPHOV, J., MICHOWITZ, M., SCHIBY, G. & LEIBOVICI, J. (2006). Efficacy of anti-angiogenic treatment of tumors in old versus young mice. *Mech Ageing Dev*, **127,** 398-409.

KAZI, A.S., LOTFI, S., GONCHAROVA, E.A., TLIBA, O., AMRANI, Y., KRYMSKAYA, V.P. & LAZAAR, A.L. (2004). Vascular endothelial growth factor-induced secretion

of fibronectin is ERK dependent. *Am J Physiol Lung Cell Mol Physiol*, **286,** L539-45.

KHOSHNOODI, J., SIGMUNDSSON, K., CARTAILLER, J.P., BONDAR, O., SUNDARAMOORTHY, M. & HUDSON, B.G. (2006). Mechanism of chain selection in the assembly of collagen IV: a prominent role for the alpha2 chain. *J Biol Chem*, **281,** 6058-69.

KIM, Y.M., HWANG, S., KIM, Y.M., PYUN, B.J., KIM, T.Y., LEE, S.T., GHO, Y.S. & KWON, Y.G. (2002). Endostatin blocks vascular endothelial growth factor-mediated signaling via direct interaction with KDR/Flk-1. *J Biol Chem*, **277,** 27872-9.

KNOX, A.J., CORBETT, L., STOCKS, J., HOLLAND, E., ZHU, Y.M. & PANG, L. (2001). Human airway smooth muscle cells secrete vascular endothelial growth factor: up-regulation by bradykinin via a protein kinase C and prostanoid-dependent mechanism. *Faseb J*, **15,** 2480-8.

KNOX, A.J., STOCKS, J. & SUTCLIFFE, A. (2005). Angiogenesis and vascular endothelial growth factor in COPD. *Thorax*, **60,** 88-9.

KO, F.W., DIBA, C., ROTH, M., MCKAY, K., JOHNSON, P.R., SALOME, C. & KING, G.G. (2005). A comparison of airway and serum matrix metalloproteinase-9 activity among normal subjects, asthmatic patients, and patients with asthmatic mucus hypersecretion. *Chest*, **127,** 1919-27.

KOKUBO, T., UCHIDA, H. & CHOI, E.T. (2007). Integrin alpha(v)beta(3) as a target in the prevention of neointimal hyperplasia. *J Vasc Surg*, **45 Suppl A,** A33-8.

KUWANO, K., BOSKEN, C.H., PARE, P.D., BAI, T.R., WIGGS, B.R. & HOGG, J.C. (1993). Small airways dimensions in asthma and in chronic obstructive pulmonary disease. *Am Rev Respir Dis*, **148,** 1220-5.

LAITINEN, A., ALTRAJA, A., KAMPE, M., LINDEN, M., VIRTANEN, I. & LAITINEN, L.A. (1997). Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med*, **156,** 951-8.

LAITINEN, L.A., LAITINEN, A., ALTRAJA, A., VIRTANEN, I., KAMPE, M., SIMONSSON, B.G., KARLSSON, S.E., HAKANSSON, L., VENGE, P. & SILLASTU, H. (1996). Bronchial biopsy findings in intermittent or "early" asthma. *J Allergy Clin Immunol*, **98,** S3-6; discussion S33-40.

LEE, C.G., LINK, H., BALUK, P., HOMER, R.J., CHAPOVAL, S., BHANDARI, V., KANG, M.J., COHN, L., KIM, Y.K., MCDONALD, D.M. & ELIAS, J.A. (2004). Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med*, **10,** 1095-103.

LEE, C.G., YOON, H.J., ZHU, Z., LINK, H., WANG, Z., GWALTNEY, J.M., LANDRY, M. & ELIAS, J.A. (2000). Respiratory syncytial virus stimulation of vascular endothelial cell growth Factor/Vascular permeability factor. *Am J Respir Cell Mol Biol*, **23,** 662-9.

LEE, J., KIM, H.R., MIN, J.W., PARK, J.S., JIN, S.M., HAN, S.K., SHIM, Y.S. & YIM, J.J. (2007). Lack of Association between Matrix Metalloproteinase 8 Promoter Polymorphism and Bronchiectasis in Koreans. *J Korean Med Sci*, **22,** 667-71.

