An Investigation of Pulmonary Vascular Remodelling in Severe Chronic Obstructive Pulmonary Disease and its Potential Relationship to Outcome Following Lung Volume Reduction Surgery

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Abbreviations

BMI = Body Mass Index

BPD = Broncho-Pulmonary Dysplasia

BV = Broncho-Vascular pair

COPD = Chronic Obstructive Pulmonary Disease

CT = Computed Tomography

DAB = DiAminoBenzidine Tetrahydrochloride

ECM = Extra-Cellular Matrix

EEL = External Elastic Lamina

EVG = Elastic van Gieson stain

FEV_1 = Forced Expiratory Volume in 1 second

FVC = Forced Vital Capacity

H&E = Haematoxylin and Eosin

HDU = High Dependency Unit

HIF-1α = Hypoxia-Inducible Factor 1α

HSI = Hue, Saturation and Intensity

IEL = Internal Elastic Lamina

IHC = immunohistochemistry

IMS = Industrial Methylated Spirits 70%

LVRS = Lung Volume Reduction Surgery

MSB = Martius Scarlet Blue

NO = Nitric Oxide

NOS = Nitric Oxide Synthase

PA = Pulmonary Artery

PAH = Pulmonary Arterial Hypertension

pCO_2 = Partial pressure of Carbon Dioxide

pO_2 = Partial pressure of Oxygen

Ppa = Pulmonary artery pressure

RV = Residual Volume

SD = Standard Deviation

SMC = Smooth Muscle Cell

TLC = Total Lung Capacity

VATS = Video Assisted Thoracoscopic Surgery

VEGF = Vascular Endothelial Growth Factor

V:Q = Ventilation : Perfusion ratio

WHO = World Health Organisation

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Contents

Chapter

Introduction

I. Background

I.i Definition and Epidemiology:

Chronic Obstructive Pulmonary Disease (COPD) is an umbrella term encompassing a range of conditions affecting airways of all sizes (*eg*. emphysema, chronic bronchitis, bronchiectasis and asthma) (Fabbri L 2003). It is widely accepted that the commonest risk factor for the development of COPD is tobacco smoking, with other risks including air pollution, occupational dusts, chemicals and genetic predisposition such as alpha-1 antitrypsin deficiency. This costly and preventable disease is also extremely common especially in those aged over 65 years. In terms of worldwide mortality, COPD was ranked sixth in 1990 (Murray CJ 1997), fifth by the WHO in 2002 (WHO 2008), and is projected to rise to the third commonest cause of death by 2020 (Murray CJ 1997). This translates into approximately 600,000 people in the UK being affected by the disease; the prevalence being up to 10% in patients aged over 75 years (Calverley P 2000). Although predominantly affecting men (2% v 1% women), the prevalence rates in women are increasing in the UK (Soriano JB 2000). In the United States, mortality in women as a result of COPD has doubled over the past twenty years, such that it now matches that seen in men (Mannino DM 2002). In both epidemiological and financial terms, COPD is a disorder responsible for a major burden in healthcare.

I.ii Diagnosis:

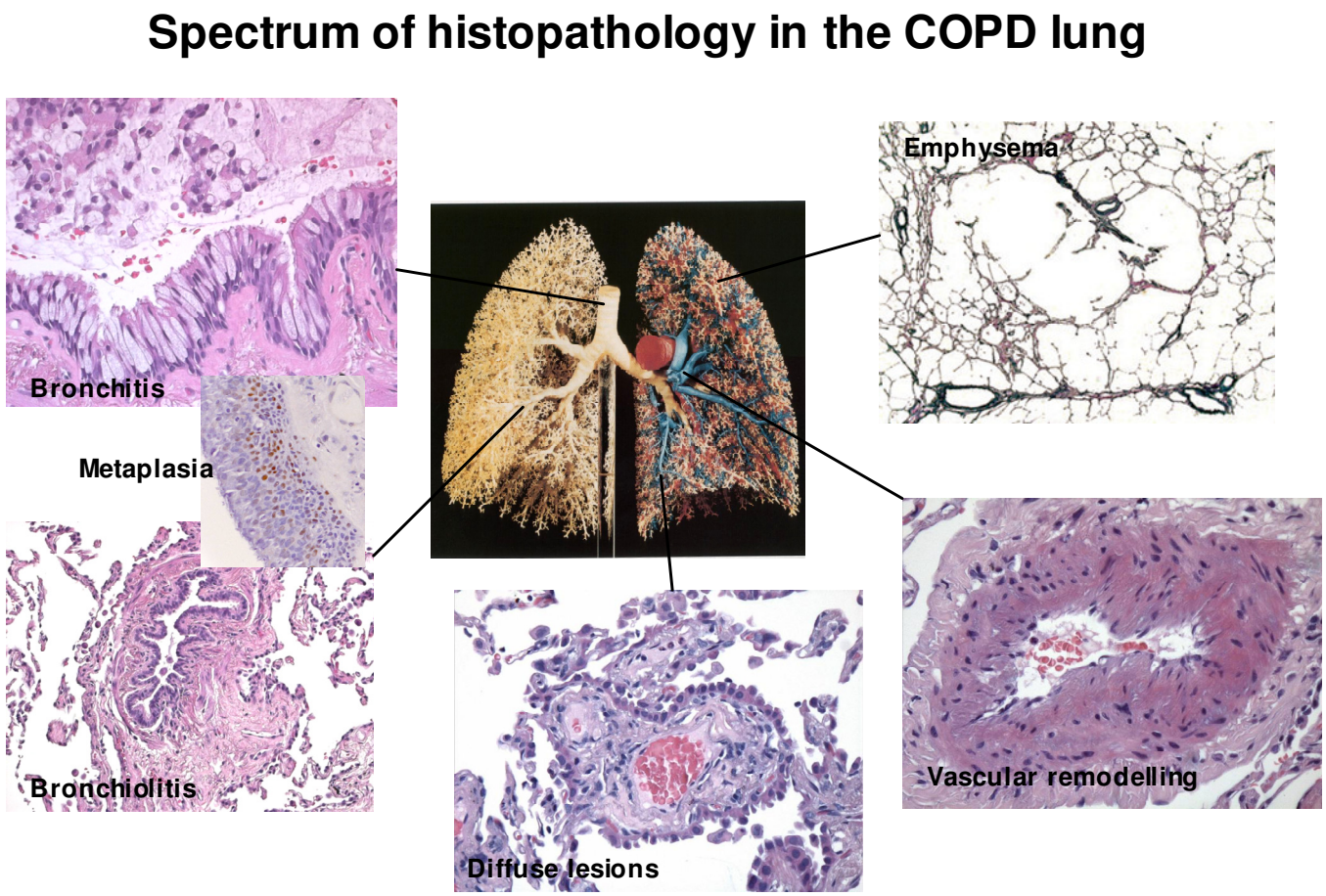
Despite such an impact upon global health, the pathology of severe COPD and its relationship with outcome has not been systematically studied in living patients. The small airways in COPD have been well characterised (Hogg JC 1993 and 2004), while research into vascular remodelling has focussed upon patients with milder disease or those with COPD associated pulmonary hypertension. There has never been a systematic study of vascular remodelling in patients with severe COPD, in whom pulmonary hypertension has been excluded. Research has been further hampered by different interpretations of the diagnostic criteria (Shirtliffe P 2007), imprecise clinical definitions based upon spirometry and respiratory function, while the pathological features are not used as diagnostic tools to investigate outcome. This has led to COPD being consistently underdiagnosed in many countries worldwide (Mannino DM 2007). The water is further muddied by the spectrum of recognised patient phenotypes, including the emphysematous "blue bloaters" and chronic inflammatory or bronchitic "pink puffers" (Filley GF 1967) are all labelled as COPD because of this "umbrella diagnosis". There is still a poor understanding of the pathogenesis of COPD. It is well known that the vast majority of COPD patients are smokers, but unclear why only a quarter of smokers develop COPD (Strachan DP 1995). It has been suggested that genetic factors may predispose individuals or determine susceptibility to developing the disease (Turato G 2001).

I.iii. Pathology:

The definition of COPD is the progressive development of irreversible airflow limitation, and evidence of an abnormal inflammatory response associated with noxious gases or particles (Fabbri L 2003). Tobacco smoke is known to contain more than 4000 chemicals (Ryter SW 2010). Exposure to such reactive compounds, leads to an exaggerated inflammatory response within the airways, involving neutrophils, macrophages, and lymphocytes. This response can be further amplified by recurrent infections, hypoxia (regional or global), oxidative stress and increased production of endogenous reactive oxygen species within the inflammatory cells. The production of inflammatory mediators in association with oxidative stress can result in an imbalance between proteases (i.e. elastases, matrix metalloproteinases) and antiproteases which is pivotal to the development of emphysema (Larsson K 2007). This is best demonstrated in α-1 antitrypsin deficiency, where emphysematous changes develop at a much younger age, due to the unopposed action of protease enzymes. COPD is manifested as a multitude of pathologies within the lung, (see Figure i) as each cell line reacts differently to the repeated insults of smoke inhalation. The characteristic feature of emphysema is destruction of the lung parenchyma and alveolar walls, whilst bronchitis and bronchiolitis are also well recognised components. The fine anatomy of these lesions, their development, and their relationship to clinical outcome has, to date, not been fully explained. To simply describe emphysema as abnormal dilation of air spaces due to alveolar wall destruction (Kumar V 1997) is a gross oversimplification, as alveolar wall destruction is only one facet of this complex and multifactorial condition.

Evidence for the involvement of the pulmonary vasculature in emphysema has been documented for many years. As early as 1866, Villemin observed that the vascularity of the alveoli was reduced in pulmonary emphysema, this being confirmed many years later with angiography (Cordasco EM 1968). More recently, Hale KA (1984) noted that in an unselected population study, smokers with COPD had a thicker tunica media, more intimal fibrosis, and twice as many fully muscularised arterioles <300μm diameter than non-smokers or smokers without COPD. They also demonstrated a significant correlation between vascular remodelling and pathological severity of airway disease and degree of emphysema, suggesting that vascular remodelling may be an initial or initiating event in the development of emphysema, rather than as a result of the airspace destruction, as is so often assumed.

Figure *i*



[Personal communication – Martyn Foster]

Pulmonary Vascular Remodelling

II.i. Definition:

Pulmonary vascular remodelling is defined as structural alterations within the wall of the pulmonary arterioles in an attempt to retain structure and function under abnormal physiological conditions (Hughes JMB 2001). These alterations are known to occur in response to a wide variety of stimuli, including hypoxia, shear stress, chronic vasoconstriction, hypertension, local or systemic inflammatory infiltrates, and potentially the repair phenotype of haematopoietic stem cells (Hughes JMB 2001). Rather than restoring normal physiology, however, it is unclear if this remodelling may further contribute to or exacerbate the underlying pathology.

Before describing the remodelling seen in COPD I would first like to consider the embryology, development and anatomy of the pulmonary arterial system.

Pulmonary Arteriolar Development and Anatomy

III.i. Development:

Prenatal human lung development is divided into four stages – embryonic, pseudoglandular, canalicular and saccular. During the embryonic phase (up to seven weeks gestation), the lung bud appears as a ventral diverticulum from the foregut and divides to form primitive main bronchi. This repeated division continues so that by the end of the pseudoglandular phase (17^{th} week of gestation) all the pre-acinar vessels are present (Hislop AA 1977). The main pulmonary arteries develop alongside the corresponding airways at a similar time by vasculogenesis *i.e. de novo* endothelial tubules coalescing from within the mesenchyme at a fixed distance from the tip of the

airway, which thus acts as a template (Hislop AA 2002). In the canalicular phase (17-27 weeks gestation), further arteriolar branching and capillary development is accomplished by angiogenesis and is accompanied by the development of the respiratory bronchioles and alveoli (Hislop AA 2000). Further alveolarisation then continues throughout the saccular phase (Hislop AA 2000).

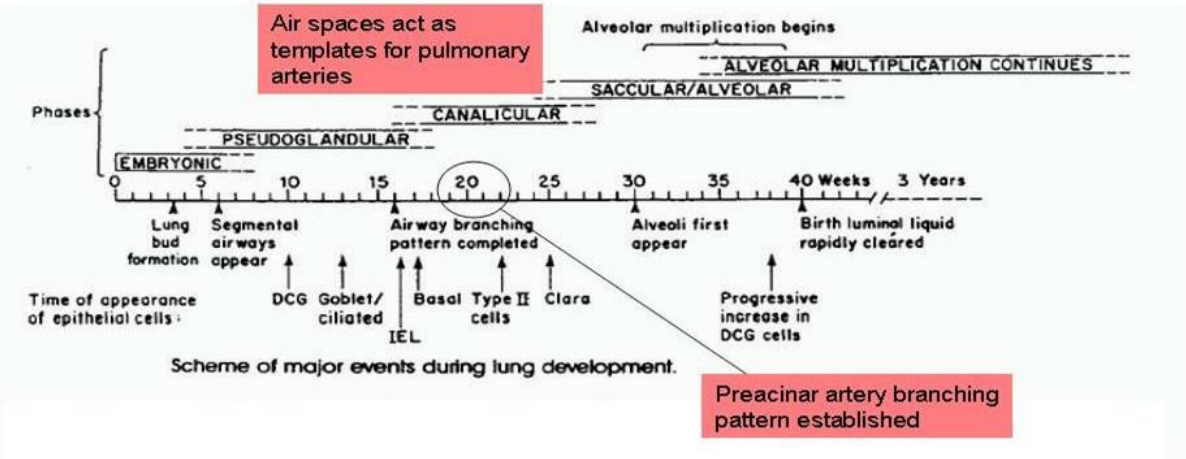


Figure *ii*. Graph of foetal lung development [modified from Jeffrey PK 1998]

The process of vasculogenesis and angiogenesis is highly regulated, principally by VEGF and fibroblast growth factors (Healy 2000), but also from within the mesenchyme by angiopoietin (Gale 1999) and hypoxia-inducible factor 1 α (HIF-1α) (Ema M 2008). Immediately after it has formed, the pulmonary artery becomes enveloped by smooth muscle cells derived from the adjacent bronchus and differentiation of endothelial cells (Hall 2000). This forms the innermost layer of the media. Fibroblasts are thought be recruited from the mesenchyme to lay down elastic laminae and collagen (Hall SM 2000), as well as line up along the outer wall of the artery and differentiate into a smooth muscle phenotype (Folkman 1996). This could

explain the heterogeneity of smooth muscle cell phenotypes within the media (Frid MG 1997a), and their markedly different rates of proliferation and matrix protein synthesis.

III.ii. Anatomy:

In the adult, a branch of the pulmonary artery accompanies the corresponding bronchus into each of the bronchopulmonary segments (10 in right lung, 9 in left lung). Approximately 16 further intrapulmonary divisions of both airway and vessel occur until finally a terminal bronchiole and arteriole supply a single acinus/lobule (Bloom & Fawcett 1986). There are approximately 65,000 acini/lobules within the lungs, which are roughly pyramidal, the base being orientated towards the visceral pleural surface (Leeson CR 1976). The lobules are incompletely separated by connective tissue, with branches of the pulmonary vein running within the septae.

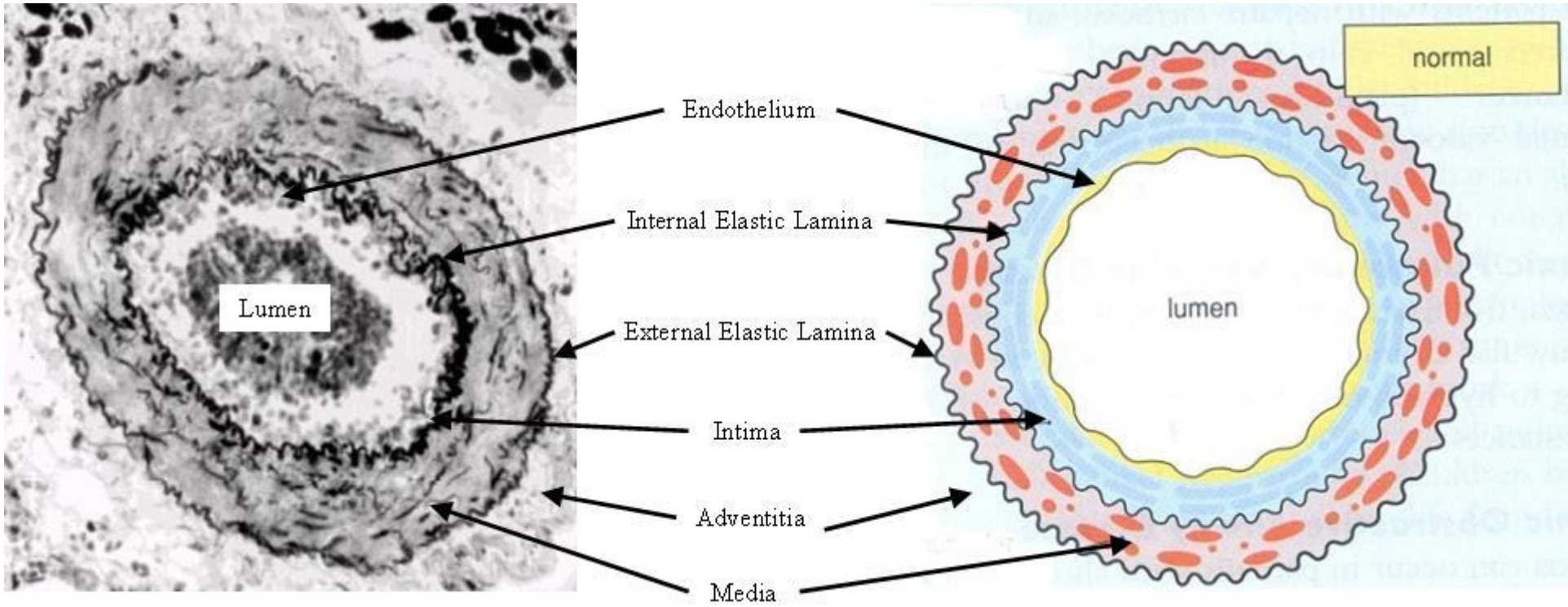
The muscular arterioles in the lung are relatively thin-walled compared to elastic pulmonary and systemic vessels however the basic structure remains the same, in that they are composed of three concentric layers – the *tunica intima* consisting of a continuous endothelium, a basal lamina, and a very thin subendothelial layer of elastic fibres - the internal elastic lamina. The underlying *tunica media* is composed mainly of concentrically orientated smooth muscle cells, with occasional fibroblasts lying in an organised matrix of connective tissue. This is separated from the loose connective tissue of the adventitia by the external elastic lamina. See Figure iii.

Elastic pulmonary arteries are classified as ranging in size from 1cm to 1mm in diameter. Muscular pulmonary arterioles range in diameter from 1000μm to 100μm, with the medial thickness diminishing with arteriole size. Muscular medial compartments are thought to terminate at 80μm – 100μm (Meyrick B et al 1980, Wilkinson M et al 1988).

Systemic blood flow to the lungs is *via* bronchial arteries. These are branches from descending thoracic aorta, which supply larger airways and supporting connecting tissue to the level of the terminal bronchiole. At the alveolar level they have become a plexus of smaller vessels (Brenner O 1935, Grays anatomy 2005). They are differentiated from pulmonary arterioles by their thick medial layer and lack of external elastic lamina (Corrin B 2006).

Figure *iii*. (Adapted from Woolf N 1998)

Demonstrating a normal muscular pulmonary arteriole stained with Elastic van Gieson(EVG)



Key Vascular Phenotypes in COPD:

Pulmonary vascular remodelling is a well recognised phenomenon. Pathological features are well described in the literature and include intimal and medial hypertrophy/hyperplasia (Wagenvoort CA 1960, Hicken P 1965, Naeye RL 1974, Meyrick B 1980, Magee F 1988, Heath D 1990, Santos S 2002), intimal sclerosis (Naeye RL 1974, Smith P 1980), and muscularisation of smaller arterioles <80μm diameter (Meyrick B 1980, Wilkinson M 1988, Heath D 1990, Smith P 1992). Although these descriptions of the lesions are correct, they are taken from patients with many different diagnoses, and suffer with poor characterisation of the patient phenotype. Heath D (1981 and 1990) described medial hypertrophy and distal arteriolar muscularisation in Bolivian Indians without COPD but with chronic hypoxaemia due to altitude. Santos S (2002) describes intimal hypertrophy/hyperplasia and sclerosis in patients with mild/moderate emphysema and pulmonary arterial hypertension (PAH). Wilkinson's study (1988) involves patients with end-stage COPD in a trial of nocturnal oxygen, while Smith (1992) describes three patients undergoing heart-lung transplantation, but again there is no documentation of the presence or absence of pulmonary hypertension.

Many of the lesions described above appear to be a generic vascular response by both pulmonary and systemic arterioles to a variety of insults, and are neither specific to nor diagnostic of emphysema. Indeed all of the above insults (hypoxia, pulmonary hypertension, inflammation, cigarette smoke products, and repeated pulmonary infections) are also well described in patients with COPD, but never conclusively demonstrated as the "driving force" behind the vascular pathology.

Hypertrophy is commonly seen in both primary and secondary pulmonary arterial hypertension (PAH), where endothelial shear stress, wall tension and capillary bed pressure, as well as the underlying causative insult could all contribute to the remodelling. Intimal and medial hypertrophy have also been demonstrated in patients with intralobar pulmonary sequestration (a congenital abnormality, where a portion of lung has a systemic instead of pulmonary blood supply), where repeated infection and acute-on- chronic inflammation are the driving forces (Corbett HJ 2004).

Intimal hypertrophy is seen as an early event in the development of atheromatous disease – affecting predominantly systemic vessels (Ross R 1992). Medial hypertrophy has also been described in many conditions including pulmonary fibrosis (Ross R 1992).

Fibrosis of both the intima and media is also well described in patients with COPD, bronchopulmonary dysplasia (BPD), sequestration and PAH. One would expect the fibrosis to be as a result of the resolution of a chronic inflammation/post-inflammatory remodelling however, pulmonary artery fibroblasts have been shown to produce collagen by simple mechanical distortion including stretching (Weiser MC 1995). Wilkinson M (1988) and Wagenvoort CA (1965) have also described intimal fibrosis of the pulmonary arterioles as an age-related phenomenon in patients with no underlying lung disease.

All of the above pathological features are well described in the literature and accepted as characteristics of pulmonary vascular remodelling in COPD. It has been suggested that this remodelling is directly correlated with disease progression (Hale KA 1980, Peinado VI 1999). However there are other issues with this interpretation. The frequency with which such lesions are encountered, and the fact that they are present before the onset of airflow limitation (Santos 2002, Hale 1984) suggests that

remodelling is an integral part of the wider spectrum of COPD development, not as a result of it. Unfortunately the vascular remodelling in severe COPD has never been systematically described in a well-defined patient population, and is frequently interpreted as secondary to pulmonary hypertension.

IV.i. Lesions of Pulmonary Arterial Hypertension:

Pulmonary hypertension (PH), like COPD, is also an umbrella term and includes a variety of diseases with different aetiologies. Unlike COPD, PH has been classified into five discrete categories based upon underlying cause and specific therapeutic interventions. Each category shares similar pathophysiological mechanisms, clinical presentation and therapeutic options (Simonneau G 2004). Within category three (PH associated with disorders of the respiratory system), the predominant cause of the arterial hypertension was ascribed to inadequate oxygenation, with only a modest increase in mean PA pressure (<35mmHg) (Weitzenblum E 1984). Despite the multitude of aetiologies, all forms of pulmonary arterial hypertension (PAH) have a similar spectrum of vascular pathological changes (Pietra GG 2004). These include medial hypertrophy and vasoconstriction of both muscular and elastic arteries (Wagenvoort CA 1960), muscularisation of smaller arterioles (Heath D 1958), and development of intimal smooth muscle cells and fibrosis (Wilkinson M 1988).

Complex lesions are also recognised and are thought to be pathognomic of PAH. These include a plexogenic arteriopathy - a focal proliferation of endothelial channels lined with myofibroblasts, smooth muscle cells, and connective tissue matrix associated with expansion and partial destruction of the arterial wall (Pietra GG 2004). In primary PAH these lesions are due to an autonomous monoclonal endothelial proliferation, with abundant vascular endothelial growth factor (VEGF) receptors. However in secondary

pulmonary hypertension these are due to a polyclonal proliferation (Lee SD 1998). Other pathological lesions found exclusively in PAH include necrotising arteritis which is often associated with other complex lesions such as pulmonary occlusive venopathy and pulmonary microvasculopathy, and dilation lesions which are thin-walled vein-like vessels usually located distal to plexiform lesions (Pietra GG 2004)

These complex lesions have never been described in COPD, suggesting that although there may be some similarities between COPD and PAH, there are differences.

IV.ii. Differential between PAH and COPD Remodelling Lesions:

In patients with COPD, the actual incidence of secondary PAH is unknown, due to lack of systematic screening, definitions of pulmonary hypertension differing between centres and the wide clinical spectrum of COPD (Barbera JA 2003). The best estimates are: up to 40% based upon right ventricular hypertrophy found at autopsy (Pietra GG 1991); up to 55% based upon tricuspid regurgitation measurable by transthoracic echocardiography (Higham MA 2000). However in a study of patients with severe emphysema 109/120 (90%) were found to have mild pulmonary hypertension at rest (mean Ppa 25-35mmHg), but only 5% of patients had mean Ppa >35mmHg (Sharf SM 2002). If these studies are to be interpreted as correct, then somewhere between 10% - 60% of patients with COPD could be mislabelled as having pulmonary hypertension.

The association between pulmonary vascular remodelling, pulmonary hypertension and COPD continues to be made despite several further observations to the contrary. Published data do suggest some pathological similarities between COPD and secondary pulmonary hypertension – namely intimal thickening, intimal fibrosis and neo-muscularisation (Smith P 1980, Barbera JA 2003, Pietra GG 2004). However the correlation between pulmonary vascular remodelling and COPD-associated pulmonary

hypertension is made despite remodelling being demonstrated in smokers with normal lung function and patients with only mild COPD (Hale KA 1984, Peinado VI 1998).