LEE, S.Y., CHO, J.Y., MILLER, M., MCELWAIN, K., MCELWAIN, S., SRIRAMARAO, P., RAZ, E. & BROIDE, D.H. (2006). Immunostimulatory DNA inhibits allergen-induced peribronchial angiogenesis in mice. *J Allergy Clin Immunol*, **117,** 597-603.

LEE, Y.C. & LEE, H.K. (2001). Vascular endothelial growth factor in patients with acute asthma. *J Allergy Clin Immunol*, **107,** 1106.

LEMJABBAR, H., GOSSET, P., LAMBLIN, C., TILLIE, I., HARTMANN, D., WALLAERT, B., TONNEL, A.B. & LAFUMA, C. (1999). Contribution of 92 kDa gelatinase/type IV collagenase in bronchial inflammation during status asthmaticus. *Am J Respir Crit Care Med*, **159,** 1298-307.

LI, X. & WILSON, J.W. (1997). Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med*, **156,** 229-33.

LOCKE, N.R., ROYCE, S.G., WAINEWRIGHT, J.S., SAMUEL, C.S. & TANG, M.L. (2007). Comparison of airway remodeling in acute, subacute, and chronic models of allergic airways disease. *Am J Respir Cell Mol Biol*, **36,** 625-32.

LOPEZ, A.D., SHIBUYA, K., RAO, C., MATHERS, C.D., HANSELL, A.L., HELD, L.S., SCHMID, V. & BUIST, S. (2006). Chronic obstructive pulmonary disease: current burden and future projections. *Eur Respir J*, **27,** 397-412.

MADRI, J.A. (1997). Extracellular matrix modulation of vascular cell behaviour. *Transpl Immunol*, **5,** 179-83.

MAESHIMA, Y., COLORADO, P.C., TORRE, A., HOLTHAUS, K.A., GRUNKEMEYER, J.A., ERICKSEN, M.B., HOPFER, H., XIAO, Y., STILLMAN, I.E. & KALLURI, R. (2000). Distinct antitumor properties of a type IV collagen domain derived from basement membrane. *J Biol Chem*, **275,** 21340-8.

MAESHIMA, Y., MANFREDI, M., REIMER, C., HOLTHAUS, K.A., HOPFER, H., CHANDAMURI, B.R., KHARBANDA, S. & KALLURI, R. (2001a). Identification of the anti-angiogenic site within vascular basement membrane-derived tumstatin. *J Biol Chem*, **276,** 15240-8.

MAESHIMA, Y., SUDHAKAR, A., LIVELY, J.C., UEKI, K., KHARBANDA, S., KAHN, C.R., SONENBERG, N., HYNES, R.O. & KALLURI, R. (2002). Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. *Science*, **295,** 140-3.

MAESHIMA, Y., YERRAMALLA, U.L., DHANABAL, M., HOLTHAUS, K.A., BARBASHOV, S., KHARBANDA, S., REIMER, C., MANFREDI, M., DICKERSON, W.M. & KALLURI, R. (2001b). Extracellular matrix-derived peptide binds to alpha(v)beta(3) integrin and inhibits angiogenesis. *J Biol Chem*, **276,** 31959-68.

MASOLI, M., FABIAN, D., HOLT, S. & BEASLEY, R. (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy*, **59,** 469-78.

MATSUI, K., TAKEDA, K., YU, Z.X., TRAVIS, W.D., MOSS, J. & FERRANS, V.J. (2000). Role for activation of matrix metalloproteinases in the pathogenesis of pulmonary lymphangioleiomyomatosis. *Arch Pathol Lab Med*, **124,** 267-75.

MCCOLLEY, S.A., STELLMACH, V., BOAS, S.R., JAIN, M. & CRAWFORD, S.E. (2000). Serum vascular endothelial growth factor is elevated in cystic fibrosis and decreases with treatment of acute pulmonary exacerbation. *Am J Respir Crit Care Med*, **161,** 1877-80.

MCDONALD, D.M. (2001). Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med*, **164,** S39-45.

MERRILEES, M.J., HANKIN, E.J., BLACK, J.L. & BEAUMONT, B. (2004). Matrix proteoglycans and remodelling of interstitial lung tissue in lymphangioleiomyomatosis. *J Pathol*, **203,** 653-60.