As mentioned earlier, PAH is characterized by complex arterial lesions such as plexiform lesions and necrotic arteritis. These lesions have never been described in COPD, where pulmonary artery pressures tend to be lower than those in PAH (Weitzenblum E 1984).

Current evidence suggests that the remodelling lesions in PAH are driven from the endothelium, as evidenced by reduced nitric oxide (NO) production, altered nitric oxide synthase (NOS) signalling, upregulation of VEGF and its receptors Flk1 &2, and CD31 positive cells occupying the thickened intima. Although there is a body of evidence (Santos S 2002, Barbera JA 1994) that suggests an endothelial role in the intimal proliferation and fibrosis seen in mild/moderate COPD, there currently is no good explanation for the medial remodelling seen in these patients, and certainly remodelling in severe COPD has never been systematically investigated.

Pulmonary hypertension in COPD is classically diagnosed in patients with more severe, and end-stage COPD, and is associated with a poor prognosis in terms of mortality and morbidity (Weitzenblum 1979, Oswald-Mammoser 1995). When measured, the mean Ppa in these patients tends to be 20-30mmHg, which is significantly lower than the pressures measured in primary PAH, or PAH secondary to congenital or valvular heart disease. Severity of remodelling has never been shown to be correlated with severity of PAH although this association suggests COPD patients with PAH do have a more aggressive vascular phenotype. The disparity between severity of COPD and PAH raises the possibility that vascular remodelling classically associated with PAH is actually part of a wider spectrum of lesions that can also occur in patients without evidence of PAH.

Lung volume reduction surgery (LVRS) patients offer a unique insight into remodelling in severe COPD, without clinical evidence of PAH at rest, as this is one of the surgical exclusion criteria. Studies have shown that the mean PA pressure increase with exercise is significantly greater in COPD than in normal subjects. Unfortunately measurement of exercise-induced PAH in these patients is not routinely performed, and as such, sub-clinical or latent PAH may still be present.

IV.iii. Regional Hypoxia v Hypoxaemia:

Pulmonary vascular remodelling has been demonstrated in many other conditions including chronic hypoxaemia. In humans living at high altitude, Heath D (1981 and 1990) described both intimal and medial hypertrophy and muscularisation of smaller arterioles in people dying from non-pulmonary causes. This hypertrophy has been ascribed to chronic vasoconstriction as a result of the low alveolar $P0_2$, and in humans chronic altitude sickness is characterised by intimal and medial hypertrophy, pulmonary hypertension and right ventricular failure (Peñalosa D 1971). Unfortunately pre-mortem secondary pulmonary hypertension was not excluded.

Regional tissue hypoxia within the lung is probably more related to hypoxic pulmonary vasoconstriction than hypoxaemia. The pulmonary vasculature responds to a low alveolar $P0_2$ by vasoconstriction of the muscular arterioles. This evolutionary response at a local level diverts blood away to those with better ventilation, and thus maintains the optimum ventilation/perfusion ratio. In COPD multiple factors are present that can disrupt such equilibrium. These can include air trapping/hyperinflation compressing smaller arterioles and capillaries; fibrosis and alveolar destruction affecting the ventilation of particular acini; whilst remodelling; vasoconstriction; polycythaemia with the related increased viscosity and microthrombi could affect perfusion. Even patients

with early stage COPD have been shown to have significantly abnormal ventilation/perfusion mismatch (Rodríguez-Roisin R 2009).

We therefore have to look further afield for other applicable models of emphysema. Interestingly, there are striking similarities between the vascular pathology of adult-onset emphysema and that seen in some neonatal and paediatric lung diseases in particular broncho-pulmonary dysplasia (BPD) and intralobar sequestration.

V. Broncho-Pulmonary Dysplasia (BPD): An embryological context for emphysema?

Broncho-Pulmonary Dysplasia and emphysema are comparable for several reasons. BPD results from impaired alveolar formation, whereas emphysema is due to progressive destruction of existing alveolar septae. Both also share a number of features in their pathophysiology. Not only do they have some of the same precipitating factors, including oxidative stress, sustained inflammation and protease–antiprotease imbalance, they are also characterised by a failure of lung maintenance (Bourbon JR 2009).

BPD is a chronic lung disease affecting preterm neonates born between 24-26 weeks gestation, and is characterised clinically by acute respiratory failure requiring mechanical ventilation and oxygen therapy, as a result of arrested peripheral lung development (usually within the late canalicular or early saccular phase) due to prematurity. The classical descriptions of "old BPD" vascular lesions are startlingly similar to those seen in severe COPD, consisting of periarteriolar thickening, elastic fibre degeneration (Northway WH Jr 1967, Bonikos DS 1976), medial smooth muscle hypertrophy, and muscularisation of arterioles <80μm in diameter (Tomashefski JF 1984). Parenchymal and airway lesions include squamous metaplasia, extensive

parenchymal fibrosis and alternating areas of hyperinflation and atelectasis (Ambalavanan N 2004). All of these features have been described in adult emphysematous lungs. As ventilatory strategies have improved and with the addition of exogenous surfactant and steroid therapy, "new BPD" has evolved and the airway lesions have almost disappeared. "New BPD" is characterised by decreased, large & simplified alveoli, minimal interstitial fibrosis and less severe arteriolar lesions (endothelial oedema, mild medial thickening and increased elastic tissue) (Coalson JJ 2003).

It is at the pseudoglandular stage of lung development that vascular growth is closely linked to alveolarisation (Jakkula M 2000, Thebaud B 2005), with studies demonstrating the importance of several angiogenic factors including VEGF (Bhatt AJ 2001) and angiopoietin (Gale NW 1999) upon lung development. This has lead to the concept of the "vascular hypothesis" for the development of 'new' BPD (Abman SH 2001). The improvement in clinical care since the original description of BPD appears to have ameliorated the inflammatory and hyperoxic components of BPD, allowing a closer inspection of the underlying responses of the lung to interrupted development and hypoxia– namely abnormal repair and development. Interesting similarities are evident between abnormal lung development and emphysema. For example, in murine models, absence of VEGF reduces capillary numbers and slows alveolarisation (Ng YS 2001), and absence of the VEGF receptor KDR/Flk-1 is not compatible with survival due to absence of blood vessel development (Shalaby F 1995). By inhibiting the KDR/Flk-1 receptor, Kasahara Y (2000) induced apoptosis of the alveolar walls and pruning of the pulmonary vascular bed. In human studies, patients with emphysema have also been shown to have a reduced number of FLk-1 receptors, and levels of VEGF (Kasahara Y 2001). As well as lung and vascular maintenance, other similar features between the

two conditions have been reported. They share similar precipitating factors including oxidative stress, sustained inflammation and protease-antiprotease imbalance and similar effects such as elastin degradation with disordered assembly and apoptosis (Bourbon JR 2009).

V.i. Pulmonary Sequestration

Intralobar sequestration is defined as a mass of lung parenchyma that is contiguous with the adjacent normal lung and enveloped in its visceral pleura, yet has no identifiable communication with the normal bronchial tree and receives its blood supply from one or more anomalous systemic arteries (Corbett HJ 2004). This congenital abnormality is thought to arise between four to eight weeks gestation (embryonic/pseudoglandular phase). Presentation is usually in late childhood due to a recurrent or chronic pneumonia, and in most cases, the overlying pleura is thickened and densely adherent to adjacent mediastinum and diaphragm (Frazier AA 1997). Macroscopically, intralobar sequestrations consists of multiple cysts of varying size, while microscopically, the lung parenchyma is affected by acute or chronic inflammation and fibrosis, with remnants of bronchi and bronchioles surrounded by dense fibrous connective tissue. The vascular changes have only been recorded in a few instances (Tandon M 1993), and include medial and intimal thickening, plexiform lesions, and dilation lesions. However despite the obvious similarity to the lesions seen in PAH, these lesions demonstrate a mesenchymal profile by strong immunoreactivity with antibodies to alpha-smooth muscle actin and vimentin, but only weak or absent reactivity with desmin antibodies. The underlying insults from hypoxia, chronic inflammation, recurrent infections and hypertension due to a systemic arterial supply are similar to those found in COPD as

well as PAH. Interestingly the presence of complex remodelling lesions in both PAH and intralobar pulmonary sequestration implies that shear stress as a result of the elevated intraluminal pressure may be the cause of these lesions rather than the underlying aetiology.

Summary

Vascular remodelling in COPD could therefore be interpreted as multifactorial. The current understanding of pulmonary vascular remodelling in COPD is complicated by the numerous insults to the lung which include regional hypoxia, recurrent infections, acute inflammation, chronic inflammation, pulmonary hypertension, oxidative stress, shear stress and the patients' reparative phenotype. There are pathological features present in PAH and BPD which are present in COPD, but do not explain the full picture. Both COPD and PAH are "umbrella" diagnoses encompassing many pathologies and patient phenotypes. The key is to tease out the relative contribution of each of the "insults" to the severity and the pathological features described in pulmonary vascular remodelling.

The remodelling within the medial compartment especially has neither been fully investigated nor fully explained by these disease models.

Table *i*. Summary of the presence or absence of particular features of pulmonary vascular remodelling.

Feature of Remodelling	PAH	Emphysema	BPD	Sequestration	Hypoxia
Intimal hypertrophy	✓	✓		✓	✓
Intimal fibrosis	✓	✓			
Complex lesions	✓			✓	
Medial hypertrophy	✓	✓	✓	✓	✓
Medial fibrosis	✓	✓	✓		
Muscularisation of arterioles <80μm	✓	✓	✓		✓

Plan of Investigation

In order to further elucidate the role of pulmonary vascular remodelling in severe non-hypertensive emphysema and it's possible role in outcome following LVRS, the investigation will proceed as follows:-

1. **Histological Characterisation of Intimal and Medial Remodelling**. The literature describing the pathological features of remodelling draws upon a heterogenous patient population. There has never been a systematic study describing the range of vascular lesions seen within the non-hypertensive, severe COPD cohort. As such it is therefore important to accurately quantify the lesions seen within our cohort of LVRS patients, using both tinctoral and immunohistochemical staining, and compare with the pathological changes described in pulmonary hypertension. The lesions will initially be characterised using tinctoral stains including Haematoxylin-Eosin (H&E) for general lesion quantification, Miller's Elastic van Gieson (EVG) to assess the elastic tissue within the arteriolar walls, and Martius Scarlet Blue (MSB) and Picrosirius Red for matrix (fibrin and collagen) staining. Immunohistochemistry using antibodies to Desmin and Vimentin (mesenchyme) and CD31 (endothelium) will be used to characterise the cellular phenotype of both the media and intima in this patient group.
2. **A Grading System for Vascular Remodelling**. All grading systems for pulmonary vascular remodelling are hampered by one single fact – they have all been developed as a diagnostic and/or therapeutic aid for clinicians to offer the most appropriate treatment to patients with pulmonary hypertension. They are

inappropriate for our purposes, because they do not accurately describe the remodelling phenotypes seen, and lack the sensitivity required to differentiate between patients in our cohort. The objective is to develop and validate a robust grading system for the vascular remodelling seen in severe COPD lung resections and to elucidate the contribution of individual pathological features to the overall phenotype. We will also attempt to differentiate between patients based upon pathological criteria and the overall severity of remodelling. This will then be correlated with clinical outcome following LVRS.

3. **Morphometric Quantification Using Image Analysis**. Most histopathological grading systems are a subjective analysis of the images seen. Following on from the grading of the vascular remodelling, we intend to objectively quantify the histopathological features seen using morphometric measurement techniques.
4. **Density Thresholds to Investigate Matrix Alterations.** We will also use density threshold techniques to objectively quantify the elastin component of the extracellular matrix.
5. **Investigation of Post-inflammatory Hypoxic Remodelling**. Although hypoxaemia is clinically well documented in patients with severe COPD, to our knowledge little or no work has been done investigating the effects of regional hypoxia in COPD at the cellular level *in-vivo*. Cellular hypoxia will be investigated using immunohistochemistry, with antibodies to Carbonic Anhydrase IX and Von Hippel Lindau protein. Post-inflammatory sclerosis will be examined by immunohistochemistry with antibodies to Collagen I & III. By grading the staining achieved, we hope to be able to correlate hypoxia with sclerosis.

Patients and Laboratory Methods

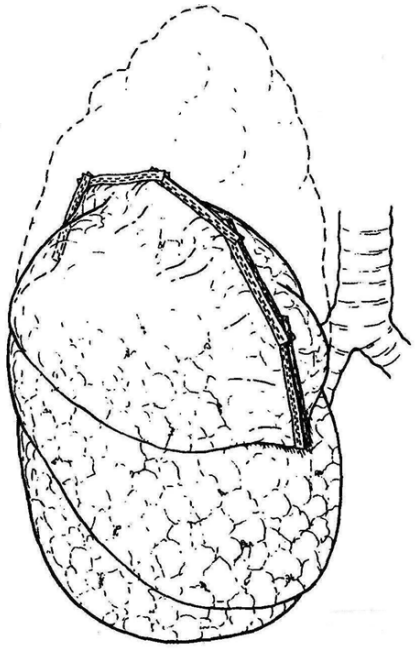
VIII.i. Patients:

Lung Volume Reduction Surgery (LVRS) has been shown to effectively palliate the symptoms associated with severe heterogenous emphysema in a highly selected cohort of patients (Geddes D 2000, Fishman A 2003). Anatomical and functional target areas are identified preoperatively using high resolution Computed Tomography(CT) and radionuclide scintigraphy respectively.

Intraoperatively single lung ventilation is employed by means of a double lumen endotracheal tube. Any adhesions between visceral and parietal pleura are divided with electrocautery. The target areas are identified as the most hyperinflated areas remaining after resorption atelectasis has deflated normally perfused lung. These target areas are then resected using linear automatic stapling devices buttressed with bovine pericardium as shown in Figure iv (adapted from Cooper JD 1995). Multiple firings of the stapling device are required with reloading after each firing. The specimen is resected usually starting anteriorly and advancing posteriorly. The mean time required to complete just this stapling was approximately 45 minutes at Glenfield Hospital. This implies that the anterior aspect of the specimen has undergone a longer period of hypoxia prior to tissue fixation than the posterior aspect. Additionally during the operation, the target area is grasped and released with clamps many times in order to facilitate visualisation. Finally the specimen is delivered through a 2-3cm skin incision often requiring extensive manipulation to achieve this. When these factors are taken into account, the prospect of artefact interfering with the pathological evaluation is a possibility.

Figure *iv*

Demonstrating "typical" lung volume reduction procedure.



[Adapted from Cooper JD 1995]

Table *ii*

Patient selection criteria for LVRS at Glenfield Hospital (Vaughan P 2005)

Indications	Contra-Indications
Pulmonary Function Tests: FEV_1 20-40% (Obstruction) RV >200% TLC >120% (Distension) DLCO>20%, pCO_2 < 7kPa (Destruction) **Symptoms:** Dyspnoea MRC 3 – 5 **"Target Areas":** Anatomical (HRCT scan) Functional (Radionuclide Perfusion Scintigraphy) **General:** Smoking cessation Compliance with pulmonary rehabilitation program Objective exercise test 6-minute-walk test, or Shuttle walk test Fitness for surgery Nutritional state	**Radiographic:** Homogenous emphysema / No target areas **Pulmonary:** Giant bulla Pulmonary nodule of unknown histology **Pulmonary hypertension (mean PAP >25mmHg)** Recurrent lower respiratory tract infections Bronchiectasis Hypercapnia (pCO_2 >7 kPa) KCO < 20% predicted Ventilator dependent **Cardiovascular:** Recent myocardial infarction (within 6 weeks) Impaired left or right ventricular function Significant arrhythmia **Others:** Disease reducing life expectancy Previous chest surgery

LVRS has been performed at Glenfield Hospital, Leicester since 1995. Inclusion and exclusion criteria for the surgery are summarised in Table *ii* (Vaughan P 2005). The selection of target areas was on an individual patient basis, following a multidisciplinary team (MDT) meeting involving surgeons, respiratory physicians, and radiologists. Pre operatively all patients also underwent 12 lead ECG and transthoracic echocardiography with pulmonary artery pressure (Ppa) estimation. Any patient with suspected PAH was referred for right heart catheterisation. Initially all patients underwent median sternotomy and bilateral LVRS. Subsequently unilateral Video-Assisted Thoracoscopy (VAT) LVRS was adopted following publication of a comparison between the two approaches showing similar spirometric outcomes (Oey IF 2002). Where bilateral target areas were present, the worst affected side was operated upon first, and the second side reserved for deterioration of symptoms.

At the commencement of this project in May 2004, 96 procedures had been performed on 92 patients. By the end of the project in January 2007, this number had risen to 132 procedures on 121 patients.

Information available from each patient includes demographic data, full spirometry and body box plethysmography, body mass index (BMI), quantification of the lung perfusion using radionuclide scintigraphy, high resolution CT Scans, operative data, and post operative follow up. This data is summarised in Table *iii*. Postoperatively all LVRS patients are reviewed with spirometry and body box plethysmography at 3 months, 6 months, 12 months and annually thereafter.

Patients studied

At the commencement of this project in May 2004, 96 procedures had been performed on 92 patients.

Patients were excluded from the study population as follows:-

- Those who had undergone bilateral surgery either as a single procedure via sternotomy or VATS (n=26) or as staged VATS procedures (n=4)
- Thirty one patients had refused consent for tissue donation
- Two patients died prior to their first review at 3 months postoperatively.

This resulted in twenty nine patients being available to study with operation dates ranging from September 1997 to February 2004. We therefore decided to study the twenty patients who had undergone first time unilateral VATS LVRS immediately prior to May 2004.

There were no significant differences found between the twenty patients in the study cohort and the seventy two excluded patients' pre operative demographic, spirometric, and nutritional data. Similarly no significant differences were found in postoperative survival, nutritional and spirometric outcomes between the two groups.

The clinical details of the twenty patients studied are summarised in Table *iii*. All patients were ex-smokers with between twenty and fifty pack-year smoking histories.

Table *iii*
Summary of pre and peri operative characteristics of the twenty patients studied compared with the seventy two excluded LVRS patients from 1995 – May 2004. Figures quoted are median (range).

Characteristic	Study Cohort	Excluded LVRS Patients	*p*-value
Age (years)	62 (39 - 70)	59 (41 - 73)	0.06
Male Gender	70 %	65%	0.61
Alpha-1 anti-trypsin deficient	5%	4%	0.84
FEV_1 (%pred)	29% (20 – 60)	26% (12 – 60)	0.36
FVC (%pred)	65% (42 - 98)	68% (25 - 121)	0.59
RV (%pred)	248% (149 – 339)	254% (142 – 399)	0.67
TLC (%pred)	142% (100 - 168)	141% (100 - 229)	0.58
BMI (kg/m^2)	24.44 (17..07 – 33.21)	23.12 (13.89 - 35.55)	0.07
TLCO (%pred)	41 (29 - 75)	44 (13-90)	0.82
pO_2 (kPa)	8.78 (7.45 – 10.41)	9.14 (6.7 – 11.42)	0.13
pCO_2 (kPa)	4.96 (3.69 - 6.25)	5.17 (3.14-6.56)	0.25
Shuttle walk distance (m)	200m (130 - 360)	205m (40-480)	0.38
Operating time (mins)	78mins (40 - 150)	80mins (27 - 255)	0.09
Duration of intercostal drains (days)	7days (2 - 37)	12days (2 - 55)	0.27
Length of post op stay (days)	12 days (7 - 89)	15days (5 - 111)	0.22
Mortality (30days : 90days)	0% : 5%	2% : 6%	0.73

VIII.ii. Outcome following LVRS

Outcome following LVRS can be measured in many ways. The percentage change in spirometry including FEV_1 is most often used, however other measures that have been studied include change in BMI (Vaughan P 2007), percentage reduction in hyperinflation (TLC), gas trapping (RV) and RV:TLC ratio, change in health status (Hamacher J 2002, Oey IF 2003, 2004), change in quality of life, change in symptoms and mortality (Fishman A 2003).

Despite such rigid inclusion and exclusion criteria resulting in a homogenous preoperative patient population, the outcome following LVRS is not uniform, as demonstrated by the results of surgery from both Glenfield Hospital (See Table *iv*) and other investigators (Geddes D 2000, Hamacher J 2002, Fishman A 2003). Although significant improvements in FEV_1, BMI, RV:TLC ratio are found, the range of changes at each time point is the most interesting feature. Even at 4 years post operatively there are still patients with significant improvements in spirometry and hyperinflation, while some patients have significant deterioration in their spirometry.

It is the preoperative identification of such patients who will benefit and the exclusion of those who will not that is the "holy grail" of lung volume reduction surgery.

Table *iv*

Post operative outcomes of all LVRS patients from Glenfield Hospital 1995 - 2007

Data expressed as median (range).

	3 months	6 months	1 year	2 years	3 years	4 years
% Change in FEV_1	20% ** (-24 - +90)	13% ** (-41 - +158)	15% ** (-35 - +87)	2% (-54 - +64)	6% (-40 - +44)	-4% (-34 - +19)
Change in BMI (kg/m^2)	0.0 (-4.5 - +3.7)	0.52 ** (-3.0 - +4.2)	0.85 ** (-2.6 - +5.0)	0.92 ** (-2.2 - +3.7)	1.2 (-8.8 - +4.0)	1.2 (-1.8 - +2.6)
Change in RV:TLC(%)	-9 ** (-39 - +10)	-7 ** (-32 - +6)	-6 * (-33 - +18)	-5.5 * (-36 - +9)	-5 (-13 - +5)	+5 (-6 - +11)

*p<0.05

**p<0.001

VIII.iii. Laboratory Methods:

All laboratory studies were performed in the Department of Pathology at AstraZeneca (R&D), Charnwood, Loughborough UK. During the study period, after appropriate training and supervision when necessary, I prepared all LVRS specimens received into the laboratory into formalin fixed, paraffin wax embedded tissue blocks. For all studies I prepared all my own slides and performed all tinctoral and immunohistochemistry staining. All photographs and measurements unless otherwise stated were taken by myself.

Tissue:

Following excision, all lung tissue was reviewed by the Department of Pathology at Glenfield Hospital. Any macroscopically abnormal areas were excised and retained for analysis. The remaining lung tissue was then placed in 10% neutral buffered formalin (Pioneer Chemicals, Surrey) and transferred to the Department of Pathology at AstraZeneca (R&D), Charnwood, Loughborough UK.

All patients gave informed consent prior to tissue being obtained (See appendix I). Approval for this research was granted by the Leicestershire and Rutland Ethics Committee (LREC).

From 1995 until 2002, only one or two blocks of tissue from each patient were available for study. After 2002, variable numbers of tissue blocks ranging from three to fifty per patient were available for study, depending upon the size of tissue available.

For the purposes of the description of lesions, characterisation of remodelling phenotypes and population grading studies samples from patients undergoing both unilateral and bilateral LVRS were utilised. However, for grading of lesions, and their potential relationship with LVRS outcome, only samples from patients undergoing

unilateral LVRS were used. For the purposes of this research project, only samples from pre-2004 were used to allow meaningful patient follow-up data to be gathered during the research period.

Tissue Preparation:

All tissue samples were fixed for 48 hours at room temperature in 10% neutral buffered formalin (Pioneer Chemicals, Surrey). The tissue was then dissected into slices approximately 3mm thick, and embedded overnight on a Leica Tissue Processor (LEICA TP 1050 fully enclosed vacuum tissue processor). Samples were dehydrated through graded alcohols and into paraffin wax, and then embedded on a Leica Histoembedder, before being positioned and set into a paraffin wax block. Blocks are stored in the dark, at room temperature, ready to use.

Microtomy:

4μm sections were cut using a Leica Jung RM2155 microtome. Cut sections were floated on water (37°C), and transferred to charged slides. The slides were then dried overnight in a warming cabinet at 45°C to assist adhesion.

VIII.iv. Tinctoral Stains:

All tinctoral stains were performed by hand except haematoxylin and eosin (H&E) stains which were performed upon an automated staining machine.

Standard optimised laboratory protocols were used throughout and based upon those described in Bancroft 5th edition 2002. Protocols for the staining of tissues and the constituents of each tinctoral stain are described in Appendix II.

Prior to staining, all slides were dewaxed in 99% xylene and rehydrated by passing through graded alcohols (Ethanol 95% x2, Industrial Methylated Spirits[IMS] 70% x2, distilled water).

The tinctoral stains used in this study are as follows:-

H&E: This stain colours nuclei dark blue/purple, cytoplasm is stained pink, while erythrocytes appear orange/red.

Millers Elastic van Gieson(EVG): This stain colours elastic tissue black/deep purple, collagen appears red, and all other tissues are a pale yellow.

Picrosirius Red: In ordinary bright- field microscopy collagen is red on a yellow background. When examined with polarised light microscopy the larger, more mature and thus more dehydrated collagen fibres are bright yellow or orange. The less mature, thinner fibres including reticular fibres are green. Birefringence is highly specific for collagen.

Martius Scarlet Blue(MSB): This trichrome stains erythrocytes yellow, fibrin red/purple depending upon maturity, collagen blue, and smooth muscle pink/red. Nuclei are stained black.

VIII.v. Immunohistochemistry:

All immunohistochemistry was performed using the standardised laboratory protocol for validated antibodies described in Appendix III. Diaminobenzidine tetrahydrochloride (DAB) was used as the chromogen throughout the studies.

VIII.vi. Primary Antibodies:

A summary of primary antibodies used, their antigen retrieval, peroxidase quench, the secondary and tertiary antibodies used are summarised in Table *v* at the end of this section

Carbonic Anhydrase IX (CA IX): rabbit polyclonal antibody, 1mg/ml, ab15086 (Abcam, Cambridge, UK), labels amino acid residues 359-459 (c-terminus) of human CA IX (Bui MH 2003).