MORAES, T.J., PLUMB, J., MARTIN, R., VACHON, E., CHEREPANOV, V., KOH, A., LOEVE, C., JONGSTRA-BILEN, J., ZURAWSKA, J.H., KUS, J.V., BURROWS, L.L., GRINSTEIN, S. & DOWNEY, G.P. (2006). Abnormalities in the pulmonary innate immune system in cystic fibrosis. *Am J Respir Cell Mol Biol*, **34,** 364-74.

NETZER, K.O., SUZUKI, K., ITOH, Y., HUDSON, B.G. & KHALIFAH, R.G. (1998). Comparative analysis of the noncollagenous NC1 domain of type IV collagen:

identification of structural features important for assembly, function, and pathogenesis. *Protein Sci*, **7,** 1340-51.

NIIMI, A., MATSUMOTO, H., AMITANI, R., NAKANO, Y., MISHIMA, M., MINAKUCHI, M., NISHIMURA, K., ITOH, H. & IZUMI, T. (2000). Airway wall thickness in asthma assessed by computed tomography. Relation to clinical indices. *Am J Respir Crit Care Med*, **162,** 1518-23.

NISATO, R.E., HOSSEINI, G., SIRRENBERG, C., BUTLER, G.S., CRABBE, T., DOCHERTY, A.J., WIESNER, M., MURPHY, G., OVERALL, C.M., GOODMAN, S.L. & PEPPER, M.S. (2005). Dissecting the role of matrix metalloproteinases (MMP) and integrin alpha(v)beta3 in angiogenesis in vitro: absence of hemopexin C domain bioactivity, but membrane-Type 1-MMP and alpha(v)beta3 are critical. *Cancer Res*, **65,** 9377-87.

NYBERG, P., XIE, L. & KALLURI, R. (2005). Endogenous inhibitors of angiogenesis. *Cancer Res*, **65,** 3967-79.

O'REILLY, M.S., BOEHM, T., SHING, Y., FUKAI, N., VASIOS, G., LANE, W.S., FLYNN, E., BIRKHEAD, J.R., OLSEN, B.R. & FOLKMAN, J. (1997). Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, **88,** 277-85.

ORTEGA, N. & WERB, Z. (2002). New functional roles for non-collagenous domains of basement membrane collagens. *J Cell Sci*, **115,** 4201-14.

PANETTIERI, R.A., JR. (2003). Airway smooth muscle: immunomodulatory cells that modulate airway remodeling? *Respir Physiol Neurobiol*, **137,** 277-93.

PAUWELS, R.A., BUIST, A.S., CALVERLEY, P.M., JENKINS, C.R. & HURD, S.S. (2001). Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med*, **163,** 1256-76.

PAVORD, I.D., COX, G., THOMSON, N.C., RUBIN, A.S., CORRIS, P.A., NIVEN, R.M., CHUNG, K.F. & LAVIOLETTE, M. (2007). Safety and efficacy of bronchial thermoplasty in symptomatic, severe asthma. *Am J Respir Crit Care Med*, **176,** 1185-91.

PENG, Q., LAI, D., NGUYEN, T.T., CHAN, V., MATSUDA, T. & HIRST, S.J. (2005). Multiple beta 1 integrins mediate enhancement of human airway smooth muscle cytokine secretion by fibronectin and type I collagen. *J Immunol*, **174,** 2258-64.

PUXEDDU, I., RIBATTI, D., CRIVELLATO, E. & LEVI-SCHAFFER, F. (2005). Mast cells and eosinophils: a novel link between inflammation and angiogenesis in allergic diseases. *J Allergy Clin Immunol*, **116,** 531-6.

RAYA, A., REVERT-ROS, F., MARTINEZ-MARTINEZ, P., NAVARRO, S., ROSELLO, E., VIEITES, B., GRANERO, F., FORTEZA, J. & SAUS, J. (2000). Goodpasture antigen-binding protein, the kinase that phosphorylates the goodpasture antigen, is an alternatively spliced variant implicated in autoimmune pathogenesis. *J Biol Chem*, **275,** 40392-9.

REVERT, F., MERINO, R., MONTEAGUDO, C., MACIAS, J., PEYDRO, A., ALCACER, J., MUNIESA, P., MARQUINA, R., BLANCO, M., IGLESIAS, M., REVERT-ROS, F., MERINO, J. & SAUS, J. (2007). Increased Goodpasture antigen-binding protein expression induces type IV collagen disorganization and deposit of immunoglobulin A in glomerular basement membrane. *Am J Pathol*, **171,** 1419-30.