Caspase 3: rabbit polyclonal anti-human/mouse caspase 3 antibody, 0.5mg/ml, AF835 (R&D Systems), labels amino acids 163-175 of human Caspase 3 (Marshman E 2001). Caspase 3 is a critical regulator of apoptosis, being activated by several pathways prior to translocating into the nucleus where it commences the apoptotic process

CD31: anti - CD31 mouse monoclonal antibody, 0.15mg/ml, ab9498 (Abcam, Cambridge, UK). Clone JC/70A reacts with a formalin-resistant epitope on CD31 (platelet endothelial cell adhesion molecule 1 [PECAM1]) on endothelial cells (Moriyama M 1997).

Collagen I: anti collagen I mouse monoclonal antibody, 6.5mg/ml, ab6308 (Abcam, Cambridge, UK). Clone COL-1 reacts specifically with the native (helical) form of collagen I found within the extracellular matrix and is prominent throughout all connective tissue, including tendons and cartilage (Decologne N 2007).

Collagen III: anti-collagen III mouse monoclonal antibody, 10mg/ml, MU167-UC (BioGenex, San Ramon, California, USA). Clone HWD1.1 reacts specifically to type III collagen found in interstitial connective tissue and basement membranes (Konomi H 1981).

Desmin: anti-human desmin mouse monoclonal antibody, 0.23mg/ml M0760, (Dako, Ely, UK). Clone D33 labels the class III intermediate filaments of smooth and striated muscle as well as mesothelial cells (Miettinen M 2000).

Ki 67: mouse monoclonal Ki 67 antibody 0.56mg/ml, Clone: MM1 VP-K452. (Vector Laboratories). Ki67 is a cell cycle associated protein expressed from G1 through the end of M phase, and is used in the assessment of cell proliferation (Gerdes J 1991).

Vascular Endothelial Growth Factor (VEGF): mouse monoclonal VEGF antibody, Sc-7269, 0.2mg/ml (Santa Cruz Biotechnology), labels amino acids 1-140 of human VEGF (Corne J 2000).

Vimentin: monoclonal mouse anti-vimentin 0.36mg/ml, M0725, (Dako, Ely, UK). Clone V9 labels the class III intermediate filaments specifically of cells of mesenchymal origin (Azumi N 1987).

Von Hippel-Lindau (VHL): mouse anti –VHL monoclonal antibody, 0.5mg/ml, 556347, (BD Pharmingen). Clone Ig32 recognises human and mouse VHL, a protein intimately associated with cellular oxygen sensing mechanisms (Kibel A 1995).

Table *v*. A Summary of Antibodies used in this project: 1

Antibody	Retrieval	Peroxidase Quench	Primary Antibody	Working Concentration	Negative Control	Secondary Antibody	Tertiary	DAB Time
CA IX	Microwave	0.5% for 10min	Rabbit monoclonal (AbCam Ab15086)	1:1000 1µg/ml	Rabbit Ig (Serotec PRABP01)	Biotinylated Goat anti-rabbit Dako E0432 1:200 20mins	TSA Biotin System	35secs
Caspase 3	Nil	0.5% for 10min	Rabbit polyclonal (R&D Systems AF835)	1:200 2.5µg/ml	Rabbit Ig (Serotec PRABP01)	Biotinylated Goat anti-rabbit Dako E0432 1:300 30mins	Streptavidin AB / HRP Dako K0377	8 mins
CD31	Nil	3% for 10min	Mouse monoclonal (Abcam ab9498)	1:40 3.75µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:300 30min	TSA Biotin System	30 secs
Collagen I	Microwave	0.5% for 10min	Monoclonal Mouse (AbCam COL-1)	1:1000 5.5µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	Streptavidin AB / HRP Dako K0377	60 secs
Collagen III	Microwave	0.5% for 10min	Monoclonal Mouse (Biogenex Clone: HWD1.1)	1:100 0.13µg/ml	Mouse IgG (Serotec PMP01)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	Streptavidin AB / HRP Dako K0377	60 secs

Summary of Antibodies used in this project: 2

Antibody	Retrieval	Peroxidase Quench	1˚ Antibody	Working Concentration	Negative Control	Secondary Antibody	Tertiary	DAB Time
Desmin	Nil	0.5% for 10min	Mouse monoclonal (Dako M0760)	1:100 2.3µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	Streptavidin AB / HRP Dako K0377	120secs
Ki67	Pressure Cook	0.5% for 10 min	Monoclonal Mouse (Vector Labs Clone: MM1 VP-K452)	1:100 0.56µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:300 20min	TSA Biotin System	60secs
VHL	Pressure Cook	0.5% for 10min	Mouse monoclonal (BD Biosciences 556347)	1:500 1µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	TSA Biotin System	90secs
VEGF	Trypsin	0.5% for 10min	Monoclonal Mouse (Santa Cruz Sc-7269)	1:150 33µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	TSA Biotin System	3mins
Vimentin	Microwave	0.5% for 10min	Monoclonal mouse (Dako M0725)	1:100 3.6µg/ml	Mouse IgG_1 (Dako X0931)	Biotinylated Goat anti mouse Dako E0433 1:200 20min	Streptavidin AB / HRP Dako K0377	30secs

VIII.vii. Microscopy:

Sections for histochemistry and immunohistochemistry were inspected at up to x40 objective magnification using light microscopy (Zeiss Axioskop plus 2 microscope). Polarised light microscopy was used to visualise the birefringence of collagen stained with picrosirius red.

VIII.viii. Photography:

All photographs were taken with a mounted Leica CD300 digital camera.

VIII.ix. Vessels Studied:

In order to quantify the severity of vascular remodelling and its possible role in the outcome following LVRS, it was first necessary to identify the vessels to be studied. Muscular pulmonary arterioles comprising a bronchiolar - arteriolar pair (BV pair) were chosen (See Figure v). These were defined as a complete muscular pulmonary arteriolar wall (intact external elastic lamina) and respiratory bronchiolar wall (intact sub-mucosa) encompassed by connective tissue. These were chosen because they are at a known anatomical point in the bronchial tree and are easily identified as arterioles and not veins/venules. They cannot be confused with bronchial arteries from the systemic circulation which have formed a vascular plexus of smaller vessels at this level (Brenner O 1935), and do not usually have an external elastic lamina (Corrin B 2006). Potentially the severity of vascular remodelling can also be correlated with the severity of adjacent airway remodelling (see Figure v and vi).

Figure *v*. H&E section of a typical bronchovascular pair (B = bronchiole, V = pulmonary arteriole)

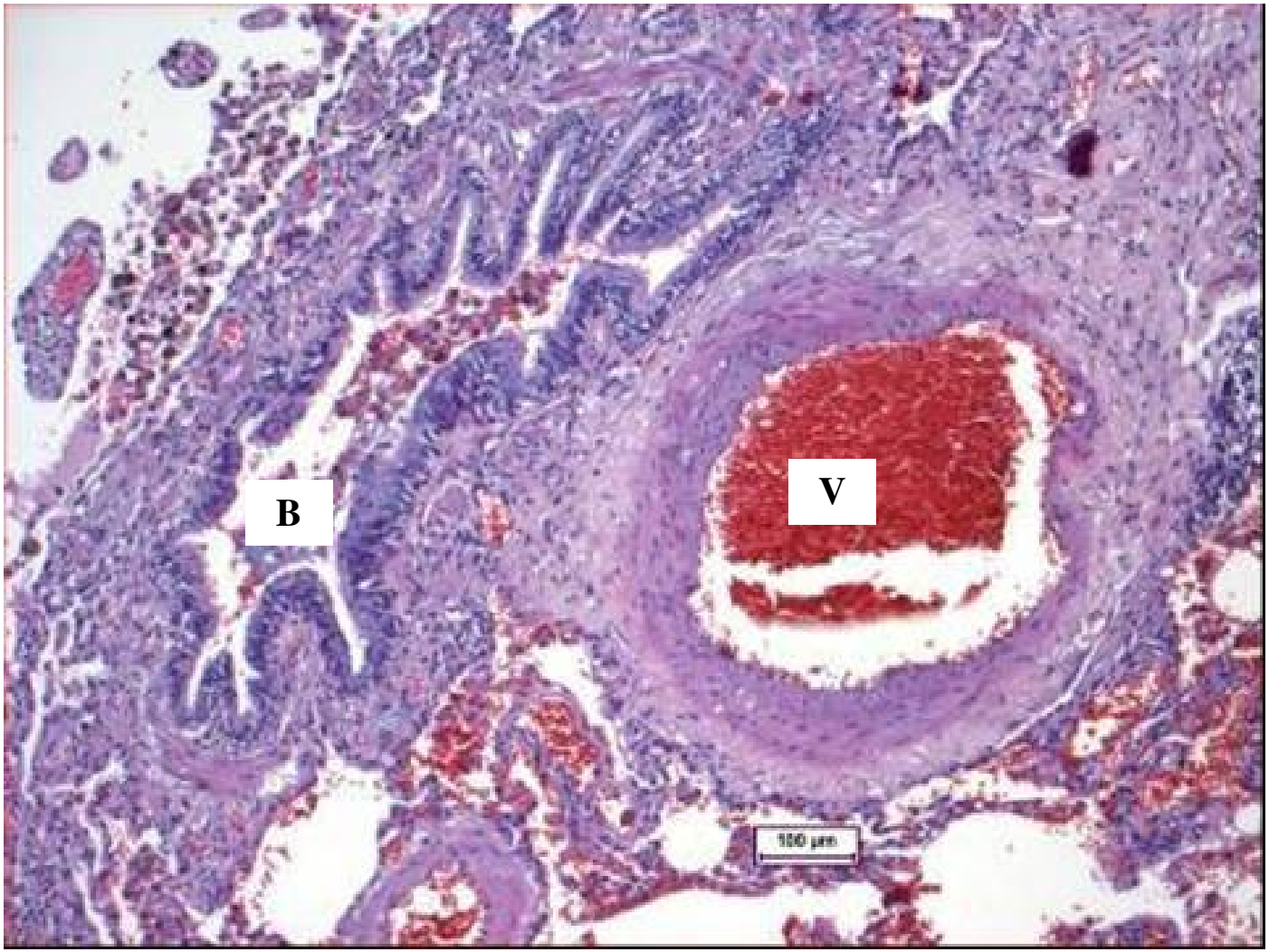
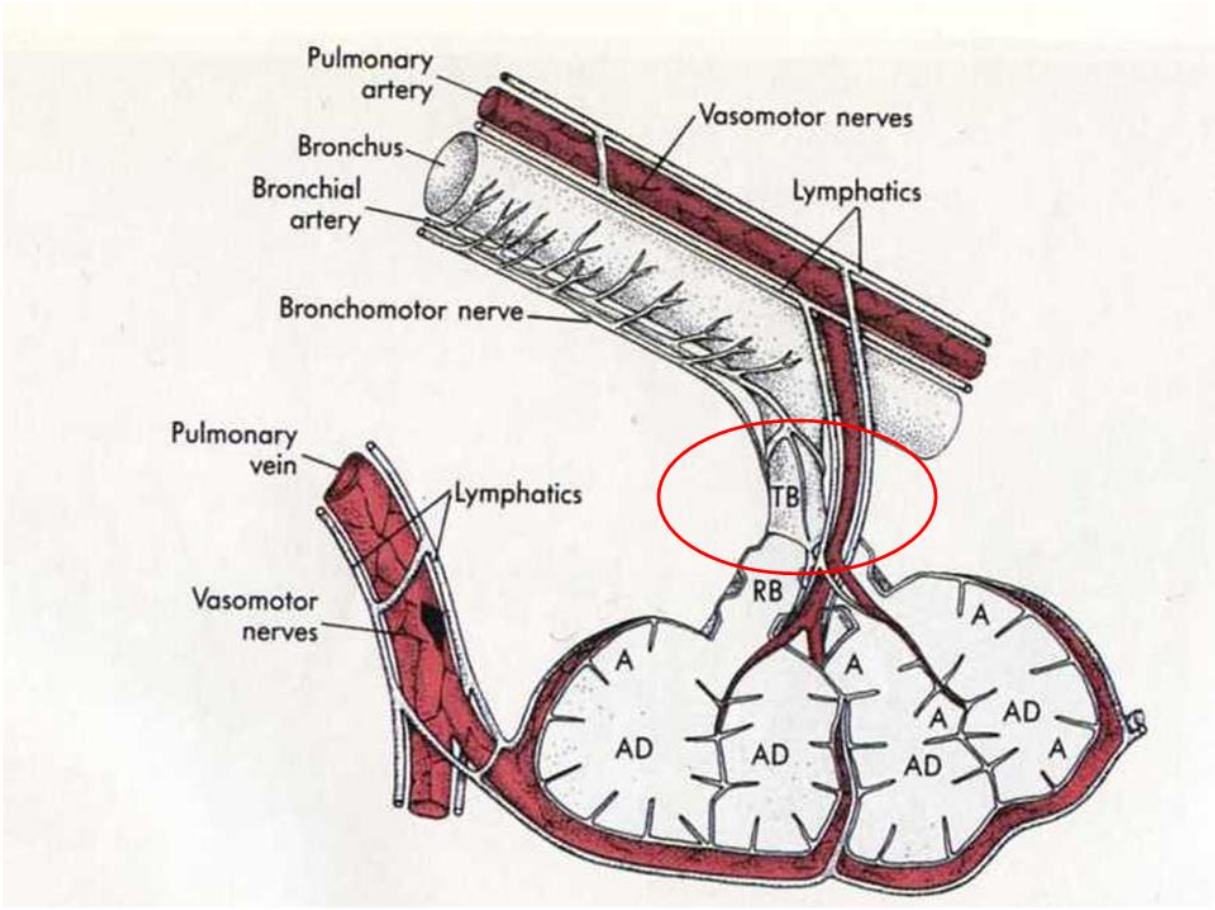


Figure *vi*

Schematic drawing of the bronchovascular pair (circled). TB=Terminal bronchiole, RB=Respiratory bronchiole, A=alveolus, AD=alveolar duct



[Adapted from Physiology 4th edition 1998: Berne RM 1998]

Grading of Lesions

Pilot Study:

All H&E stained slides from the first five patients were reviewed and assessed for the presence of bronchovascular (BV) pairs. BV pairs were identified and photographed to enable repeated identification and assessment.

The cumulative scoring system (See Table *vi*) was applied to each vessel. All the features present in the scoring system are regularly seen in the vessel walls of our cohort of patients. Two points were awarded to each pathological feature that involved more than 50% of the vessel wall, one point, if less than 50% of the vessel wall was affected, and zero points if the pathological feature was absent. A maximum score of 23 can be generated, for the severity of remodelling of the whole vessel.

Intra-observer variation was assessed by choosing fifty vessels from these five patients (the first 10 BV pairs in each patient) and repeatedly applying the scoring system, blinded to the previous scores. Ten randomly chosen BV pairs from this sample were also assessed by an experienced pathologist blinded to the initial scores and thus inter-observer variation was assessed.

Population Study:

All 309 H&E slides from the twenty patients were assessed for the presence of BV pairs. The same cumulative scoring system was applied to every muscular arteriole within a BV pair. Intra and inter-patient variability was then calculated.

Table *vi*

The Cumulative Scoring System. Two points were awarded to each pathological feature that involved more than 50% of the vessel wall; one point, if less than 50% of the vessel wall was affected, and zero points if the pathological feature was absent.

Pathological Feature	Score Achieved
Intima:	
Hypertrophy / Hyperplasia	**0 / 1 / 2**
Roughened / fibrillated Endothelium	**0 / 1 / 2**
Luminal Thrombus	**0 / 2**
Fibrosis / Collagen Deposition	**0 / 1 / 2**
New Internal Elastic Lamina	**0 / 1 / 2**
Media:	
Hypertrophy / Hyperplasia	**0 / 1 / 2**
Reorientation of medial smooth muscle cells into a radial alignment	**0 / 1 / 2**
Apoptosis	**0 / 1 / 2**
Fibrosis / Collagen Deposition	**0 / 1 / 2**
Leucocytes present within wall	**0 / 1**
Fragmented Internal Elastic Lamina	**0 / 1 / 2**
Fragmented External Elastic Lamina	**0 / 1 / 2**
Total Score:	**/ 23**

Heterogeneity of Remodelling Studies:

In order to assess the ability of the grading system to differentiate between vessels with different severities of remodelling, we performed two further studies.

First we compared the most severely remodelled vessel on each slide (the "worst" vessel) with the BV pairs on that slide. These "worst" vessels did not form part of a broncho-vascular pair.

Secondly, we compared the severity of arteriolar remodelling in ten LVRS patients with nine patients without emphysema or with mild/moderate disease who had undergone lung resection for malignancy during the same time period. All slides were taken from areas distant from the tumour.

Agglomerative Hierarchical Clustering

This is a form of cluster analysis where each patient or each pathological feature starts as an individual observation. The two most closely related observations are merged into a single cluster and the similarities calculated. The process is repeated as the two most similar clusters successively merge until only one cluster, containing all data, remains (MacQueen JB 1967). The similarity (or difference) between each cluster is known as the Euclidean distance, which is shown as the height or distance between clusters on a dendrogram. A heatmap can also be constructed to further demonstrate the relationships.

We used this technique to investigate both the distribution of pathological features within individual arterioles and also between patients, to ascertain if certain pathological features represented a particular phenotype.

Morphometry

Morphometric analysis of the arterial walls was performed using Qwin v3 image analysis system (Leica Systems, US). This is an image capture system using a CCD (Charge-Coupled Device) of 2088x1550 pixels with eight bits/channel. The system was calibrated using a standard microscope graticule at x10 objective magnification. The image of the graticule was captured, and using the software's measurement tool, a line was drawn along the length of the graticule image corresponding to 50µm. This distance was then saved on the computer as 50µm at x10 objective magnification. The process was repeated at x5, x20 and x40 objective magnifications with corresponding distances being 100µm, 50µm or 10µm (whichever was most appropriate for the magnification).

A pre-requisite for using the system calibrated in this way, was that following the capture of every image, the magnification used was inputted to allow accurate measurements to be calculated.

Morphometric analysis was only performed upon EVG stained slides that had been serially sectioned with previously graded H&E slides. Severely remodelled vessels with a discontinuous Internal Elastic Lamina (IEL), a discontinuous External Elastic Lamina (EEL) and/or multiple elastic laminae are a potential source of error. Those vessels with these features were excluded from morphometric studies investigating medial area.

The following measurements were made on every graded vessel:-

- The circumference and the area contained within the lumen, the IEL and EEL by using the cursor to draw around them (Fernie JM 1985a) (Fig vii).
- The diameter of the artery between external elastic laminae in the long and short axis was measured perpendicular to each other, and the medial thickness calculated at each of these four points (Morrell NW 1995) (Fig viii).

From these measurements, the following were calculated.

- Intimal area – by subtracting the luminal area from the area encompassed within the IEL
- Medial area - by subtracting the area encompassed within the IEL from the area encompassed within the EEL (*ie* medial area = EEL area – IEL area).
- Mean medial thickness as an average of the medial thickness at the four points
- The ratio of medial area : length of lumen, IEL and EEL.
- Medial area as a percentage of the vessel wall area (intima + media), and the vessel wall and luminal area

Figure *vii*

EVG stained vessel (x20 objective magnification) demonstrating the luminal, IEL and EEL circumferences that are traced, which also provides the area encompassed data.

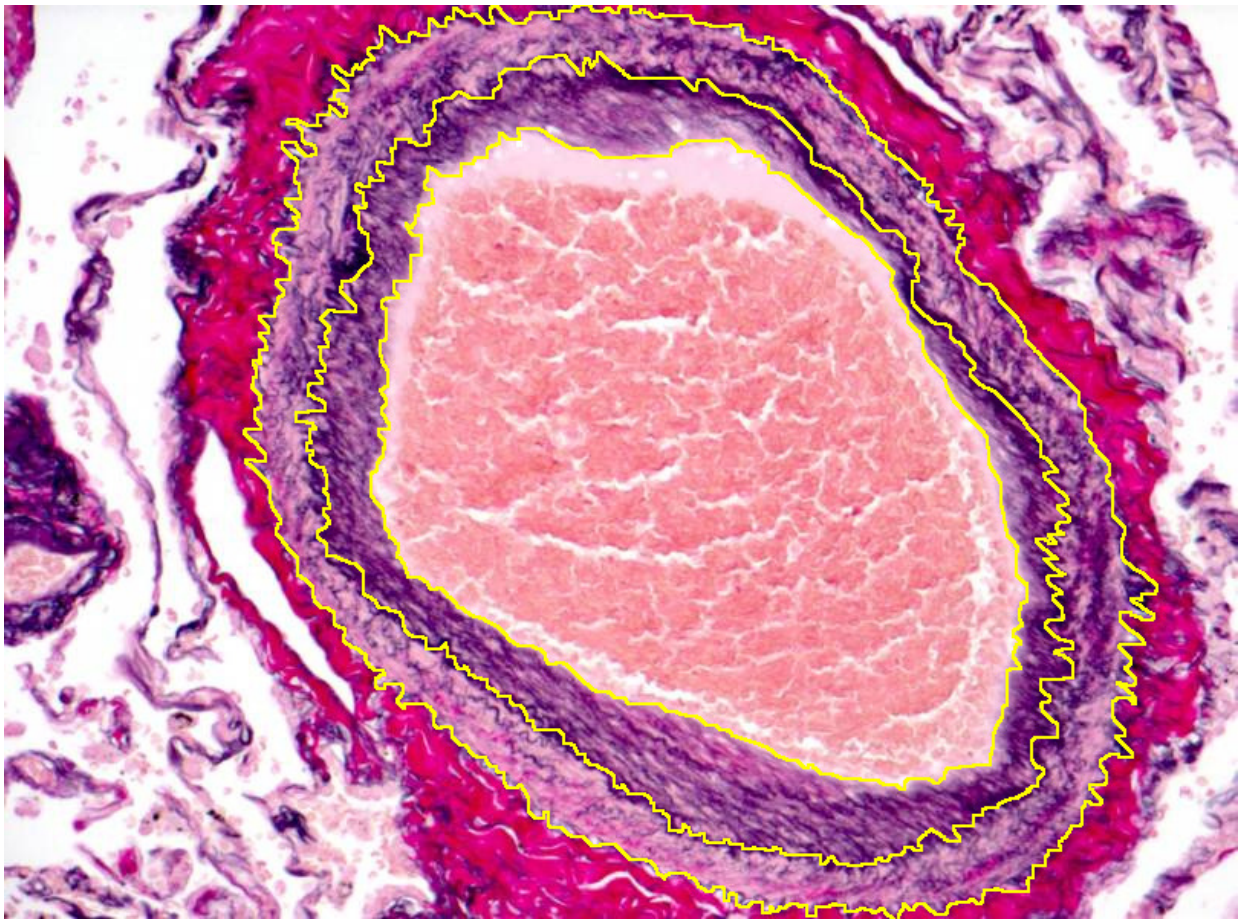
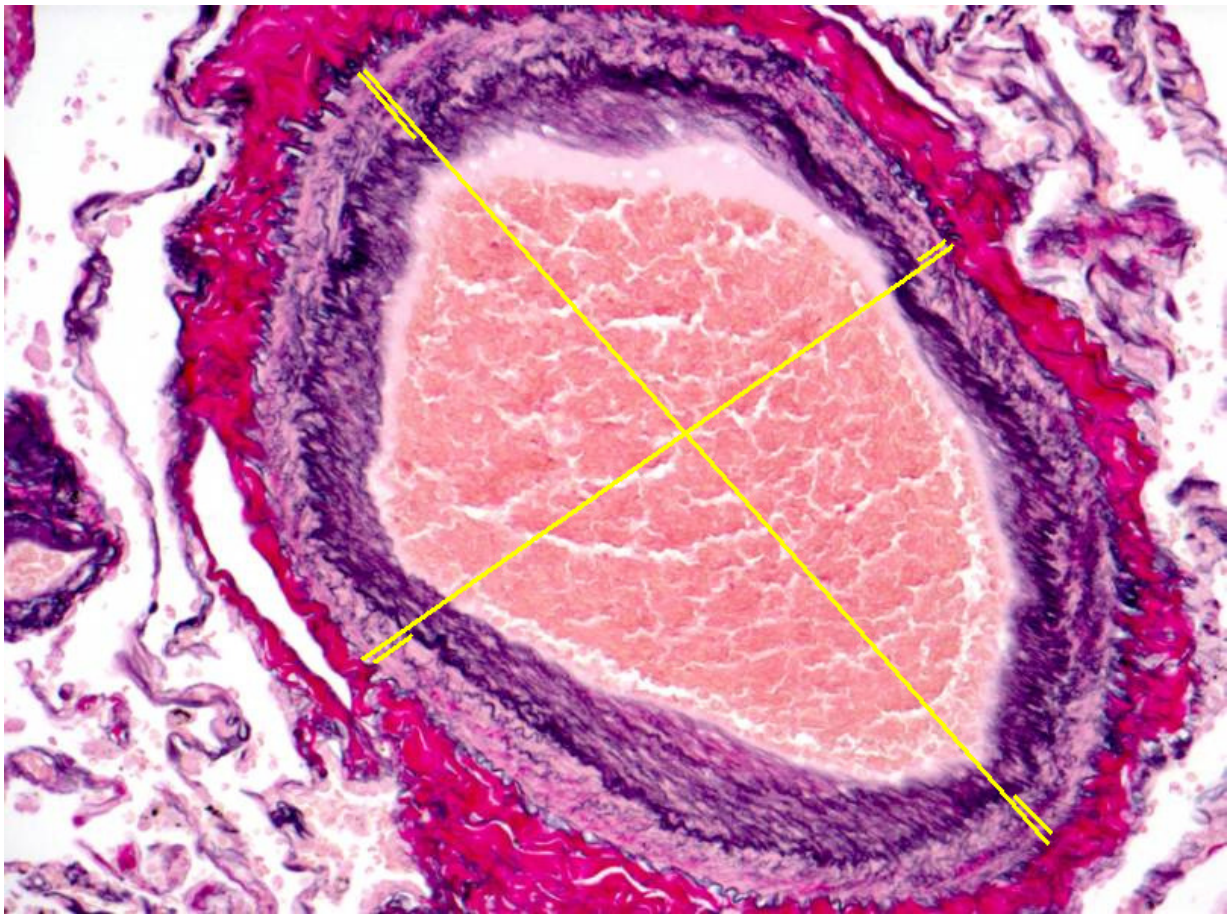


Figure *viii*

EVG stained vessel (x20 objective magnification) demonstrating perpendicular long and short axis diameter measurements, as well as medial thickness measurements in the four quadrants.



Density Threshold

Density thresholding is a format where the desired level of colour (hue), shade (saturation) and illumination (intensity) are specified, and all pixels above this threshold are highlighted by the computer. The HSI is a format based upon hue, saturation and intensity. Hue describes the attribute of pure colour, *i.e.* the proximity of a pixel within the image to a primary colour *eg.* pure red blue or green. The measurement of hue therefore distinguishes between the primary colours. The saturation parameter characterises the shade of that particular colour, and defines how much white has been added to the pure colour, *eg.* pale red will have a greater white component than pure red and thus a *lower* saturation score. The intensity is a measure of the overall brightness of the colour. (Weymes JG 1995).

The HSI is automatically calculated by the computer for every image displayed to allow the best colour visualisation. Using the Qwin v3 image analysis system (Leica Systems, US), the units of hue, saturation and intensity for each image are set on a scale based upon pixel density, that is used consistently for every image displayed. The scale used by the Qwin v3 system is from 0 – 255. Although the numerical scale appears arbitrary, with no SI unit, the same scale is consistently used for every image, thus allowing comparisons to be made between images.

Using only slides stained with EVG, the hue and saturation were altered using the slider adjacent to the image until all the elastic tissue, and only the elastic tissue that was stained black was highlighted (see Figures i*x* and *x*). We were then able to document the new hue, saturation and intensity scores displayed upon the Qwin scale, and therefore able to quantitatively assess the purity and shade of colour of the elastin tissue within the field of view.

It is well known that different factors affect the shades of colours that can be achieved when using the elastic van Gieson stain. In order to minimise the differences in shading and the subsequent analysis of these slides, we undertook the following steps:

- All slides were stained in a single batch to minimise differences in hue and saturation that could result from the staining process
- All slides were reviewed on a single microscope, camera and computer
- All slides were reviewed at a fixed brightness to standardise intensity
- All slides were reviewed by a single observer, in a single batch, who determined when all elastic tissue had been highlighted

Figure *ix*

Pulmonary arteriole (x20 objective magnification) stained with Elastic van Gieson. All elastic tissue within the arteriolar wall is black and adventitia/collagen is magenta (black arrows). Extra-arteriolar carbon deposits are also black (black arrowheads)

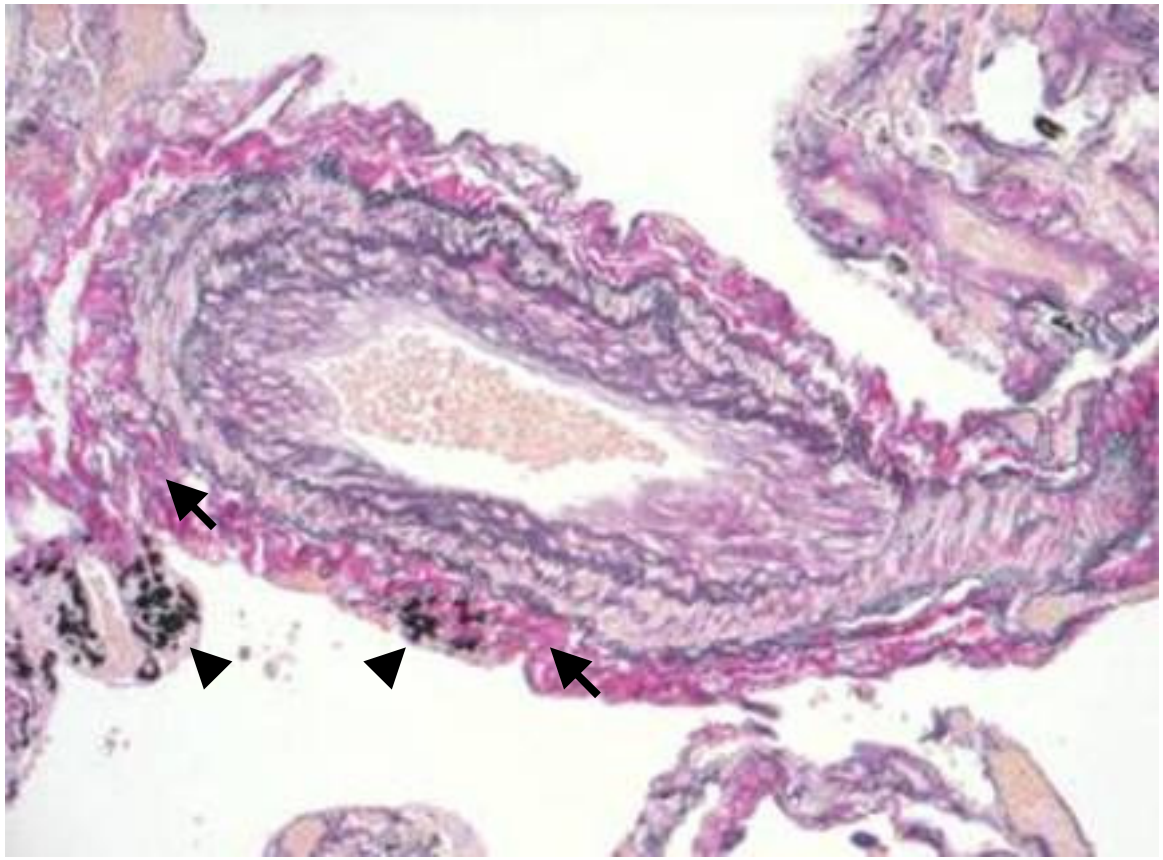
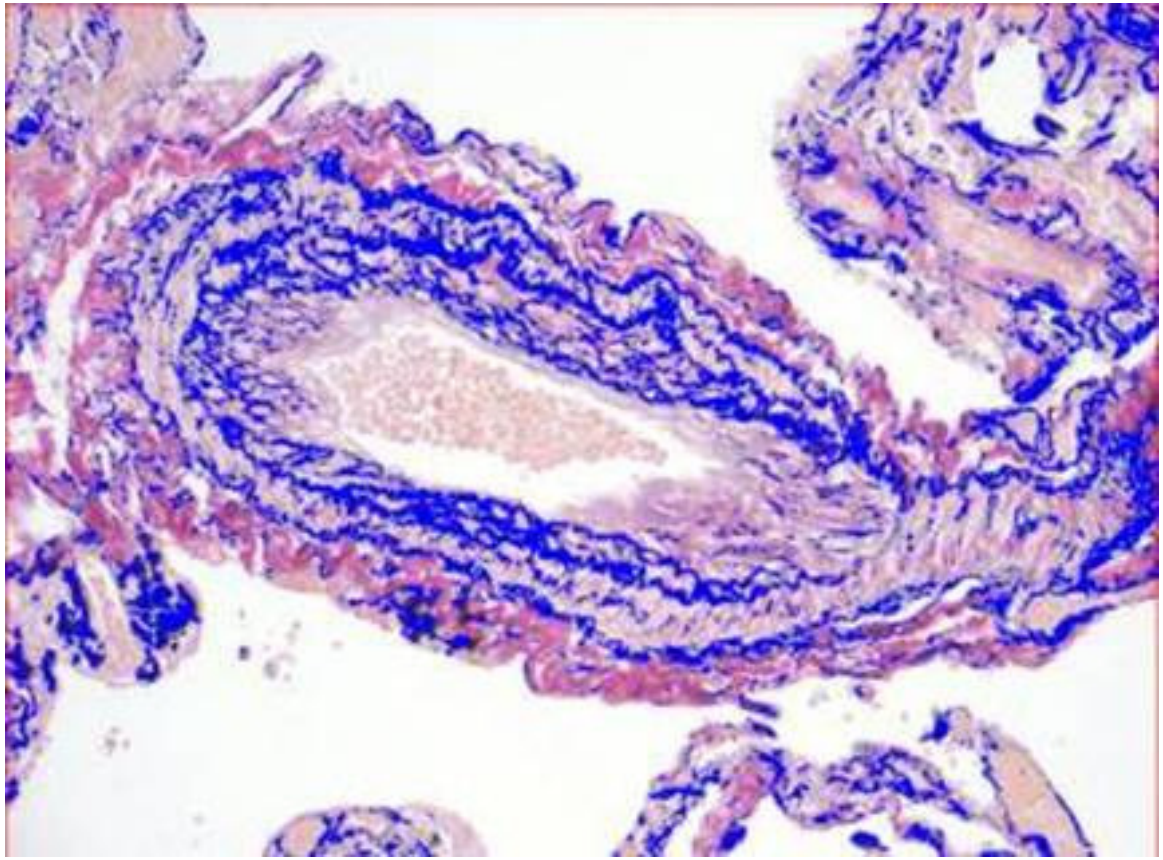


Figure *x*

The same arteriole with all the black elastic tissue, and only the black elastic tissue highlighted by altering the HSI.



Statistical Analyses

All data are presented as median (range) or as a percentage unless otherwise stated. All statistical analyses were performed using SPSS v16 (SPSS Inc, Chicago), except the production of the heatmaps in the hierarchical cluster analysis which was performed with Spotfire Decision Site 10.0.

A *p*-value of <0.05 was considered to be statistically significant throughout the thesis.

Qualitative variables were assessed using the Mann-Witney U test. Quantitative variables were compared using the un-paired student's t-test.

Correlations were assessed using Pearson's correlation coefficients for quantitative variables, or Spearman's rank test for qualitative variables.

Chi squared test was used to compare two categorical variables.

A one-way ANOVA was used to compare more than two groups of quantitative data, as well as analysing variance between the patients remodelling scores.

Inter-observer variation was assessed using the coefficient of variance.

Chapter One

Histopathological Characterisation of Intimal and Medial Remodelling

Introduction

Alterations in pulmonary vascular morphology occur in both COPD and pulmonary hypertension, where chronic hypoxia, inflammation, cigarette smoking and increased shear stress are the primary stimuli (Barbera JA 2003, Hopkins N 2002). The pathological features of this remodelling are well described in the literature, however all the studies suffer from flaws in their methodology. These include a poor description of the patient phenotype, heterogenous patient groups studied in terms of COPD severity or lack of documentation of the presence/absence of pulmonary hypertension. Therefore their results should be interpreted with caution.

Smith P (1992) describes the ultrastructural changes within the pulmonary arteriolar walls of three patients who had undergone heart-lung transplantation for COPD. There is no description of the patients and no information about the presence or absence of pulmonary hypertension in these patients. Balk AG (1979) describes intimal fibrosis and hypertrophy in COPD patients, while Smith P (1980) and Wilkinson M (1988) describe this finding as age –related.

Most studies into COPD arteriolar remodelling are affected by poor patient characterisation. The association between arteriolar remodelling in emphysema and pulmonary hypertension continues to be made despite several observations to the contrary. Remodelling has been demonstrated in smokers without COPD (Hale KA 1980), and also in patients with mild COPD who wouldn't be expected to have developed COPD-related PAH (Santos 2002). Nevertheless medial and intimal hypertrophy and muscularisation of smaller arterioles are well recognised features of both COPD and PAH remodelling, suggesting that the COPD cohort is nested within the PAH population.

Hake KA (1980) also correlated the degree of intimal and medial hypertrophy with severity of COPD in smokers and patients with no, mild, or moderate disease. There has been no such correlation in PAH, nor have there been any such studies in severe COPD.

There are other differences between COPD and PAH not explained by the current literature. Primary pulmonary hypertension has been shown to be associated with a monoclonal expansion of the intima (Lee 1998), while secondary PAH is a polyclonal intimal expansion. Certainly intimal hypertrophy in COPD has been demonstrated by many authors (Barbera JA 1994, Magee F 1988), and this has been shown to be due to endothelial expansion in some studies (Santos S 2002). The complex arteriolar lesions that are pathognomic of PAH have never been described in any cohort of patients with COPD, regardless of the severity of airways disease or the presence of secondary pulmonary hypertension. The cause and the cellular phenotype of the medial hypertrophy has never been satisfactorily explained by previous studies.

The aims of this study are therefore to accurately describe the cellular and extracellular lesions in severe non-PAH COPD, and to ascertain the cellular phenotype of the remodelling reactions in our population.

Hypothesis:

There are no differences between the vascular remodelling seen in our cohort (severe COPD without pulmonary hypertension) to that described in COPD and PAH.

Methods

The patient characteristics, surgical procedure and tissue preparation are all described in the Patients and Methods section.

Tinctoral Stains used:

- Haematoxylin and Eosin (H&E)
- Miller's Elastic Van Gieson (EVG)
- Picrosirius Red
- Martius, Scarlet, Blue (MSB)

Immunohistochemistry:

This was performed using monoclonal mouse antibodies to desmin, vimentin, CD31, Ki 67 and polyclonal rabbit antibody to caspase 3 as described in the Patients and Methods section.

Microscopy:

Sections for histochemistry and immunohistochemistry were inspected at up to x40 objective magnification using light microscopy (Zeiss Axioskop plus 2 microscope). Polarised light microscopy was used to visualise the birefringence of collagen stained with picrosirius red.

Results

Haematoxylin & Eosin (H&E) stained slides were examined from every patient, for general lesion staging. Miller's Elastic van Gieson (EVG) was used to stain elastic tissue, Martius Scarlet Blue (MSB) to assess fibrin and collagen staining, and Picrosirius Red for collagen birefringence under polarised light microscopy.

General Pathology

Parenchyma:

Throughout all the samples, carbon deposits and the presence of moderate or large numbers of tissue macrophages were consistent features. Small bullae were commonly seen, and in most patients, evidence of parenchymal fibrosis was found to varying degrees. Granulomata and foci of calcification were also occasionally seen within the alveolar septae and interstitial connective tissue. Occasional patients had large numbers of neutrophils within the alveoli and septæ, suggesting recent infection.

Airways:

Airway remodelling was present in all slides to varying degrees. Airway remodelling consisted of muscularisation of smaller airways, bronchiolar epithelial cell hypertrophy, goblet cell hypertrophy, fibrosis and evidence of inflammation. Mucus plugs within the bronchiolar lumen were found in approximately half of samples.

Vasculature:

Arteriolar remodelling also affected nearly every vessel observed. The normal flattened endothelial cells adjacent to the internal elastic lamina was a rare finding. The "normal" tunica media consisting of one-four layers of concentric smooth muscle cells sandwiched between the internal and external elastic laminae was also uncommon. Heterogeneity of severity of vascular remodelling was also a consistent feature. Even within a single slide,

arterioles showed a wide range of remodelling reactions, such that a severely remodelled vessel could lie adjacent to one that was only mildly affected.

Haematoxylin and Eosin

Arterioles (n=441) from all twenty patients were graded as mild, moderate or severely remodelled based upon the overall presence of the pathological features as described below. The presence of more features was assumed to indicate increased severity of remodelling. Mild lesions affected part of the arteriolar wall, while moderate lesions affected the whole vessel circumference and severe lesions had the most pathological features, frequently with loss of the usual vessel wall structure.

Tunica Intima

Endothelium/lumen:

Very few arterioles had normal endothelial cells. Most vessels demonstrated endothelial activation (422/441 95.7%), characterised by endothelial cell degeneration, resulting in a fibrillated appearance to the luminal surface. The nuclei of these endothelial cells appeared crescent shaped and darker compared to the underlying intimal cells. Endothelial cell degeneration was always accompanied by intimal hypertrophy.

Luminal thrombi affected only 8/441 (1.8%) of vessels, and were only found in the smallest arterioles. Leukocytes were commonly seen adhering to the endothelial surface and in some cases entering the intima through the fibrillated endothelium. The above changes rarely seemed to affect the calibre of the vessel lumen.

Intima:

Hypertrophy was the commonest pathological feature affecting the intima, being present in 88% (388/441) of arterioles often as the only pathological feature. Intimal hypertrophied cells retained their normal circumferential orientation in mild remodelling, however their nuclei appeared larger, and more rounded than endothelial cells, and the cellular appearance was of smooth muscle cells. When endothelial cell degeneration was present, the intimal cells appeared to adopt a more radial appearance, with the nucleus flattened, paler and deep within the intima. In severely remodelled vessels, intimal hypertrophy and an activated endothelium were always present however apoptosis of the intimal cells was evident. This was demonstrated by obvious tissue defects without staining, and pale nuclei in the adjacent cells. In one third of vessels (141/441 32%), a homogenous grey/purple extracellular stain, indicating sclerosis, was present surrounding the apoptotic areas. The normal orientation of the intimal cells was also completely lost, with cells adopting both radial and circumferential appearances within the same vessel wall.

Tunica Media

Internal Elastic Lamina (IEL):

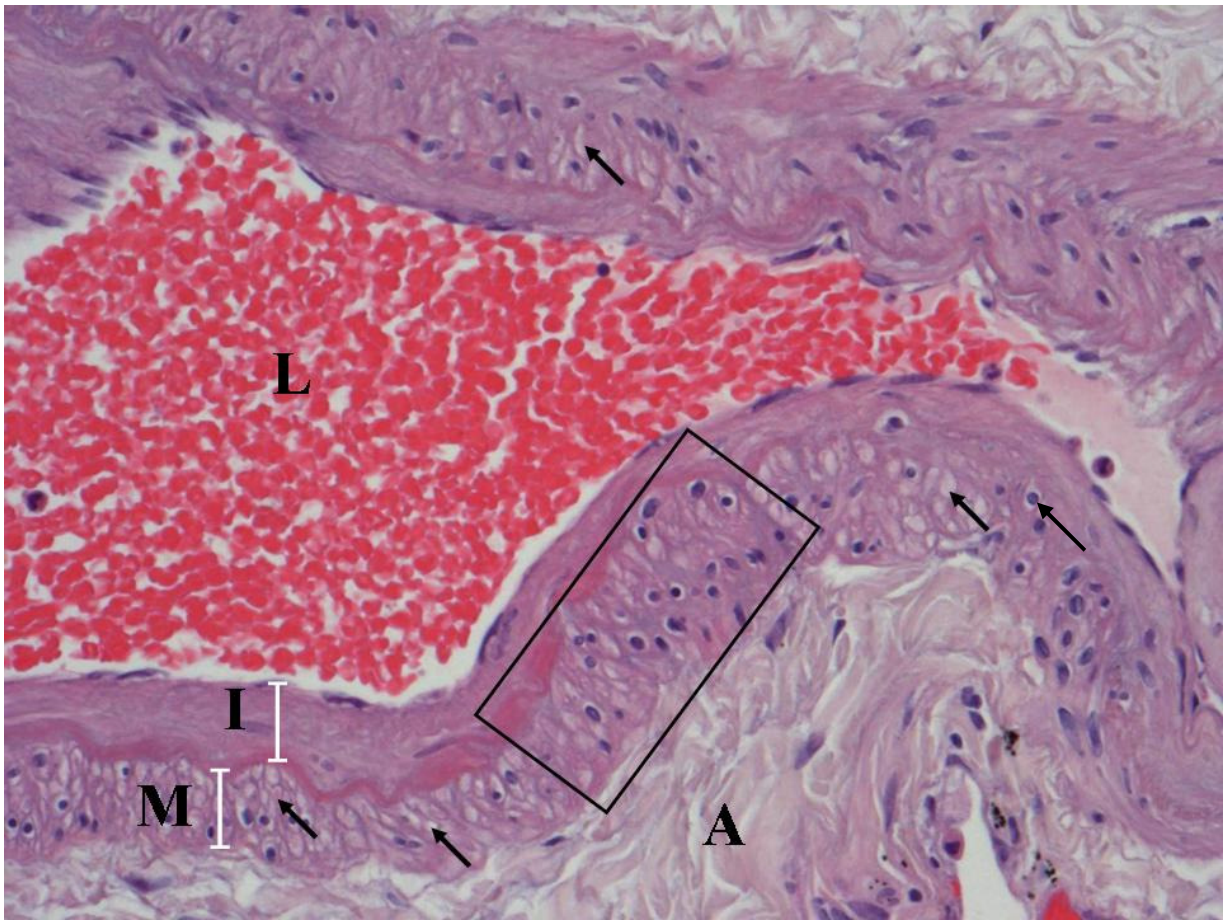
The internal elastic lamina was readily identifiable as a crenellated and occasionally birefringent brighter pink line separating the tunica intima from the tunica media. In both mild and moderately remodelled vessels the IEL was normally circumferential, however it became indistinct when intimal and/or medial sclerosis, or medial matrix and cellular re-orientation were present (117/441 26.5%). In severely remodelled vessels, multiple elastic laminae were present (75/441 17%) within these walls, making differentiation between intima and media extremely difficult.

Smooth Muscle Cells:

One of the most frequent findings was hypertrophy, with this affecting 312/441 (70.8%) of arterioles, and it was therefore interpreted as an early and mild lesion. Intimal and medial hypertrophy did not consistently co-localise with each other in mildly remodelled vessels, but did when moderate and severe remodelling was present. With increasing severity of remodelling, medial smooth muscle cells adopted a radial alignment instead of the usual circumferential/concentric appearance. Although occasionally affecting part of the arteriole's media, this cellular re-orientation more commonly affected the entire circumference, suggesting that the medial matrix of these vessels is similarly re-orientated (see Figure 1.1). This medial re-orientation was found in most moderate and all severely remodelled vessels (363/441 82.3%). Frequently encountered with this medial re-orientation was apoptosis (327/441 74.2%) of the smooth muscle cells. This was characterised by pale pyknotic nuclei in surrounding cells and an obvious tissue defect or absence of staining corresponding to a cellular outline. Apoptotic areas were never confluent, and were always separated by smooth muscle cells regardless of apoptosis severity. Within the media of vessels with apoptosis, leukocytes with very dark nuclei were often found (309/441 70.1%) sandwiched between smooth muscle cells, suggesting an inflammatory process. Occasionally, a focal area of hypoplasia of the arteriolar wall was seen, affecting all layers and suggested impending rupture. This was found only in moderate or severely remodelled vessels. In the most severely remodelled vessels, all of the above features affecting the smooth muscle cells were present. In addition, when the most severe remodelling was present, the usual structure of the vessel wall (lumen-endothelium/intima-IEL-media-EEL) was completely lost, with multiple elastic laminae being seen throughout the vessel wall and a lack of organisation of cellular and matrix

Figure 1.1

H&E x20 objective magnification demonstrating medial apoptosis (black arrows), and cellular & matrix re-orientation of the media (boxed). (L=lumen, I=intima, M=media, A=adventitia)



orientation. These most severely remodelled arterioles tended to be larger, with thicker walls than their less severely remodelled counterparts.

Matrix:

Moderate and severe remodelling of the media was characterised by extracellular sclerosis affecting 102/441 (23.1%) of arterioles. This sclerosis was typified by a homogenous grey/purple extracellular stain similar to that seen in the intima. This sclerosis was rarely found without other features such as apoptosis or re-orientation.

External Elastic Lamina (EEL):

The external elastic lamina was identifiable as a crenellated brighter pink line separating tunica media from the adventitia.

Examples of mild, moderate and severely remodelled arterioles are demonstrated in Figures 1.2, 1.3 and 1.4. The remodelling lesions are summarised in Figure 1.7.

Figure 1.2.