RIES, A., ENGEL, J., LUSTIG, A. & KUHN, K. (1995). The function of the NC1 domains in type IV collagen. *J Biol Chem*, **270,** 23790-4.

ROCHE, W.R., BEASLEY, R., WILLIAMS, J.H. & HOLGATE, S.T. (1989). Subepithelial fibrosis in the bronchi of asthmatics. *Lancet*, **1,** 520-4.

RUNDHAUG, J.E. (2005). Matrix metalloproteinases and angiogenesis. *J Cell Mol Med*, **9,** 267-85.

SAADEH, C. & SAADEH, C. (2007). Asthma remission in a patient with rheumatoid arthritis while on antiangiogenesis therapy during a rheumatoid arthritis trial demonstrated by forced oscillation and spirometry. *J Asthma*, **44,** 281-3.

SASAKI, S., ZHOU, B., FAN, W.W., KIM, Y., BARKER, D.F., DENISON, J.C., ATKIN, C.L., GREGORY, M.C., ZHOU, J., SEGAL, Y., SADO, Y., NINOMIYA, Y., MICHAEL, A.F. & KASHTAN, C.E. (1998). Expression of mRNA for type IV collagen alpha1, alpha5 and alpha6 chains by cultured dermal fibroblasts from patients with X-linked Alport syndrome. *Matrix Biol*, **17,** 279-91.

SCHIAVINA, M., DI SCIOSCIO, V., CONTINI, P., CAVAZZA, A., FABIANI, A., BARBERIS, M., BINI, A., ALTIMARI, A., COOKE, R.M., GRIGIONI, W.F. & D'ERRICO-GRIGIONI, A. (2007). Pulmonary lymphangioleiomyomatosis in a karyotypically normal man without tuberous sclerosis complex. *Am J Respir Crit Care Med*, **176,** 96-8.

SCHMIDT, A., WENZEL, D., THOREY, I., SASAKI, T., HESCHELER, J., TIMPL, R., ADDICKS, K., WERNER, S., FLEISCHMANN, B.K. & BLOCH, W. (2006). Endostatin influences endothelial morphology via the activated ERK1/2-kinase endothelial morphology and signal transduction. *Microvasc Res*, **71,** 152-62.

SHI, W., BELLUSCI, S. & WARBURTON, D. (2007). Lung development and adult lung diseases. *Chest*, **132,** 651-6.

SIDDIQUI, S., SUTCLIFFE, A., SHIKOTRA, A., WOODMAN, L., DOE, C., MCKENNA, S., WARDLAW, A., BRADDING, P., PAVORD, I. & BRIGHTLING, C. (2007). Vascular remodeling is a feature of asthma and nonasthmatic eosinophilic bronchitis. *J Allergy Clin Immunol*.

SODER, S. & POSCHL, E. (2004). The NC1 domain of human collagen IV is necessary to initiate triple helix formation. *Biochem Biophys Res Commun*, **325,** 276-80.

SUND, M., HAMANO, Y., SUGIMOTO, H., SUDHAKAR, A., SOUBASAKOS, M., YERRAMALLA, U., BENJAMIN, L.E., LAWLER, J., KIERAN, M., SHAH, A. & KALLURI, R. (2005). Function of endogenous inhibitors of angiogenesis as endothelium-specific tumor suppressors. *Proc Natl Acad Sci U S A*, **102,** 2934-9.

SUND, M., XIE, L. & KALLURI, R. (2004). The contribution of vascular basement membranes and extracellular matrix to the mechanics of tumor angiogenesis. *Apmis*, **112,** 450-62.

SUZAKI, Y., HAMADA, K., SHO, M., ITO, T., MIYAMOTO, K., AKASHI, S., KASHIZUKA, H., IKEDA, N., NAKAJIMA, Y., IWASE, M., HOMMA, I., KOBZIK, L. & KIMURA, H. (2005). A potent antiangiogenic factor, endostatin prevents the development of asthma in a murine model. *J Allergy Clin Immunol*, **116,** 1220-7.

TIAN, S., HAYES, A.J., METHENY-BARLOW, L.J. & LI, L.Y. (2002). Stabilization of breast cancer xenograft tumour neovasculature by angiopoietin-1. *Br J Cancer*, **86,** 645-51.