H&E x20 objective magnification. An example of a mildly remodelled arteriole demonstrating intimal hypertrophy (I), and endothelial fibrillation (arrows). There is some evidence of apoptosis and reorientation (black arrowheads) within the media (M). L=lumen, A=adventitia

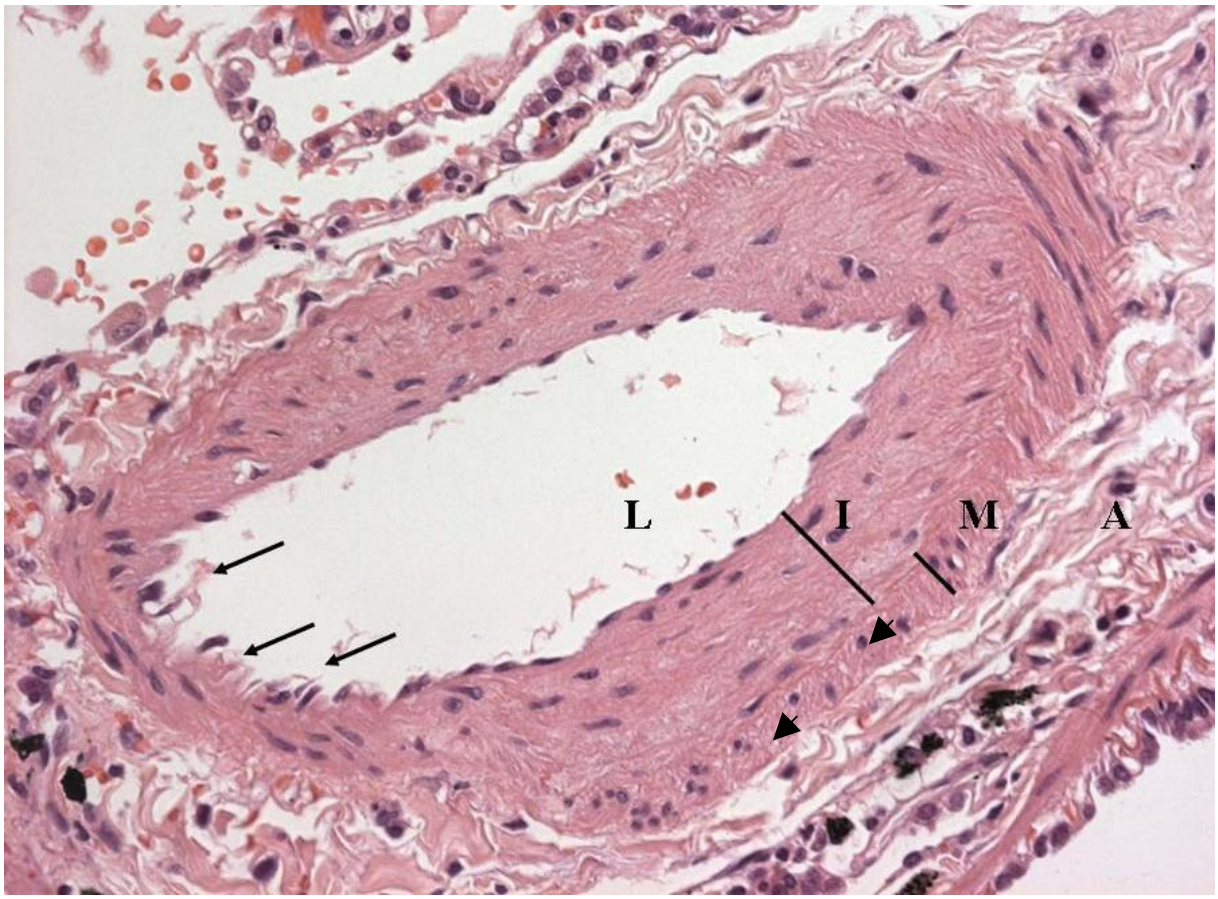


Figure 1.3

H&E x20 objective magnification. A moderately remodelled arteriole demonstrating intimal hypertrophy (I), and circumferential endothelial fibrillation (arrows). There is almost circumferential medial apoptosis and reorientation (boxed), as well as medial hypertrophy (M). L=lumen, A=adventitia

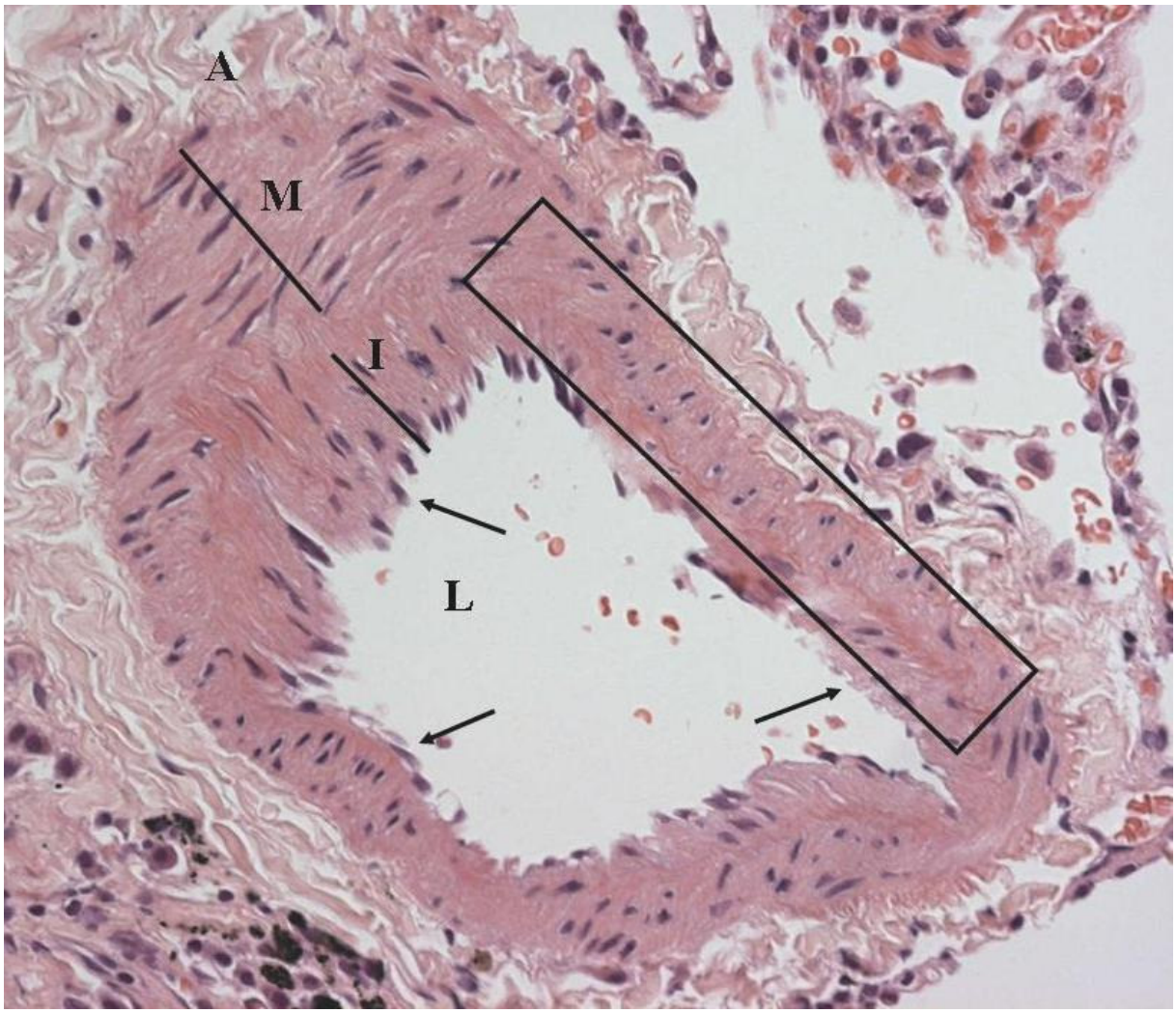
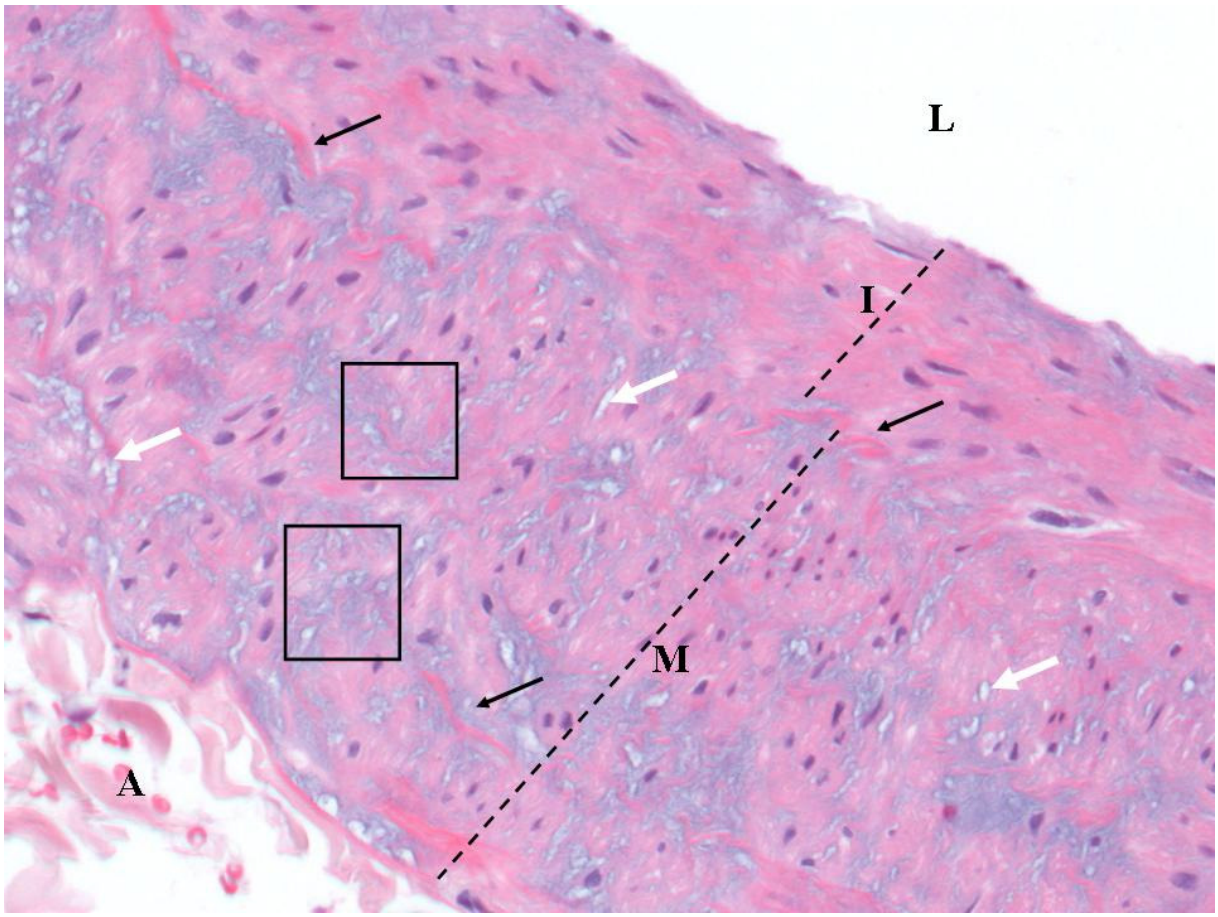


Figure 1.4

H&E x20 objective magnification. Severely remodelled arteriole demonstrating complete loss of usual muscular arteriolar wall structure. Multiple elastic laminae are visible (black arrows), with acellular grey/blue sclerosis (boxed). Intimal hypertrophy (I) and medial hypertrophy (M) are indicated by the dotted lines. White arrows represent apoptosis. L=lumen, A=adventitia



Miller's Elastic van Gieson (EVG)

The Miller's EVG stained the internal and external elastic laminae dark purple/black in all but the most severely remodelled arterioles. These laminae were readily identifiable as a thin, crenellated, circumferential black line. Smooth muscle cells were uniformly stained a pale yellow, and adventitial collagen stained red. See Figures 1.5 and 1.7.

Tunica Intima

Endothelium/lumen:

No staining identified

Intima:

In mildly remodelled arterioles with only hypertrophy, a slightly increased number of elastic fibres were evident. These lay between the internal elastic lamina and the endothelium and had a corkscrew appearance. With increasing severity of remodelling demonstrated by endothelial activation, fibrillation and radially-orientated intimal cells, the density of elastic fibres increased significantly. They formed a dense lattice-work pattern within the extracellular matrix with the fibres appearing shorter, thinner and outlining the intimal smooth muscle cells. When this dense lattice-work was present, the internal elastic lamina became indistinct (as it was exactly the same shade of purple/black), although the junction of intima and media was still readily evident. Severely remodelled arterioles demonstrated multiple elastic laminae as well as a dense lattice-work, with increased elastic staining in areas corresponding to the sclerosis seen on the H&E slide.

Tunica Media

Internal Elastic Lamina (IEL):

In all mild vessels the internal elastic lamina was readily identifiable as a black crenellated circumferential line separating intimal and medial compartments. With increasing density of the lattice-work staining within the intima, the internal elastic lamina could only be identified as the junction between the yellow medial smooth muscle cells and the black lattice of the intima. In severely remodelled vessels the differentiation between intima and media was not possible due to cellular disorganisation (117/441 26.5%), the presence of multiple laminae (75/441 17%), and the deposition of large amounts of elastic tissue throughout the vessel wall.

Smooth Muscle Cells:

These were uniformly stained pale yellow.

Matrix:

In mildly remodelled arterioles, with only hypertrophy, the occasional elastic fibres were, long, fatter, wavy and scattered throughout the media. Moderately remodelled arterioles, with hypertrophy and some evidence of re-orientation with apoptosis, had more elastic fibres within the media. This increased density of elastic fibres was characterised by shorter and thinner fibres, some of which were straighter and between two adjacent smooth muscle cells, while others appeared as a corkscrew. Although there was an increased density of elastic fibres in the media, this never appeared as dense as seen in the intima, nor as a lattice-work pattern. In arterioles with extensive re-orientation and hypertrophy, the elastic fibres coalesced almost circumferentially at a point midway between the internal and external laminae, with some fibres connecting the new lamina to both the IEL and EEL like spokes in a wheel.

In severely remodelled arterioles fibres of different sizes and thickness outlined the smooth muscle cells, and multiple elastic laminae were visible. These were dissimilar to the IEL, in that they were wavy rather than crenellated, and did not involve the full circumference of the vessel. They were not always concentric, nor a uniform distance from the lumen for their entire course, starting almost at the EEL and terminating in the middle of the vessel wall. Increased elastic staining was noted in the corresponding areas of sclerosis as seen on the H&E slide. Within the extracellular matrix, the elastic fibres had lost their usual regularity, suggesting either degradation of existing fibres, or uncoordinated production of new fibres.

External Elastic lamina:

The external elastic lamina remained preserved in all but the most severely remodelled vessels (16/441 3.6%).

Martius Scarlet Blue

MSB stained all erythrocytes pale yellow. Collagen was stained a light blue within the matrix and more intense/brighter blue in the adventitia. Fibrin appeared only occasionally as a red stain. See Figure 1.5.

Tunica Intima

Endothelium/lumen:

Erythrocytes were stained a uniform yellow. When severe endothelial activation occurred, the nuclei stained a deeper purple than the normal endothelium.

Intima:

Little staining occurred in mildly remodelled intimae, however with increasing severity of endothelial activation and apoptosis, there was a corresponding increase in light blue extracellular collagen staining. The areas staining light blue correlated with the grey/purple sclerosis seen on the H&E slides. In a few samples, fibrin appeared in the severely remodelled intima, as a red stain, however it appeared to selectively stain the cytoplasm.

Tunica Media

Internal Elastic Lamina (IEL):

This was not stained / seen in any slide regardless of severity of remodelling.

Smooth Muscle Cells:

Smooth muscle cells were not stained although the matrix surrounding these cells was stained.

Matrix:

In mild vessels with hypertrophy only, there was no collagen or fibrin staining within the media. However when apoptosis and re-orientation were present indicating moderate remodelling, a light blue extracellular collagen staining was noted, similar to that seen within the intima. In the most severely remodelled arterioles, this staining was present throughout the full thickness of the vessel wall, and again corresponded with the areas of homogenous grey/purple sclerosis seen on the H&E slides. When fibrin staining was present, it tended to be closer to the adventitia than the intima.

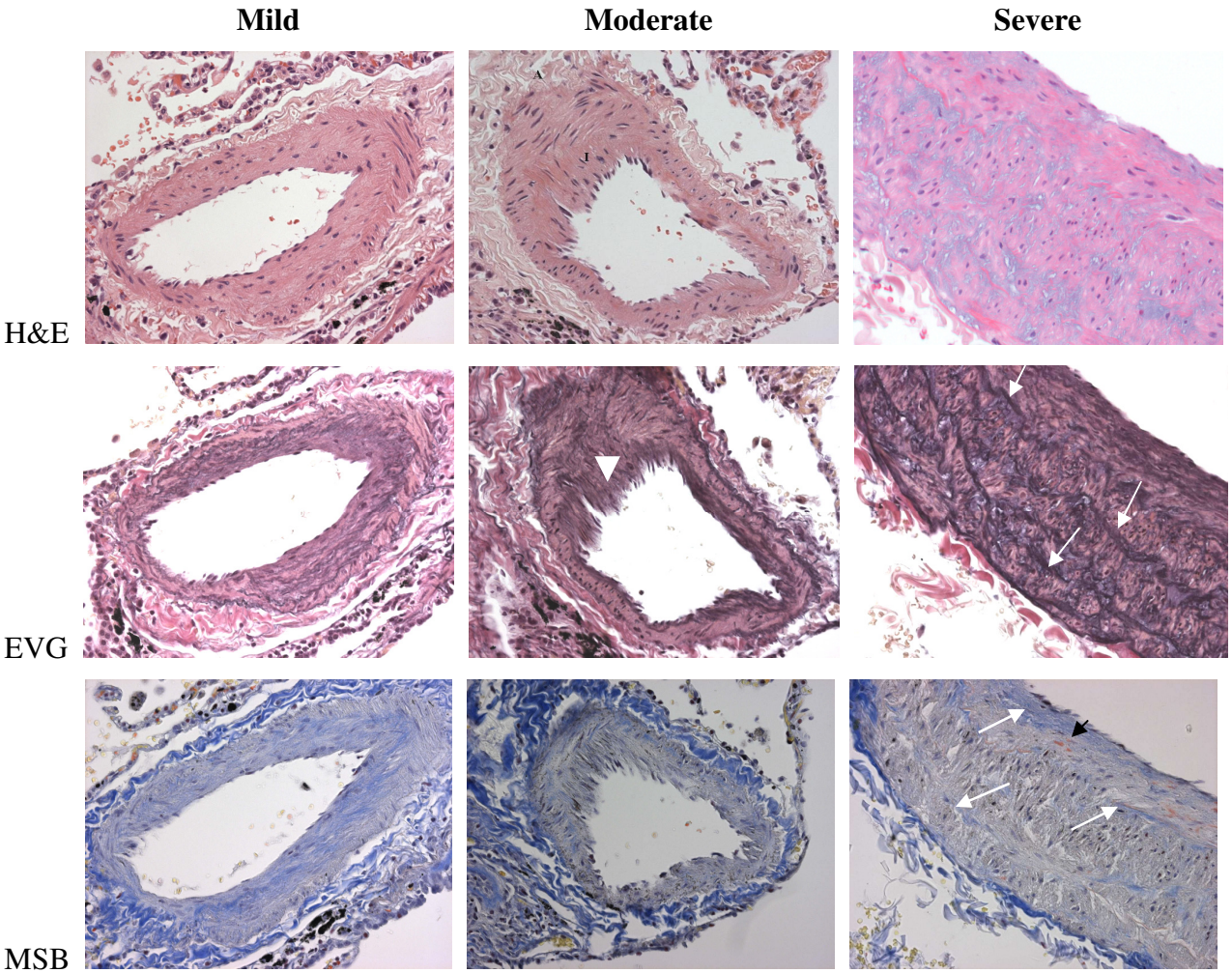
External Elastic lamina:

This was not stained in any slide regardless of severity of remodelling.

Adventitia

This was stained an intense, bright blue colour in all cases. No fibrin staining was seen within the adventitial compartment in any vessel.

Figure 1.5
Serial sections of mild moderate and severely remodelled vessels stained with H&E, EVG and MSB. Magnification x20 objective for all images.



H&E staining as demonstrated in figures 1.2, 1.3 and 1.4.

Deposition of elastin within the intima (white arrowhead) is clearly seen in moderately remodelled vessels. Multiple laminae and elastic fibre destruction seen in severe remodelling (white arrows).

Fibrin (black arrow) and collagen (white arrows) seen close to the lumen and adjacent to the elastic laminae in severe remodelling

Picrosirius Red

Using bright light microscopy all collagen was stained red upon a yellow background. Using crossed polarisation light microscopy, collagen appears as a woven pattern of bi-refringent fibres (yellow, white and green), which corresponds to the density, water content, orientation, and maturity of the collagen fibres – yellow fibres are more mature, green fibres have a greater water content and are thus more immature.

In mildly remodelled vessels there was collagen staining in the adventitia only. Collagen deposition was observed only in the intima at an early stage of remodelling, in a pattern that varied from punctate to a lattice-work, with a relative absence of staining in the media.

Non-polarising microscopy of Picrosirius Red sections showed that in moderate lesions collagen fibres were distributed throughout the vessel wall, following the orientation of the adjacent smooth muscle cells. Within the intima, they were hyperplastic, compacted and orientated perpendicularly to the lumen whilst in the media they were shorter, straight and less dense. Polarisation showed varying fibre brightness and density. The medial fibres were lighter than the intimal fibres, whilst strongly stained collagen fibres were visualised in the adventitia. In vessels with apoptotic and re-orientated medial cells, the collagen fibres were also found to be re-orientated perpendicular to the lumen. See Figures 1.6 and 1.7.

In severe lesions, collagen was distributed throughout the vessel wall with no distinction between the adventitia and the medial layers. Polarised light outlined the intima in these vessels as a darker band along the innermost portion of the arteriolar wall.

Figure 1.6

Picrosirius Red stained arteriole, x20 objective magnification, demonstrating bi-refringent, reorientated collagen (red box) within the medial compartment using polarised light microscopy. (L=lumen, I=intima, A=adventitia)

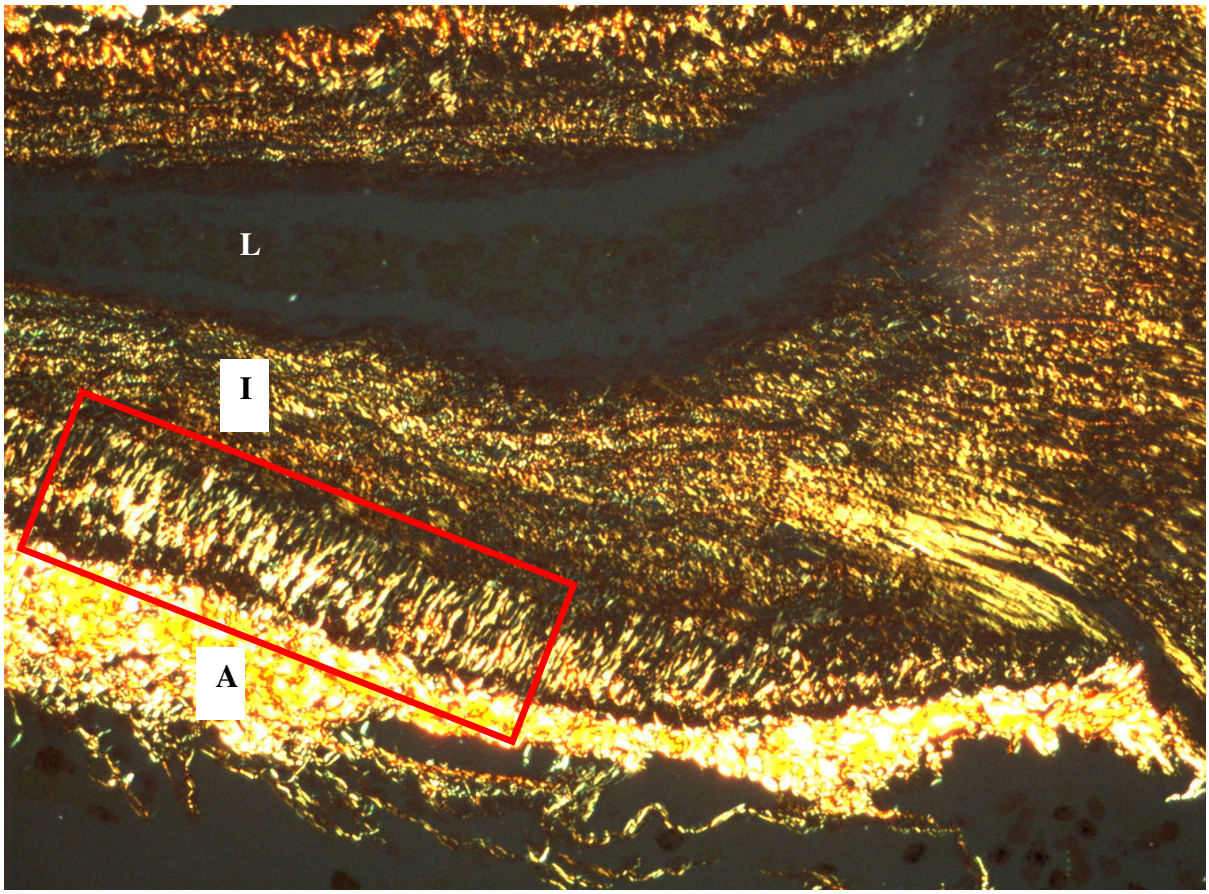
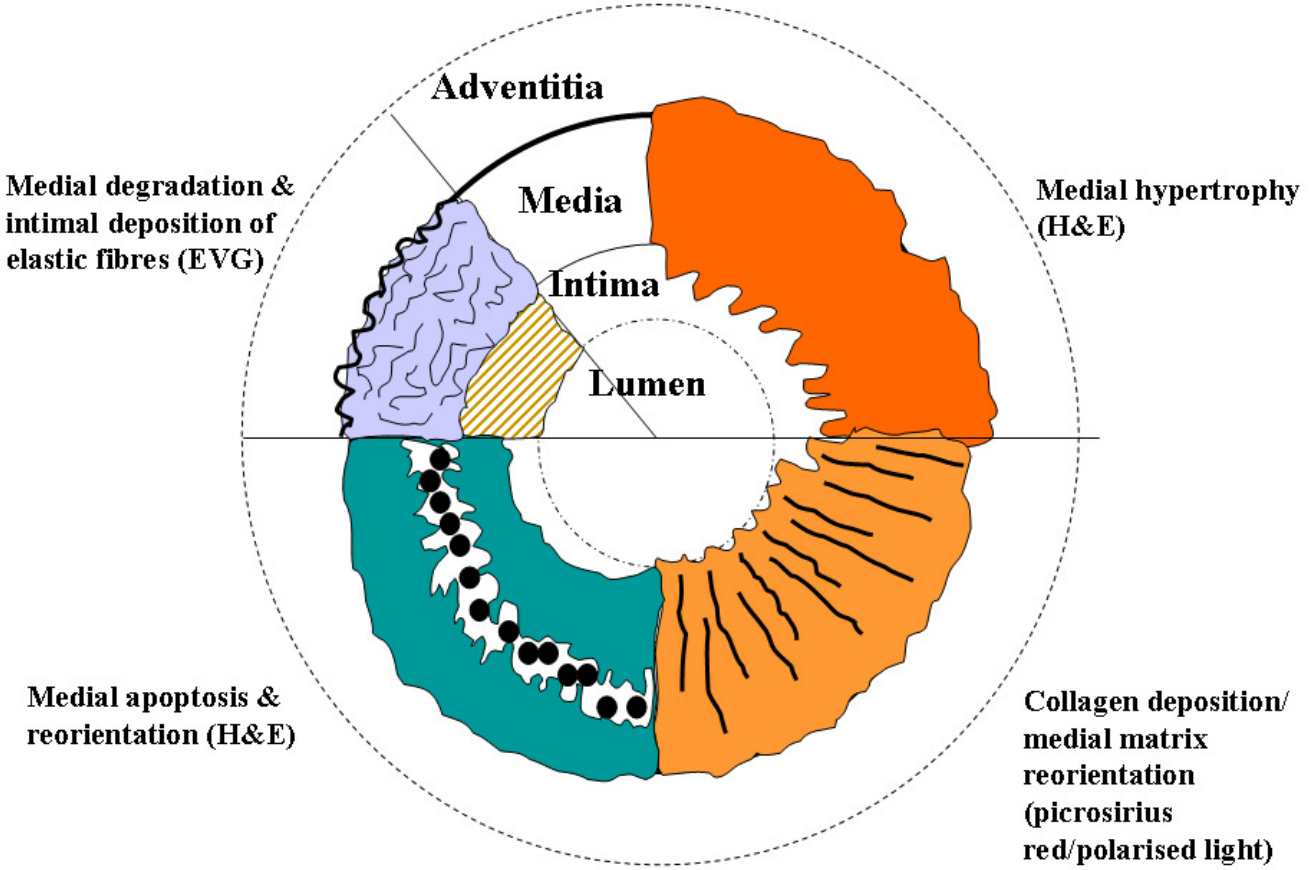


Figure 1.7

Diagram summarising the arteriolar medial remodelling lesions seen in the severe COPD population with tinctoral staining.



Immunohistochemistry

CD31:

CD31 antibody stained cells were strictly localised in the endothelium in mildly, moderately and severely remodelled arterioles and outlined a single endothelial layer in the innermost portion of the pulmonary arterioles. See Figure 1.8. There was no staining within the intima or media regardless of severity of remodelling. We found CD31 in the adventitia demonstrating either the endothelium of new capillaries or outlining the vasa vasorum.

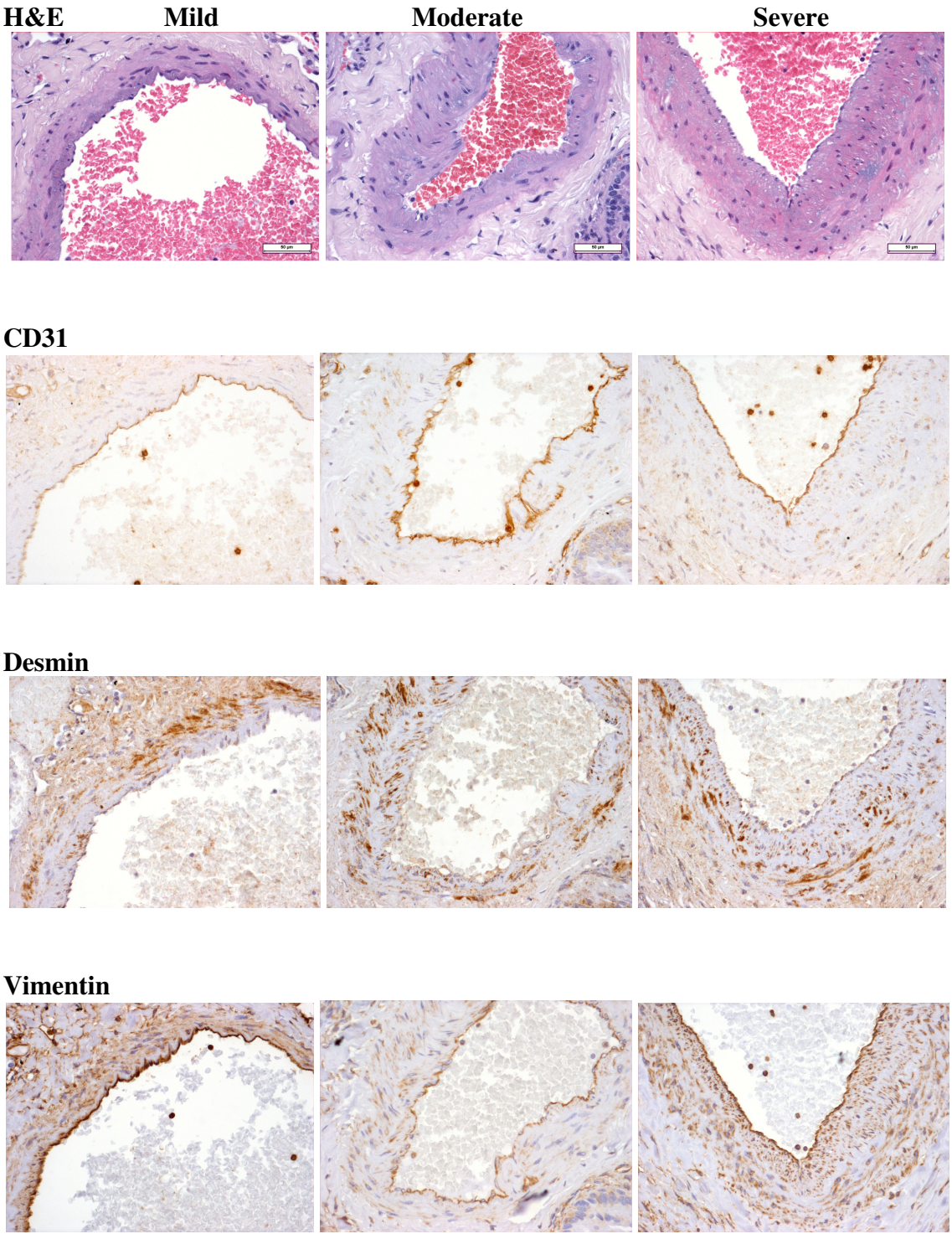
Desmin:

Desmin stained the majority of smooth muscle cells in both the vasculature and airways, appearing as a punctate pattern, most intense in the cytoplasm and perinuclear region. See Figure 1.8. Endothelial cells were never stained. In mildly remodelled arterioles, desmin only stained the intima when hypertrophy/hyperplasia was present. With increasing severity of remodelling and fibrillation/re-orientation of the intima, desmin staining subjectively appeared more intense. When sclerosis of the intima was present, the number of desmin positive cells appeared to decrease.

Desmin stained smooth muscle cells were seen in the media of every arteriole, regardless of the presence or absence of hypertrophy/hyperplasia. When re-orientation and apoptosis were present, desmin highlighted these features in a fibrillar or a punctate pattern respectively. In moderately remodelled vessels, desmin was more uniformly distributed in both the intimal and medial layers, even though a lower proportion of smooth muscle cells showed desmin in the intima. In the most severely remodelled vessels, where loss of vessel wall structure and sclerosis were features, desmin staining was markedly reduced.

Figure 1.8

Serial sections of mild, moderate and severely remodelled vessels stained with CD31, desmin and vimentin immunohistochemistry. Magnification x20 objective for all images.



In some cases this was to the point of being limited to a few smooth muscle cells adjacent to the external elastic lamina, and completely absent from the rest of the vessel wall.

Vimentin:

Vimentin intermediate filaments were expressed in endothelial cells, adventitial fibroblasts, tissue macrophages, background lung parenchyma and in smooth muscle cells where it exhibited a stippled or a wavy pattern. See Figure 1.8. In mildly remodelled arterioles, with hypertrophy of the intima and/or medial compartments, vimentin was localised in the endothelium and media only with sparing of the intimal smooth muscle cells. When re-orientation and fibrillation of the intima was present, then these cells also stained positive for vimentin. Medial staining by vimentin was present throughout all grades of remodelling, and again outlined medial apoptosis and re-orientation of the smooth muscle cells. In the most severely remodelled arterioles with sclerosis and loss of all structure to the vessel wall, uniform distribution of cellular staining remained throughout the full thickness of the vessel wall.

Caspase 3

Overall there was little variability in staining with this marker of apoptosis. Intense staining was noted in the cytoplasm of hyperplastic bronchial epithelial cells and nuclei of type I pneumocytes. The endothelium tended to have one or two positive cells in most arterioles however the staining was washy / dilute in the mildly remodelled vessels. Most, if not all SMC's appeared to be positive, both in the intima and media. The most subjectively intense uptake was found in the medial SMC's of arterioles with medial cellular/matrix re-orientation and apoptosis. In severely remodelled vessels with

complete loss of structure every cell within the wall appeared positive. Adventitial fibroblasts were frequently positive

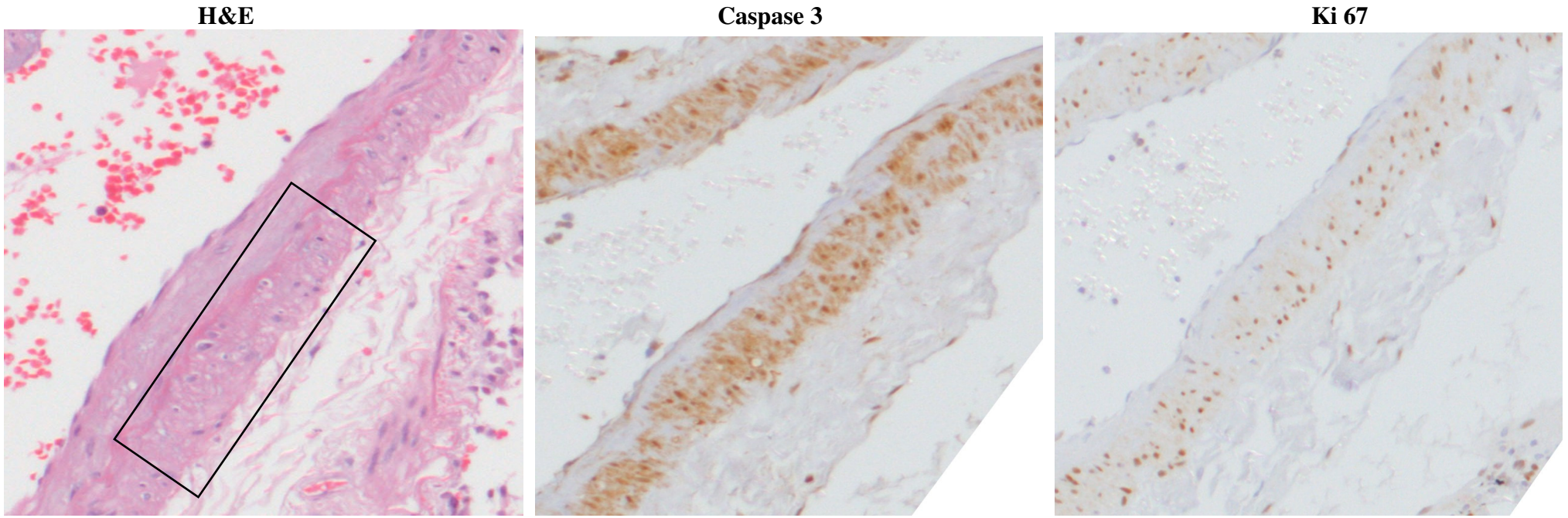
See Figure 1.9.

Ki 67

The staining across the panel of slides with this proliferation marker was also consistent. The most intense staining was found in the nuclei of most hyperplastic bronchial epithelial cells, tissue macrophages and type II pneumocytes. Regardless of remodelling severity, only occasional endothelial cells and intimal SMC stained positive. More proliferative cells were noted within the medial SMC especially when matrix re-orientation/apoptosis was present. When end-stage sclerosis was present, proliferating cells were seen randomly scattered throughout the arteriolar wall. Occasional adventitial fibroblasts were stained regardless of remodelling.

See Figure 1.9.

Figure 1.9. Confirming that apoptotic and proliferative medial SMC co-exist nested within the same arteriole. All magnifications x20 objective. The black box highlights the medial cellular/matrix reorientation, while the brown chromagen stains the Caspase 3 and Ki 67 positive cells.



Discussion

This study has demonstrated that there are many unexplained and several previously unrecognised features of arteriolar remodelling in severe COPD. The most startling feature was the range of remodelling pathologies encountered – even within a single patient. The samples studied are taken from the worst affected areas of lung, from patients at the most severe end of the COPD spectrum. If a simple correlation existed between remodelling and severity of COPD, then one would expect all vessels to be severely remodelled in our study. However, on a single slide, remodelling varied from a vessel with mild intimal and / or medial hypertrophy to one with end-stage fibrosis, apoptosis and cellular re-orientation within a few millimetres. This heterogeneity extended across all slides from every patient and has not been previously described.

This has several possible explanations. We may have observed different phenotypic responses by pulmonary arterioles within the same area of lung, or observed regional changes affecting each acinus differently. Each tissue block from which slides were taken contains several acini and thus bronchovascular pairs from different origins. We therefore hypothesise that the observed heterogeneity is as a result of differing responses within different acini. This response is certainly multifactorial with the role of recurrent infections, sputum plugging at the bronchiolar level, regional hypoxia and post-inflammatory remodelling all affecting the outcome.

The description of the lesions as mild, moderate and severe was based solely upon appearances of each arteriole and the subjective integration of the many different factors by the investigator. Such a grading naturally assumes there is a temporal relationship between each grade, and that mild lesions will become moderate and ultimately severe. The evidence for such a temporal relationship is weak, but is described as follows.

Hypertrophy was an extremely common pathological feature with only 28/441 (6.3%) vessels having neither intimal nor medial hypertrophy. Hypertrophy was never associated with only sclerosis without other remodelling features being present. We can therefore assume that sclerosis occurs later and hypertrophy earlier. Even if a vessel had completely repaired itself leaving just hypertrophy, there would be evidence of such a repair by way of sclerosis. Other extremely common lesions were medial apoptosis and SMC re-orientation (327/441 74.2% and 363/441 82.3% respectively). Sclerosis was rarely present without apoptosis or re-orientation (6/441 vessels [1.4%]), suggesting that sclerosis also occurred after medial apoptosis and SMC re-orientation. This implies that both hypertrophy and apoptosis/reorientation need to be present before sclerosis occurs. There is no evidence however that a fibrotic / hypertrophied / apoptotic arteriole progressed to one where there was complete loss of vessel wall structure, with multiple elastic laminae and an unstructured cellular architecture.

It is possible that we have observed different phenotypes of vascular repair, rather than a temporal relationship. Hypertrophy was the commonest lesion seen and affected most vessels. Given the frequency with which it was observed in our population and also the reported frequency in the COPD literature, we attributed these lesions to be a generic vascular response to injury. If so, the three different repair phenotypes would be:-

1 Medial and intimal fibrosis: collagen and elastin deposition within the matrix of the vessel wall, possibly demonstrating a co-ordinated post-inflammatory remodelling.

2 SMC apoptosis / radial re-orientation: the frequency with which these lesions were seen, suggests a pathological process rather than the vessel being sectioned tangentially instead of perpendicularly.

3 Complete loss of vessel wall structure: a proliferative phenotype with uncoordinated repair, involving features of both the previous two phenotypes.

Although intimal and medial hypertrophy is well described in COPD, apoptosis and re-orientation of the medial smooth muscle cells and the associated matrix has never been described in COPD before. These lesions were encountered so frequently that they were considered pathognomic of the severity of COPD, just as complex vascular lesions such as plexogenic arteriopathy and necrotising arteritis are considered pathognomic of PAH. Although the plane of cut through a vessel would affect the apparent orientation of the smooth muscle cells, the frequency with which this lesion occurred would suggest otherwise. The re-orientation of the medial matrix was also demonstrated using picrosirius red, where the collagen fibres within the matrix were radially aligned and crossed the external elastic lamina.

Similar lesions have however been described in BPD (Hislop AA 2002) and described as changes in SMC "length/width ratios" or "cells becoming more brick-shaped". The presence of re-orientation of medial SMC has also never been described in PAH, although Burton AC (1954) suggested that "longitudinal muscle bands, connecting the internal and external elastic laminae" within the media would have better biomechanical properties to effect persistent vasoconstriction in the presence of chronic hypoxia and to withstand higher intraluminal pressures. Apoptosis of the vascular smooth muscle cells has never been described in COPD. Caspase 3 immunohistochemistry confirmed the H&E appearance of apoptosis within the media however Ki 67 demonstrated a population of proliferating SMC nested within these apoptotic cells. This suggests ongoing repair of the vessel wall, although the prevalence of such apoptosis, is evidence of the failure of vascular maintenance and homeostatic mechanisms suggested in the development of COPD (Kasahara Y 2000).

In this study we described the most severely remodelled vessels as having lost all normal muscular arteriolar wall structure, with sclerosis, multiple elastic laminae, apoptosis, and cellular disorganisation. This phenotype of remodelling affected only the vessels with a larger lumen, and with greater wall thickness than other remodelled arterioles. Although this greater wall thickness is undoubtedly due in part to vessel wall oedema, these vessels appear larger than most other remodelled arterioles in the study. Despite the appearances of uncoordinated collagen deposition and cellular disorganisation, these severely remodelled vessels resemble the much larger, more proximal elastic arteries. Much like the well-described extension of medial smooth muscle cells into arterioles <80μm diameter, this could be evidence of the distal extension of elastic arteries.

The cellular phenotype of the remodelling in our population is also of interest. It is well known that primary PAH is as a result of abnormal endothelial proliferation (Lee SD 1998). Many studies (Santos, Peinado, Barbera JA) have also demonstrated the intimal hypertrophy in COPD also to be endothelial in origin. However, these studies are not in patients with severe COPD, nor is the possibility of COPD associated PAH excluded. Our study has shown that the intimal and medial hypertrophy is due to expansion of the mesenchymal component as demonstrated by the positive staining to both desmin and vimentin and lack of CD31 staining. The desmin positive cells within the intima are most likely to be due to SMC migration through gaps in the elastic lamina (Aiello VD 2003, Smith P 1992, Dingemans KP 1976), however differentiated reparative myofibroblasts, or differentiated endothelial cells (Frid MG 2002) cannot be completely excluded.

CD31 staining was limited to endothelium in all arterioles, regardless of severity or phenotype of remodelling, while positive desmin and vimentin staining of both media and intima suggest mesenchymal led remodelling. Therefore pulmonary arteriolar remodelling in COPD, particularly the medial lesions appear dissimilar to classical descriptions of PAH.

Different staining patterns with desmin and vimentin immunohistochemistry were also noted. Arterioles with apoptosis and reorientation stained intensely positive for both desmin and vimentin in both the intima and media, however the cells in those arterioles with loss of vessel wall structure, appeared to lose desmin positivity yet retain vimentin positivity with proximity to the lumen. This suggests that either: the SMCs close to the lumen have been replaced by fibroblasts; or they have de-differentiated and lost their desmin positive intermediate filaments. Pulmonary artery SMC's have been shown to originate from two different sources during foetal lung development, namely the inner layers from the bronchus (Hislop AA), and the outer layers from the mesenchyme/fibroblasts (Frid MG 1997b). It is possible that the different repair phenotypes demonstrated in this study are related to these different SMC origins.

In summary, we have demonstrated that a wide range of remodelling reactions are present within the arterioles of patients with severe COPD, and that they involve proliferation of the mesenchymal elements of the media. This is possibly a response to inflammation. The medial changes are morphologically distinct from the classical descriptions of PAH. The role of post-inflammatory events, including regional hypoxia, will form the basis of future investigations within the context of this thesis.

Chapter Two

A Grading System for Vascular Remodelling

Introduction

Studies on pulmonary vascular remodelling in COPD have been limited by the lack of a standardised grading system. Given the heterogenous nature of the disease, it could be argued that a unified system embracing all levels of pathology is unrealistic. The vessel wall changes seen in severe COPD are repeatedly interpreted as COPD-associated PAH. There is data to suggest similarities between the lesions seen in PAH, and those seen in severe COPD (Barbera JA et al 2003). Many of the histopathological features described in the previous chapter, such as intimal and medial hypertrophy, muscularisation of arterioles of less than 80μm diameter and intimal and medial sclerosis, are generic to pulmonary vascular remodelling, and not specific to any particular diagnosis. In PAH, other histopathological features which are not seen in COPD have also been documented. These include multilayer endothelial cell proliferation, arterial necrosis, and the formation of plexiform lesions. Similarly in the previous chapter we have described arteriolar wall pathology never previously documented in severe COPD or indeed PAH.

Several grading systems have been published in an attempt to quantify severity of pulmonary vascular remodelling based upon pathological features (Heath D 1958, Wagenvoort CA 1974, Roberts WC 1986). All these grading systems were developed to guide management and predict clinical outcome in patients with pulmonary hypertension either primary or secondary to congenital heart disease. All are based upon wall hypertrophy and include complex lesions. Criticisms aimed at these grading systems in the published literature are numerous. They include a lack of correlation with disease progression by the Heath-Edwards classification (Rabinovitch M 1978), or

being too simplistic (Roberts classification) (Wagenvoort CA 1987). The patient grade in all of these classifications is also based upon the most remodelled vessel seen, with no account of heterogeneity within the sample.

For the purposes of this study, the existing grading systems do not accurately describe the remodelling phenotypes and hence are not applicable. None has the required sensitivity to accurately differentiate between the patients in our study population. As demonstrated in the previous chapter, we have a range of phenotypes present, suggesting differences in the severity of remodelling which do not translate into changes in overt wall thickness. None of the existing classification systems has the required sensitivity to elucidate the contribution of individual pathological features to the remodelling seen in each arteriole.

One of the startling features of the previous chapter was the heterogeneity of remodelling seen. Even within a few millimetres of lung tissue, arteriolar remodelling ranged from mild to severe, despite the samples being the most severely affected areas of lung from the patients at the worst end of the COPD spectrum. This heterogeneity has never been described or fully investigated.

We hypothesise that certain histopathological features of pulmonary vascular remodelling in severe COPD, and their sequelae, are related to outcome following LVRS. This study describes the development and validation of our histology-based grading system, and its correlation with clinical data.

Methods

Patients

In total 309 LVRS tissue blocks were studied in twenty patients. A variable number of BV pairs were available for study from each patient depending upon the size of the specimen. In ten patients, only a small number of tissue blocks, and hence a small number of BV pairs were available for study.

Samples of lung tissue were also obtained with ethical consent from nine patients who underwent lung resection for malignancy (n=8 lobectomy for Non Small-Cell Lung Cancer, n=1 pleuro-pneumonectomy for mesothelioma) during the same time period. Patients in this group were subject to standard surgical inclusion and exclusion criteria for patients undergoing lung resection for malignancy (Armstrong P 2001). Only a single block of tissue was available from each patient in this malignancy group.

Tissue

Lung tissue sections from both the LVRS and the malignancy patients underwent identical processing as previously described. Only grossly normal areas of peripheral lung distant from the tumour were sampled.

A single slide was taken from each block and stained with H&E.

Grading Algorithm

The grading algorithm (see Table 2.1) was developed to encompass all pathological features (both intimal and medial) of pulmonary vascular remodelling seen in our patient cohort. A cumulative score for each vessel was chosen, rather than an overall mild/moderate/severe grading that is so often seen with pathological scoring. This is because a cumulative score has unity variables, which removes the subjectivity of the mild/moderate/severe scoring system, and also allows the contribution of every pathological feature to the overall score to be assessed. The cumulative scoring system was designed to investigate the both the heterogeneity of pulmonary arteriolar remodelling, as well as to assess inter-patient differences in severity of remodelling. The scoring system was weighted in favour of the medial features, as the data from the previous chapter suggested a mesenchymal origin for the pathological features seen.

Development of the Grading System

Pilot Study

Intra-observer variation was assessed by choosing fifty vessels from the first five patients (the first 10 BV pairs in each patient) and applying the scoring system, blinded to the previous scores. The grading of individual pathological features was then compared, and the scores achieved by the intima, media and entire vessel were correlated.

Ten randomly chosen BV pairs from these vessels were also assessed by an experienced pathologist blinded to the initial scores and inter-observer variation assessed.

Table 2.1

The Cumulative Scoring System. Two points were awarded to each pathological feature that involved more than 50% of the vessel wall; one point, if less than 50% of the vessel wall was affected, and zero points if the pathological feature was absent.

Pathological Feature	Score Achieved
Intima:	
Hypertrophy / Hyperplasia	**0 / 1 / 2**
Roughened / fibrillated Endothelium	**0 / 1 / 2**
Luminal Thrombus	**0 / 2**
Fibrosis / Collagen Deposition	**0 / 1 / 2**
New Internal Elastic Lamina	**0 / 1 / 2**
Media:	
Hypertrophy / Hyperplasia	**0 / 1 / 2**
Reorientation of medial smooth muscle cells into a radial alignment	**0 / 1 / 2**
Apoptosis	**0 / 1 / 2**
Fibrosis / Collagen Deposition	**0 / 1 / 2**
Leucocytes present within wall	**0 / 1**
Fragmented Internal Elastic Lamina	**0 / 1 / 2**
Fragmented External Elastic Lamina	**0 / 1 / 2**
Total Score:	**/ 23**

Heterogeneity of Remodelling Studies

In order to assess the ability of the grading system to differentiate between vessels with different severities of remodelling, we performed two further studies:

Firstly, in patients 9-14 inclusive, we identified every slide that contained one or more BV pairs. On these slides we also identified the most severely remodelled vessel upon each slide (the "worst" vessel). The worst vessel did not form part of a BV pair. We then compared the severity of remodelling between these worst vessels and the BV pairs.

Secondly, we identified the ten LVRS patients who had contributed the least number of BV pairs (patients 2, 3, 6, 7, 8, 16, 17, 18, 19, 20). They were chosen because the number of patients, the total number of BV pairs, and the number of BV pairs/patient approximated to the nine patients undergoing lung resection for malignancy. The severity of remodelling was then compared between the two groups.

Population Study

All BV pairs from the twenty LVRS patients' samples were then graded as part of the population study.