TIMPL, R., WIEDEMANN, H., VAN DELDEN, V., FURTHMAYR, H. & KUHN, K. (1981). A network model for the organization of type IV collagen molecules in basement membranes. *Eur J Biochem*, **120,** 203-11.

TRIAN, T., BENARD, G., BEGUERET, H., ROSSIGNOL, R., GIRODET, P.O., GHOSH, D., OUSOVA, O., VERNEJOUX, J.M., MARTHAN, R., TUNON-DE-LARA, J.M. & BERGER, P. (2007). Bronchial smooth muscle remodeling involves calcium-

dependent enhanced mitochondrial biogenesis in asthma. *J Exp Med*, **204,** 3173-81.

URBICH, C., REISSNER, A., CHAVAKIS, E., DERNBACH, E., HAENDELER, J., FLEMING, I., ZEIHER, A.M., KASZKIN, M. & DIMMELER, S. (2002). Dephosphorylation of endothelial nitric oxide synthase contributes to the anti-angiogenic effects of endostatin. *Faseb J*, **16,** 706-8.

WARE, L.B. (2007). Clinical Year in Review III: Asthma, Lung Transplantation, Cystic Fibrosis, Acute Respiratory Distress Syndrome. *Proc Am Thorac Soc*, **4,** 489-93.

WEI, G., ZHIHONG, L., HUIPING, C., CAIHONG, Z., ZHAOHONG, C. & LEISHI, L. (2006). Spectrum of clinical features and type IV collagen alpha-chain distribution in Chinese patients with Alport syndrome. *Nephrol Dial Transplant*, **21,** 3146-54.

WEINBERGER, M. (2003). Clinical patterns and natural history of asthma. *J Pediatr*, **142,** S15-9; discussion S19-20.

WILSON, J. (2003). Angiogenesis and increased airway vascularity in airway diseases. *Arch Physiol Biochem*, **111,** 361-3.

WILSON, J.W. & HII, S. (2006). The importance of the airway microvasculature in asthma. *Curr Opin Allergy Clin Immunol*, **6,** 51-5.

WILSON, J.W. & ROBERTSON, C.F. (2002). Angiogenesis in paediatric airway disease. *Paediatr Respir Rev*, **3,** 219-29.

WILSON, J.W. & STEWART, A.G. (1999). Airway vascularity in asthma. *Clin Exp Allergy*, **29,** 1295-7.

YACH, D., HAWKES, C., GOULD, C.L. & HOFMAN, K.J. (2004). The global burden of chronic diseases: overcoming impediments to prevention and control. *Jama*, **291,** 2616-22.

YAMAMOTO, Y., MAESHIMA, Y., KITAYAMA, H., KITAMURA, S., TAKAZAWA, Y., SUGIYAMA, H., YAMASAKI, Y. & MAKINO, H. (2004). Tumstatin peptide, an inhibitor of angiogenesis, prevents glomerular hypertrophy in the early stage of diabetic nephropathy. *Diabetes*, **53,** 1831-40.

YAMAUCHI, K. (2006). Airway remodeling in asthma and its influence on clinical pathophysiology. *Tohoku J Exp Med*, **209,** 75-87.

YANCOPOULOS, G.D., DAVIS, S., GALE, N.W., RUDGE, J.S., WIEGAND, S.J. & HOLASH, J. (2000). Vascular-specific growth factors and blood vessel formation. *Nature*, **407,** 242-8.

ZEISBERG, M., BONNER, G., MAESHIMA, Y., COLORADO, P., MULLER, G.A., STRUTZ, F. & KALLURI, R. (2001). Renal fibrosis: collagen composition and assembly regulates epithelial-mesenchymal transdifferentiation. *Am J Pathol*, **159,** 1313-21.

ZHENG, L., LAM, W.K., TIPOE, G.L., SHUM, I.H., YAN, C., LEUNG, R., SUN, J., OOI, G.C. & TSANG, K.W. (2002). Overexpression of matrix metalloproteinase-8 and -9 in bronchiectatic airways in vivo. *Eur Respir J*, **20,** 170-6.

ZHOU, J., DING, M., ZHAO, Z. & REEDERS, S.T. (1994). Complete primary structure of the sixth chain of human basement membrane collagen, alpha 6(IV). Isolation of the cDNAs for alpha 6(IV) and comparison with five other type IV collagen chains. *J Biol Chem*, **269,** 13193-9.