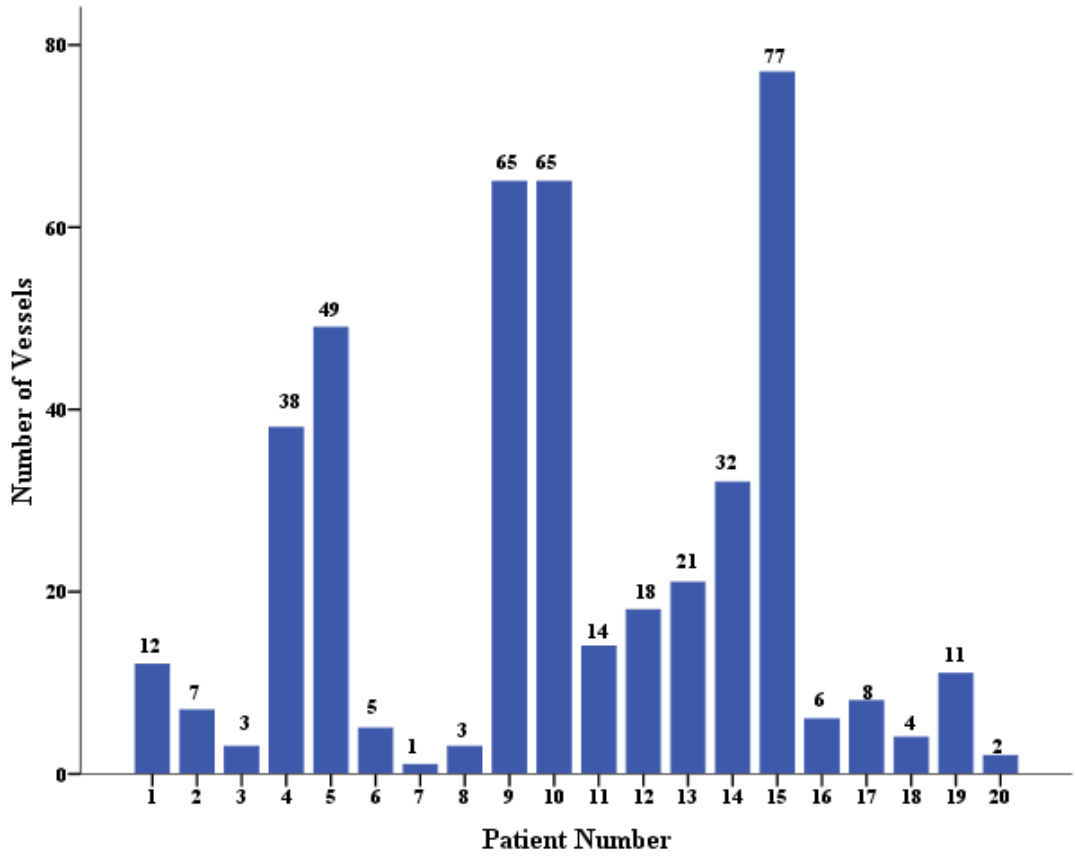
Results

Pathology of Lesions

Specimens from the twenty LVRS patients in the population study resulted in 301 H&E slides, which generated 441 broncho-vascular pairs (Figure 2.1). Most vessels from the LVRS population showed evidence of intimal and/or medial hypertrophy, and endothelial activation. With increasing severity of remodelling, apoptosis and radial re-alignment of the medial cells and matrix became a prominent feature. The most severe remodelling was demonstrated by complete disruption of the normal vessel wall architecture. Multiple new elastic laminae were present, with disorganisation of the alignment of the medial smooth muscle cells and sclerosis affecting both intimal and medial compartments. Intimal sclerosis alone, without medial sclerosis was rarely seen, however medial sclerosis alone was seen in vessels with severe medial remodelling. Both medial and intimal sclerosis was only present in the more severely remodelled vessels.

Figure 2.1

Bar chart demonstrating the number of vessels contributed by each patient



Pilot Study

Intra-observer variation was assessed by repeated examination of the 50 chosen vessels, blinded to previous scores. No significant difference was found in the scores of individual pathological features from these 50 vessels (Table 2.2) when repeatedly assessed ($p=0.92$, $p=0.79$ and $p=0.65$ for intima score, media score and overall vessel score respectively). A good correlation between assessments was also seen (Figure 2.2) in terms of the total intima score ($p<0.001$, $r=0.56$), the total media score ($p<0.001$, $r=0.80$) and the overall vessel score ($p<0.001$, $r=0.76$).

Inter-observer variation was assessed when ten randomly chosen slides were independently examined by a second observer (MLF). There was no significant difference found in scores achieved for pathological features except the assessment of sclerosis. This resulted in the overall co-efficient of variance being 14%. Despite this high coefficient, it must be noted that sclerosis can only be subjectively assessed with H&E staining, whereas all other pathological features are more objectively assessed. It is probable that this factor that is the main contributor to the variance.

Table 2.2

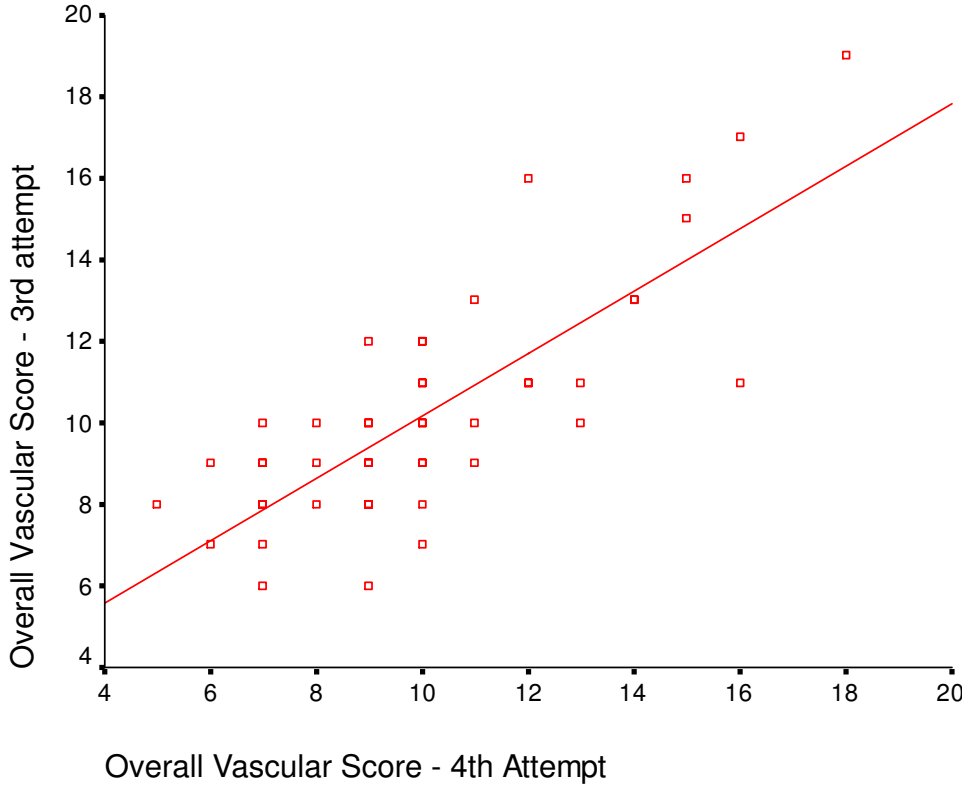
Demonstrating no significant intra-observer variation between the pathological features.

Scores quoted as median (range)

Intima	**4th Scoring**	**5th Scoring**	***p* value**
Hypertrophy	2 (0 – 2)	2 (0 – 2)	0.56
Roughened Endothelium	2 (1 – 2)	2 (1 – 2)	0.62
Thrombosis	0 (0 – 2)	0 (0 – 2)	0.56
Sclerosis	0 (0 – 2)	0 (0 – 2)	0.86
New I.E.L. formed	0 (0 – 1)	0 (0 – 1)	0.34
Total Intima Score	4 (2 - 9)	4 (2 - 9)	0.92
Media			***p* value**
Hypertrophy	1 (0 – 2)	1 (0 – 2)	0.57
Re-orientation of SMC	2 (0 – 2)	2 (0 – 2)	0.88
Apoptosis	2 (0 – 2)	1 (0 – 2)	0.85
Sclerosis	0 (0 – 2)	0 (0 – 2)	0.35
Leucocytes	1 (0 – 1)	1 (0 – 1)	0.41
Fragmented I.E.L	0.5 (0 – 2)	0 (0 – 2)	0.49
Fragmented E.E.L	0 (0 – 1)	0 (0 – 1)	0.57
Total Media Score	6 (1 – 10)	6 (3 - 10)	0.79
Total Score			***p* value**
Total Vascular Score	9 (5 - 18)	9.5 (6 - 19)	0.65

Figure 2.2

Scatterplot demonstrating the correlation between repeated scoring ($p<0.01$, $r=0.76$)



Heterogeneity of Remodelling Studies

"Worst" Vessel Study

The cumulative scoring system was applied to all 215 BV pairs from the six patients being studied and the results compared with 87 "worst vessels". These results are summarised in Figure 2.3. A wide range of remodelling reactions and thus a large degree of heterogeneity is demonstrated in the histogram with scores ranging from 3 to 19 out of a possible 23. When a comparison is made of the intima, media and overall vessel scores between the two groups, the "worst vessels" have significantly higher intima, media and total scores than the BV pairs (Table 2.3).

Figure 2.3

Graph demonstrating the heterogeneity of remodelling within the LVRS samples, as well as the difference between broncho-vascular pairs and the worst vessels

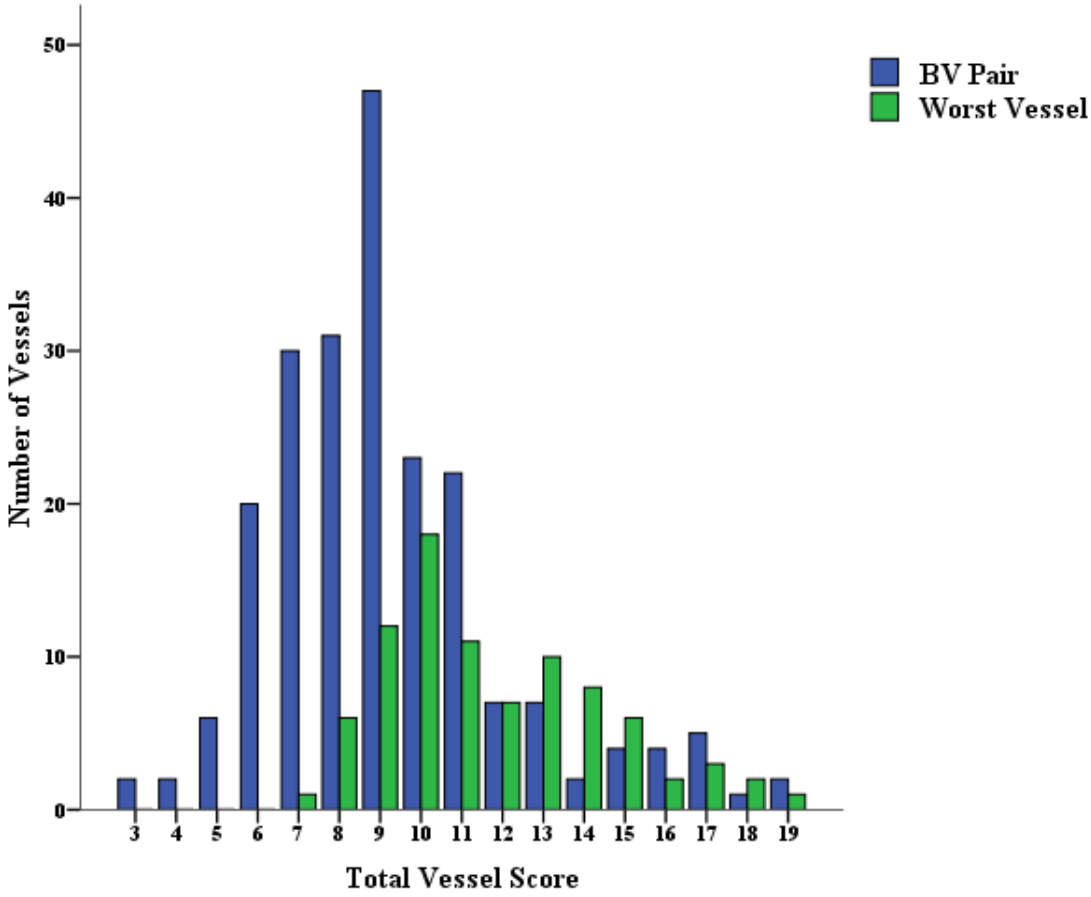


Table 2.3

Table comparing the severity of remodelling between the broncho-vascular pairs and the "worst vessels" in the LVRS cohort.

	Broncho-vascular Pair	"Worst Vessel"	p value
Intima Score	4 (0 – 9)	5 (2 – 8)	< 0.001
Media Score	5 (0 – 11)	6 (3 – 11)	< 0.001
Overall Vessel Score	9 (3 – 19)	11 (7 -19)	< 0.001

LVRS v Malignancy Study

In the second study, samples from the nine patients in the malignancy group revealed 53 BV pairs. These were graded and compared with the 50 BV pairs from the ten LVRS patients. The preoperative demographic data of the two groups are compared in Table 2.4. The LVRS group is shown to be significantly younger, with worse spirometry than patients undergoing lung resection for malignancy. The results are summarised in figure 2.4 and table 2.5. Heterogeneity is a feature of the LVRS cohort with total vessel scores ranging from two to twenty for the LVRS group, but only four to eleven in the malignancy group. When compared to the remodelling seen in the malignancy group, the LVRS cohort demonstrated significantly worse intimal and medial remodelling (Table 2.5).

When the remodelling in the ten LVRS patients was compared to the remodelling in the other ten LVRS patients, no statistically significant differences in intima, media, and total scores were found.

Table 2.4

Comparison of the pre operative characteristics of the LVRS v malignancy patients.

Variable	LVRS n = 10	Malignancy n = 9	p value
Age (Years)	60 (45 - 70)	74 (53 - 84)	0.02
Gender (M:F)	8:2	5 : 4	0.35
FEV_1 (%pred)	25 (17-33)	70 (44-93)	<0.001
FVC (%pred)	58 (42-74)	107 (99-112)	=0.007

Figure 2.4

Graph demonstrating the heterogeneity of remodelling within the LVRS samples, as well as the difference in total vessel scores between BV pairs in LVRS and malignancy cohorts.

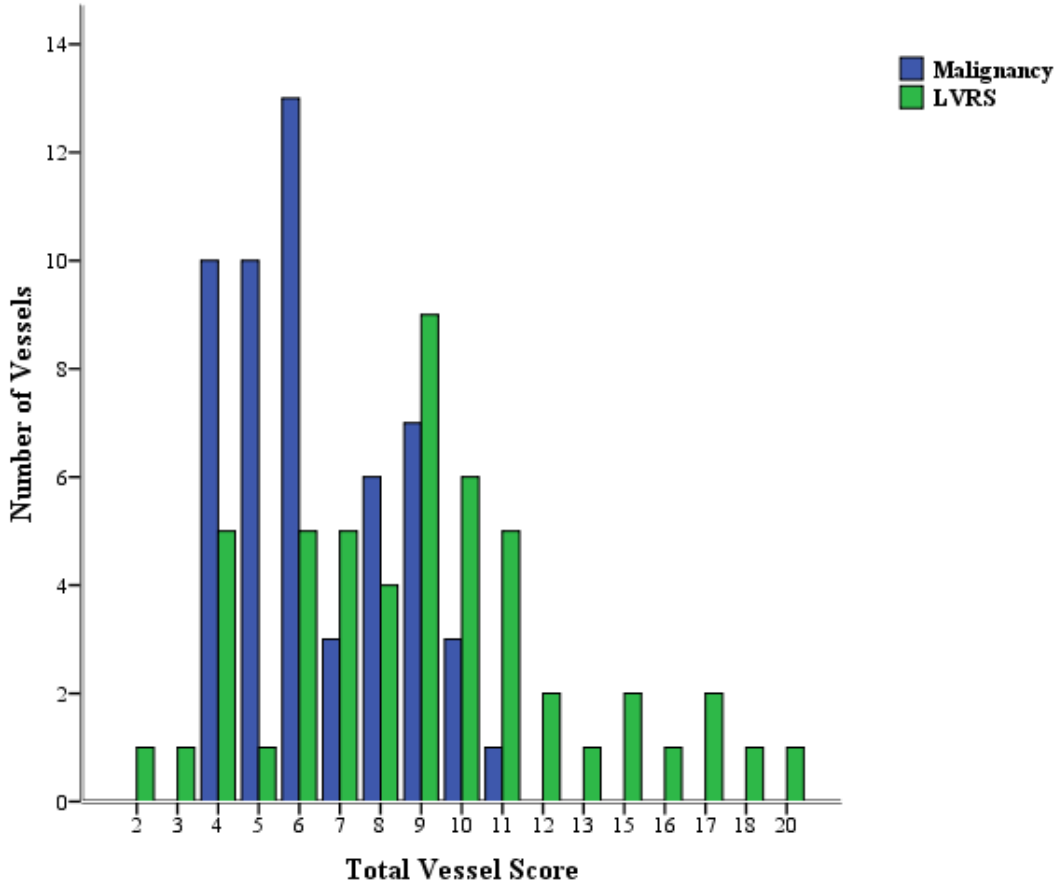


Table 2.5

Table comparing the severity of remodelling between the LVRS and malignancy cohorts.

	Broncho-vascular Pairs	Malignancy	p value
Intima Score	4 (1 - 9)	3 (1 - 6)	0.006
Media Score	5 (1 - 11)	3 (0 - 7)	< 0.001
Overall Vessel Score	9 (2 - 20)	6 (4 - 11)	< 0.001

Analysis of Variance Study

We excluded the five patients who had contributed less than 5 vessels (patients 3, 7, 8, 18, 20) to the population study, and performed a one way ANOVA on the remaining 15 patients (428 vessels) to determine if there was significant inter-patient variation for the intima, media and overall vessel scores. The variance of the mean intima, media and total scores is demonstrated in figures 2.5 - 2.7. Significant variance was found for all three scores (all $p<0.001$), suggesting that it may be possible to differentiate between patients. Unfortunately when the same test was applied to the twelve individual pathological features graded, only luminal thrombosis and a new IEL were not significant.

Figure 2.5

Scatterplot demonstrating the variance of mean intima scores. Horizontal lines indicate mean (3.58) and 1 standard deviation (SD)

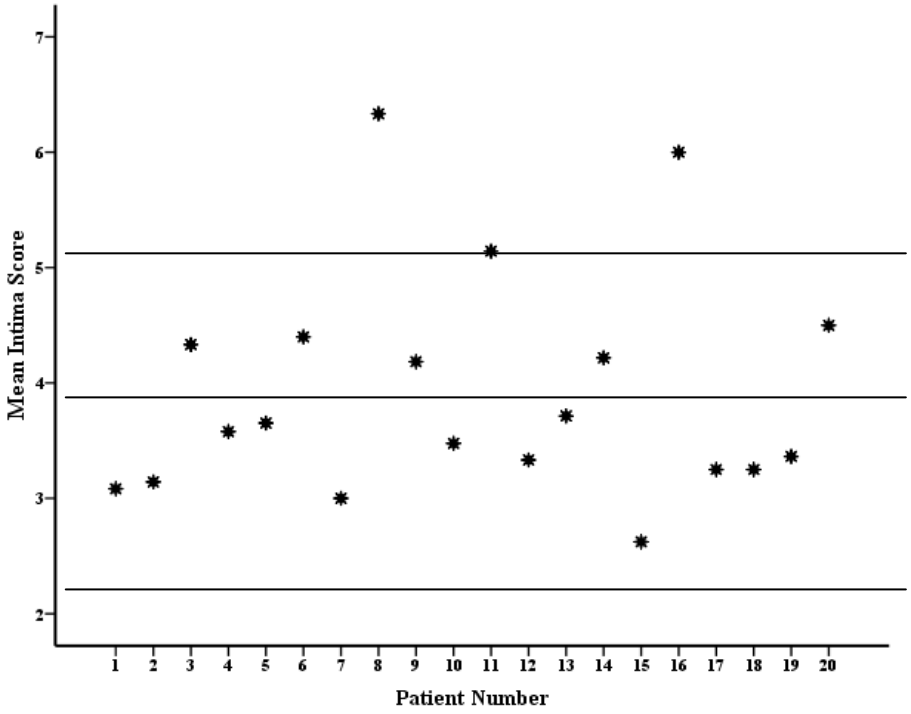


Figure 2.6

Scatterplot demonstrating the variance of mean media scores. Horizontal lines indicate mean (4.8) and 1 SD

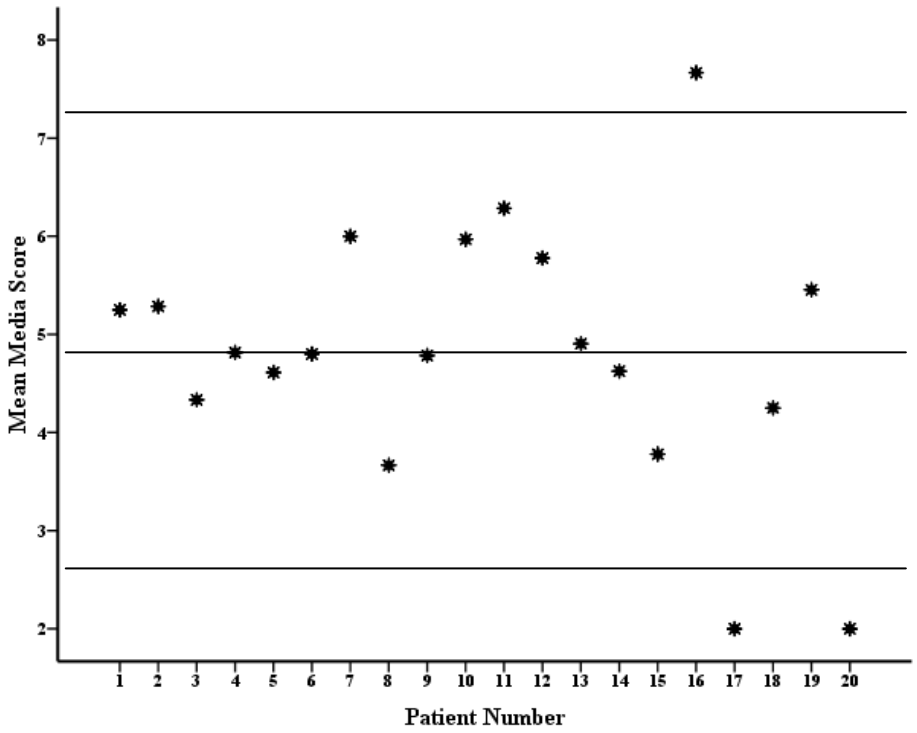
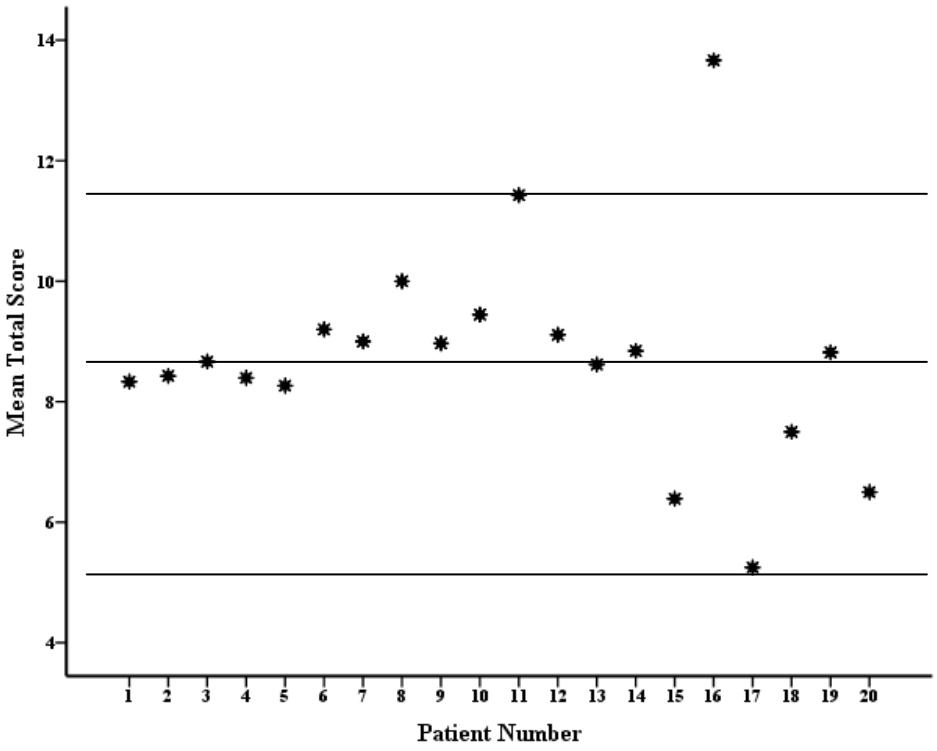


Figure 2.7

Scatterplot demonstrating the variance of mean total scores. Horizontal lines indicate mean (8.8) and 1 SD



Contribution of Each Pathological Feature to Overall Severity of Remodelling

In order to determine the relative contribution of each individual pathological feature to the overall severity of remodelling and the potential for combinations of features to predict severity, we performed two further studies:

The first divided the population into two groups – above and below the median total vessel score. The percentage of vessels that displayed each feature in both groups was determined and a *chi*-squared test performed (see table 2.6). There were significantly

more vessels displaying each feature in vessels above the median total score ($p<0.001$ in all cases), except for fragmented external elastic lamina where no significant difference between the two groups was noted ($p=0.8$).

Table 2.6

Demonstrating that vessels with above median remodelling scores have significantly more of every pathological feature (except fragmented EEL) than below median remodelled vessels

Pathological Feature	Below median Total score	Above median Total score	p value
Intimal Hypertrophy	80	100	<0.001
Roughened endothelium	92	100	<0.001
Thrombosis	0	4	0.007
Intimal sclerosis	16	48	<0.001
New IEL present	5	29	<0.001
Medial hypertrophy	55	87	<0.001
SMC repolarisation	67	98	<0.001
SMC apoptosis	52	96	<0.001
Medial sclerosis	5	42	<0.001
Leucocytes present	63	77	0.001
Fragmented IEL	16	37	<0.001
Fragmented EEL	3	4	0.8

The second study involved a multivariate and linear regression analysis using forward stepwise addition to ascertain which features or combination of features would predict severity of remodelling. All pathological features except fragmented EEL were again found to be significant ($p<0.001$).

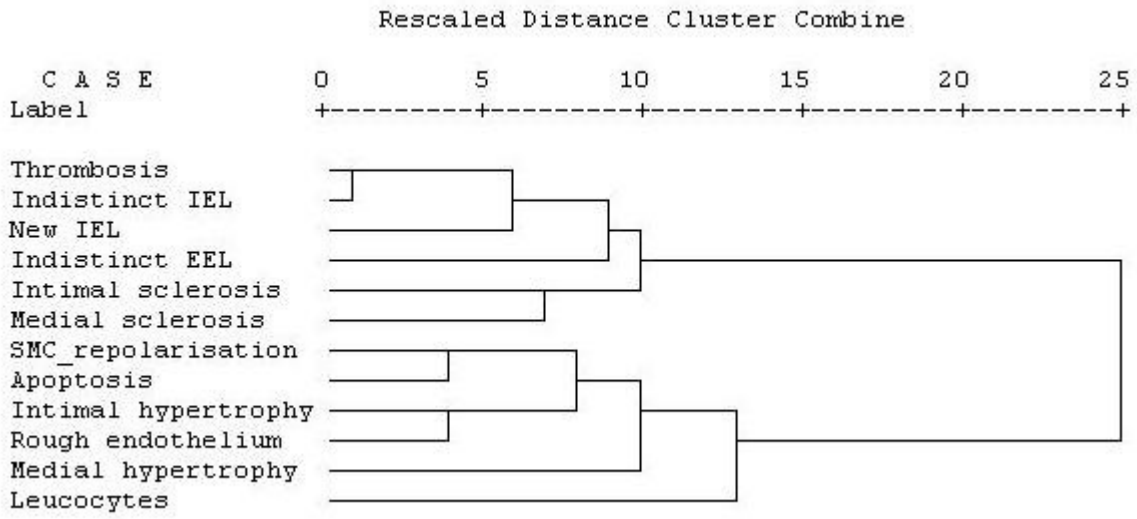
Hierarchical Cluster Analysis

In order to try to elucidate the role of individual pathological features to the overall remodelling score, and to attempt to further differentiate between patients we performed a hierarchical cluster analysis. This was achieved by agglomerative clustering using between-groups linkage.

We performed two analyses. The first involved clustering all 441 vessels based upon the presence or absence of the twelve pathological features to produce a dendrogram and heat map. The results of this first cluster are shown in Figures 2.8 and 2.9.

Figure 2.8

Dendogram showing the cluster relationship between pathological features

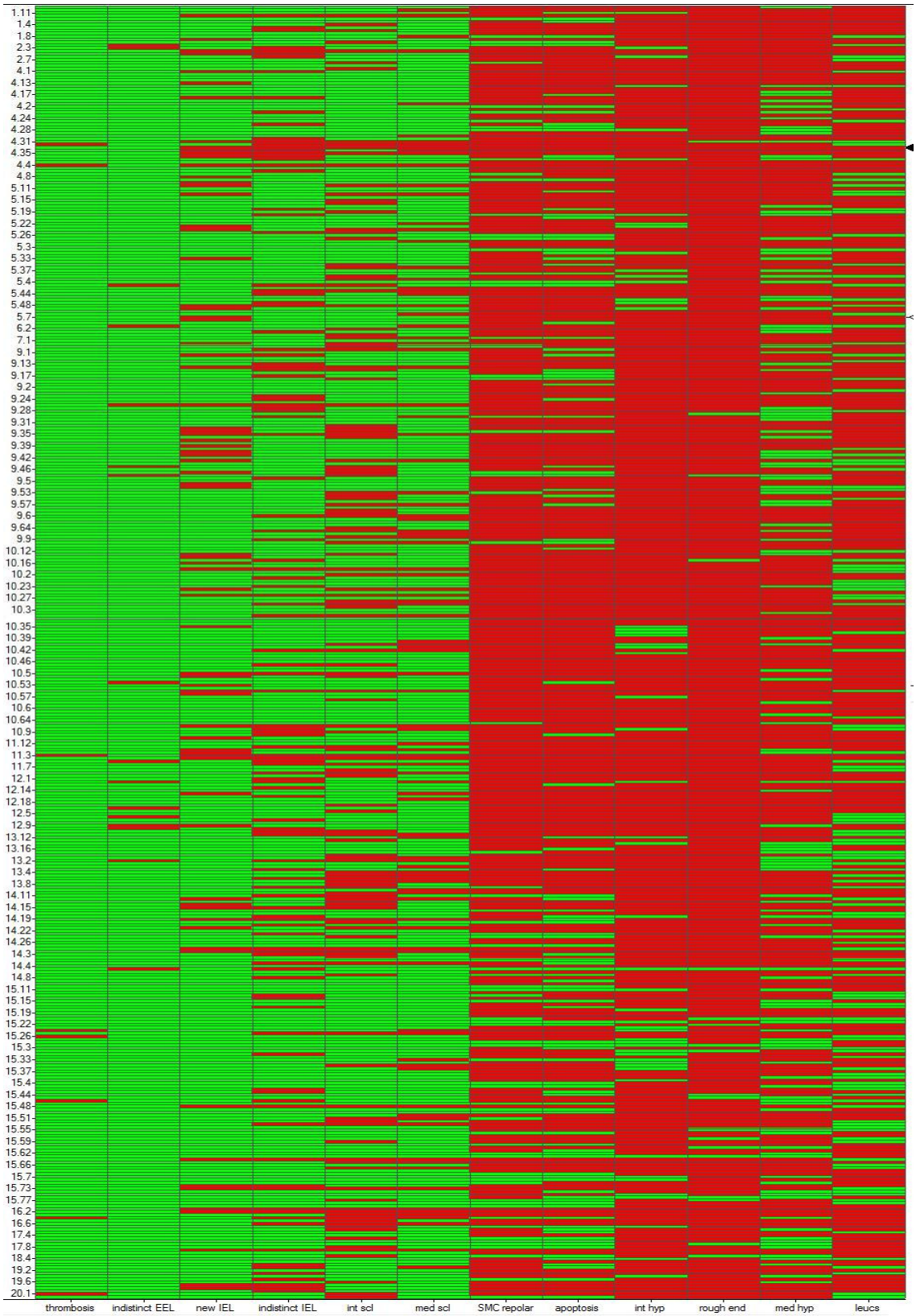


The analysis demonstrates that the pathological features belong to two main clusters, which have distinctly different characteristics, as shown by the distance between the two. In the first cluster, thrombosis was most commonly associated with an indistinct IEL. While all the pathological features within the second cluster were the most commonly found within the study population. The combinations of intimal hypertrophy with activated endothelium and SMC repolarisation with SMC apoptosis were the features most commonly associated with each other.

The results of this clustering determined the columns of the following heatmap (figure 2.9), while all 441 vessels form the rows. The incidence of each pathological feature can easily be seen, confirming that the second cluster contained the most frequently observed features.

Figure 2.9

Heatmap showing the distribution of pathological features in every vessel. Green is where a feature is absent, red where present.



The second study involved clustering the average score per arteriole achieved for each pathological feature in all twenty patients.

The dendrogram (figure 2.10) shows that patients 4, 19 and 5 are most closely associated with each other. Patients 1 and 2 are also closely linked. The patients that are the least closely linked with each other are patients 16, 8, 20, 17 and 7. The clustering demonstrated in this dendrogram formed the basis of the rows in the following heatmap (figure 2.11). The twelve pathological features constituted the columns. The spectrum of blue represents the mean score of each pathological feature, with dark blue indicating a higher mean score and white representing zero.

There did not appear to be any significant clustering of patients according to the presence or severity of a particular pathological feature.

Figure 2.10

Dendrogram demonstrating the cluster relationships between the twenty patients

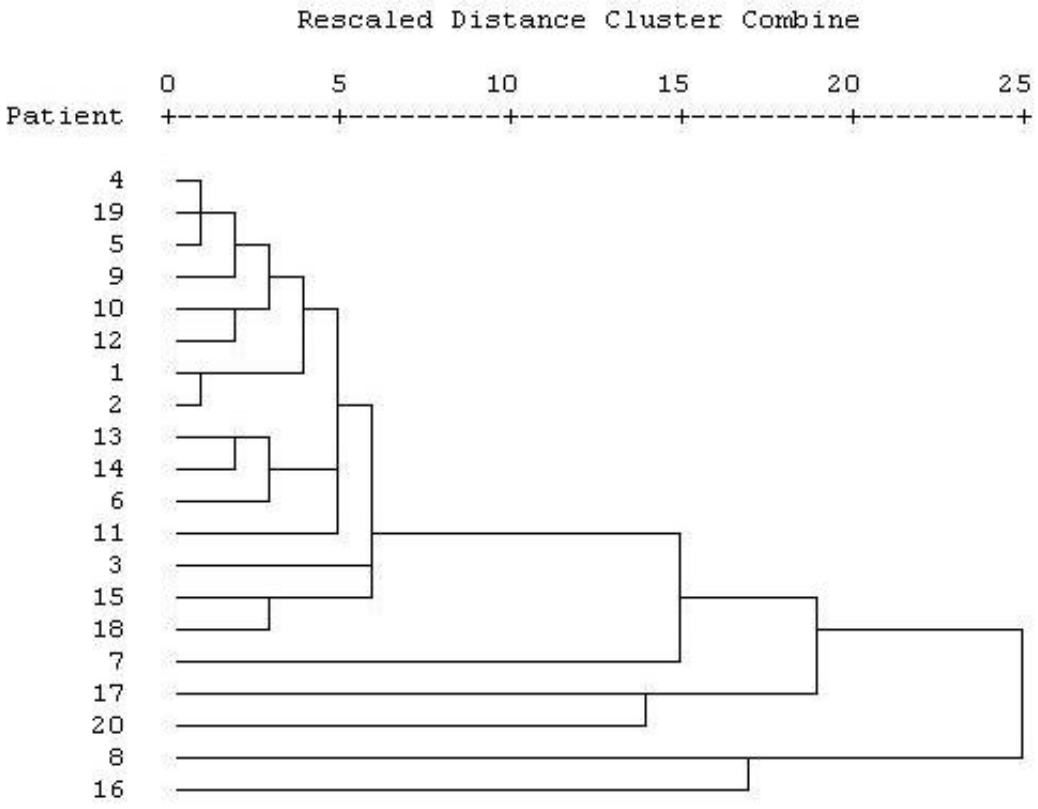
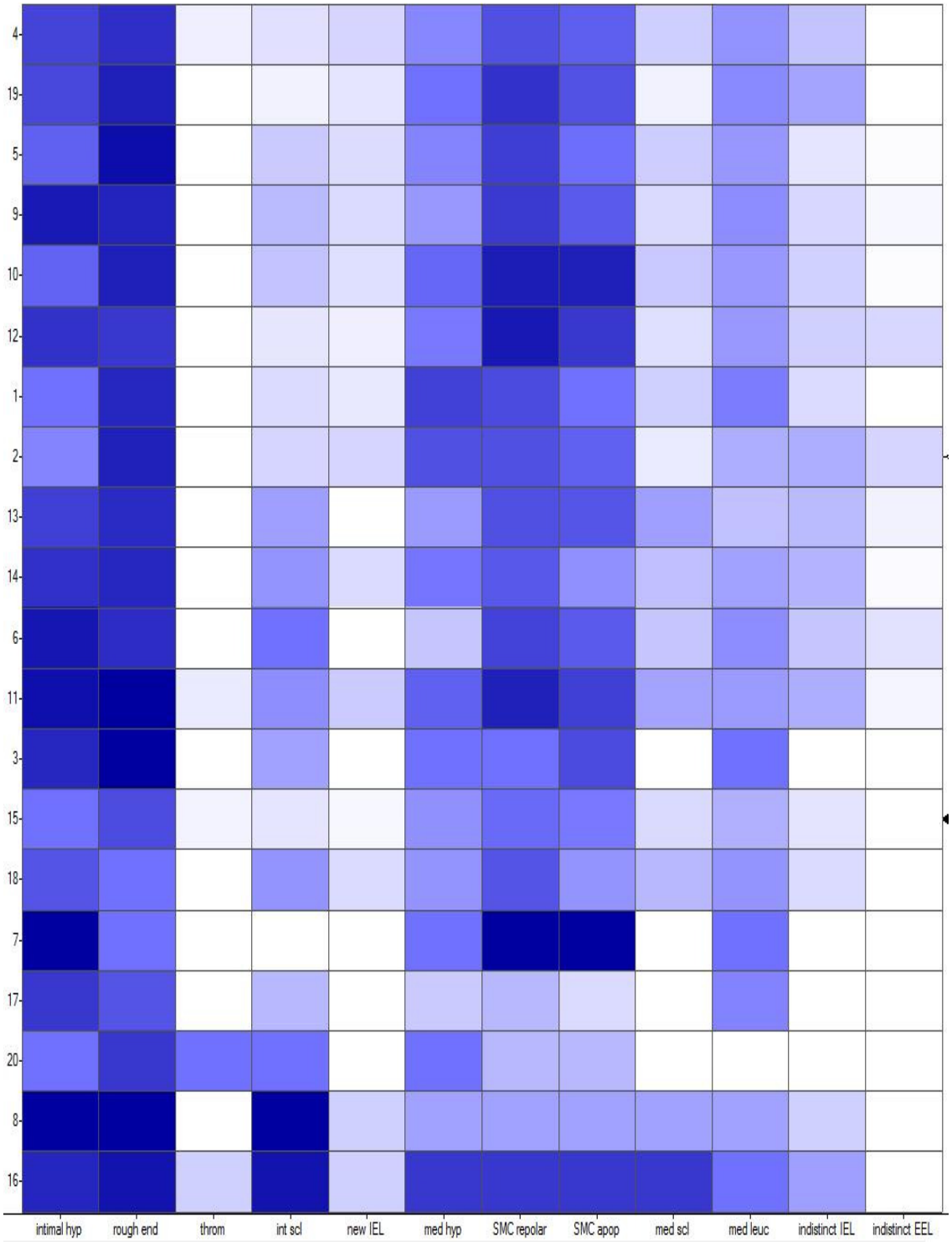


Figure 2.11

Heatmap of mean scores achieved for each individual pathological feature in every patient. A score of zero is shown as white, light blue equates to a mean score of 1, while dark blue equates to a score of 2



Correlation with Clinical Outcome:

The one way ANOVA suggested significant variations between the patients' mean intima, media and total remodelling scores. As a result of this, we then attempted to correlate each patient's mean scores with clinical outcome following their LVRS procedure. We used change in body mass index (BMI) and percentage change in FEV_1 at three, six and twelve months post operatively as markers of clinical outcome. These were chosen because of their significant differences from preoperative values. A summary of the twenty patients' post-operative outcomes are shown in table 2.7.

There were no peri-operative deaths amongst the twenty patients. Although four had died post operatively, only one was within a year of their operation (34 days). The other patients died at 20, 53 and 59 months postoperatively.

Table 2.7

Variable	3 months	6 months	12 months
% Change in FEV_1	17.6 (-13.3 – +72.7)	12.2 (-16.1 – 38.2)	0 (-30 – 54.5)
Change in BMI	-0.74 (-4.52 – 0.71)	1.23 (-3.55 – 1.77)	-0.26 (-2.57 – 2.13)

The relationship between time post operatively and changes in FEV_1 is more linear than the relationship between time post op and BMI. A significant correlation was found between the media score and change in FEV_1 at three and twelve months (see table 2.8). There was no correlation between the intima score or total score and the change in FEV_1. There were also no correlations with the intima, media and total scores and change in BMI (see tables 2.8 and 2.9).

Table 2.8

Table showing correlation coefficients between intima score, media score, total vessel scores and percentage change in FEV_1. Significant correlations are highlighted

Variable	% Change in FEV_1 at 3 months	% Change in FEV1 at 6 months	% Change in FEV_1 at 12 months
Intima Score	0.18	0.27	0.07
Media Score	0.66*	0.58	0.79*
Total Score	0.59	0.56	0.65

* $p < 0.05$

Table 2.9

Table showing correlation coefficients between intima score, media score, total vessel scores and change in BMI

Variable / Time	Change in BMI at 3 months	Change in BMI at 6 months	Change in BMI at 12 months
Intima Score	0.07	-0.21	0.26
Media Score	0.48	0.83	0.51
Total Score	0.40	0.53	0.52

We hypothesised that simply using intima, media and total vessel scores was potentially masking the effect of a particular phenotype of patient. For example, some patients may have more fibrosis contributing to the score, while others may have more apoptosis. We then investigated if any particular pathological feature of remodelling was associated with outcome following LVRS.

We calculated the mean score achieved by each individual pathological feature in each patient and correlated this with the outcome measures. These are demonstrated in tables 2.10 and 2.11.

Table 2.10

Correlation coefficients between individual pathological features and percentage change in FEV_1. Significant correlations are highlighted

Pathological feature	% Change in FEV1 at 3 months	% Change in FEV1 at 6 months	% Change in FEV1 at 12 months
Intimal hypertrophy	0.20	-0.01	-0.11
Roughened endothelium	0.09	0.29	0.25
Thrombosis	-0.23	-0.09	-0.29
Intimal fibrosis	0.16	0.17	0.08
New IEL	0.40	0.91*	0.57
Medial hypertrophy	0.56	0.57	0.71*
SMC repolarisation	0.36	0.46	0.57
SMC apoptosis	0.37	0.36	0.46
Medial fibrosis	0.67*	0.54	0.70*
Leucocytes	0.68*	0.22	0.61
Indistinct IEL	0.48	0.51	0.52
Indistinct EEL	-0.29	-0.35	0.20

*p<0.05

Table 2.11

Correlation coefficients between individual pathological features and change in BMI. Significant correlations are highlighted.

Pathological feature	Change in BMI at 3 months	Change in BMI at 6 months	Change in BMI at 12 months
Intimal hypertrophy	0.57	0.63	0.68
Roughened endothelium	-0.09	-0.24	-0.35
Thrombosis	-0.55	-0.92 *	-0.13
Intimal fibrosis	0.01	-0.18	0.33
New IEL	-0.03	0.5	0.21
Medial hypertrophy	0.13	0.16	0.34
SMC repolarisation	0.43	0.91*	0.38
SMC apoptosis	0.42	0.70	0.52
Medial fibrosis	0.26	0.40	0.43
Leucocytes	0.71*	0.97*	0.37
Indistinct IEL	0.16	0.43	0.12
Indistinct EEL	0.21	0.00	-0.54

*p<0.05

When the individual pathological features were analysed, scattered significant correlations were found. Medial fibrosis correlated with percentage change in FEV_1 at three and twelve months post operatively, while medial hypertrophy and leucocytes correlated with change in FEV_1 at twelve and three months respectively. The presence of leucocytes also correlated with change in BMI at three and six months.

Discussion

A recurrent interpretation of pulmonary vascular remodelling in severe COPD is that of pulmonary hypertensive changes. Indeed some of the pathological features seen in this study are identical to those seen in pulmonary arterial hypertension (PAH), namely intimal and medial hypertrophy and intimal sclerosis. Clinical evidence of PAH at rest, however, is an exclusion criterion applied to all the patients in our cohort (Fishman A 2003, Oey IF 2003). We have never experienced complex lesions characteristic of PAH such as arteritis, intimal concentric laminar thickening, or plexiform lesions (Pietra GG 2004). These observations further support the hypothesis that the remodelling seen in our LVRS patients is due to factors other than PAH. We have distinguished our study from previous work (Hicken P 1965, Naeye RL 1974), where the patient phenotype is less well defined, by studying patients with severe COPD who specifically do not have evidence of PAH at rest.

The grading systems described by Heath and Edwards (1958), Roberts (1986) and Wagenvoort (1974) were all developed to categorise patients with PAH according to clinical outcome. They are all inappropriate for our purposes. If our observations were entered into these grading systems, most vessels would be identically scored, without any impression of heterogeneity both within and between patients. This grading system was developed as a research tool, in order to quantify the severity of remodelling in a well-characterised patient group. It was designed specifically as a hierarchical system encompassing all the pathological features of vascular remodelling we encounter in severe COPD. By allocating a score to each individual feature to produce an overall score, not only does it demonstrate the overall severity of remodelling in an arteriole, but also the number of pathological features and severity of each individual feature.

Thus the relative contribution of each feature to the overall remodelling picture can be dissected out.

The purpose of any grading system is to record the pathological features that are thought to indicate severity of disease (Ishak K 1995). This usually produces a single label (eg. 0, 1 or 2, or mild/moderate/severe) for the whole tissue examined, which unfortunately reduces the amount of information available (Cross SS 1998), and is reliant upon the "morphological integration by the pathologist of many different features" (Ishak K 1995). By scoring each pathological feature individually, our grading system does not suffer this relative subjectivity and lack of sensitivity.

An important factor when developing a grading system is reproducibility (Cross SS 1998). Our grading system is based upon a unity score that is easily reproducible using the standard histopathological stain – Haematoxylin and Eosin. Every feature, apart from the assessment of sclerosis, is objectively present or absent. Our inter-observer coefficient of variance of 14% could be regarded as high. However it should be noted that the single feature of bias was the assessment of sclerosis. This is almost certainly because H&E is not the optimum stain to assess the presence or absence of collagen, elastic and fibrous tissue, which strengthens the objective nature of the assessment of the other pathological features. Basing the grading system on the MSB stain rather than H&E, in order to objectively evaluate sclerosis, would unfortunately reduce the amount of data available on the cellular remodelling. The correlation between this grading system and the assessment of matrix changes will be further discussed in subsequent chapters.

The identification of medial apoptosis and medial matrix and cellular re-orientation, not only distinguishes LVRS patients from those with PAH, but also raises more questions about the aetiology and pathophysiology of severe COPD.

We attempted to dissect out the relative contribution of each pathological feature, to ascertain if the presence or absence of a particular characteristic or combination of features would predict more severe remodelling. The cluster analysis demonstrated that six features were present in most arterioles – namely intimal and medial hypertrophy, activated endothelium, SMC repolarisation, SMC apoptosis and the presence of leucocytes implying active inflammation. The other six features were less commonly seen, implying that post-inflammatory remodelling (sclerosis - both intimal and medial), thrombosis and disorganised elastin homeostasis (indistinct IEL and EEL, and new internal elastic laminae) are predictors of more severe remodelling. Unfortunately it was not possible to identify one individual feature that predicted severity.

As previously stated LVRS involves the excision of hypoperfused, hyperinflated, functionless areas of peripheral lung tissue, that is, the most severely affected area of lung from patients with severe emphysema (GOLD stage III and IV) (Fabbri L 2004). Although the ANOVA suggested significant variation between patients, this was almost certainly due the four patients that were greater than one standard deviation from the mean. When analysed using hierarchical clustering, the dendrogram (figure 2.10) demonstrated that patients could not be clustered according to individual pathological features, and thus we were unable stratify the patients.

Using this grading system we have demonstrated that even within such samples there is some evidence of normality of the vasculature and within a few millimetres are the most severely remodelled vessels. The reason why such heterogeneity of arteriolar remodelling exists is unclear, however we hypothesise that it is due to regional hypoxia as a result of post-inflammatory remodelling, the investigation of this will be the focus of future work. Unfortunately due to the heterogeneity within this study, the intra-patient variation appears greater than the inter-patient variability.

Clinical outcome following lung volume reduction surgery is dependent upon the interaction of a multitude of different pre, peri and postoperative factors, including BMI (Vaughan P 2007), distribution of emphysema throughout the lung (Russi EW 1999), the amount of lung resected and post operative complications. This study was certainly not powered to control for factors such as these. Despite this, there were several interesting observations. Medial remodelling was correlated with percentage change in FEV_1 at three and twelve months, with active inflammation (leucocytes) and post inflammatory remodelling (medial sclerosis) being the most important features. Active inflammation was also correlated with changes in BMI within the first six months. This suggests that inflammation and post inflammatory remodelling may have a role in the development of severe emphysema.

The severity of vascular remodelling in severe emphysema does not appear to have an effect upon peri-operative mortality or longer term survival. Again the numbers of patients affected was extremely small, the study was not powered to fully investigate this, and a number of biases are present. If this is indeed the case, other factors including systemic co-morbidities, alveolar destruction and bronchiolar remodelling must be affecting outcome.

Although there is some evidence that the effects of LVRS are more durable than suggested by spirometry alone (Lim E 2006), the size of the study cohort, and the incompleteness of follow up data greater than 2 years post op limited the opportunity to perform these analyses.

In summary we have demonstrated a robust grading system that can be used as a research tool to quantify the severity of pulmonary arteriolar remodelling in patients with severe COPD, and also identify vessels with particular remodelling features to aid future work.

Chapter Three

Image Analysis for:

Morphometric quantification of Arteriolar Remodelling

Introduction

Thickening of the pulmonary arteriolar wall is well recognised in both COPD and PAH (Wagenvoort CA 1960). There is considerable debate however regarding both the aetiology and responsible part of the vessel wall (i.e. intima or media or both). As previously mentioned there are limited studies in well characterised patient groups which have demonstrated intimal expansion in patients with mild/moderate COPD (Santos S 2002) however the role of pulmonary hypertension in these patients is unclear. Other studies have demonstrated medial expansion without intimal hypertrophy (Hale KA 1984), which corresponds to our work.

For many years, the components of vessel walls, particularly the medial layer have been quantified, using a variety of techniques, to establish the response of the vessel to various disease states. The commonest methods used to assess hypertrophy have historically been measurements of "wall thickness" (Kernohan 1929, Barrat Am 1963). Subsequently, with the introduction of image analysis - the extraction of quantitative data from an image, several indices of medial thickness have been derived in order to relate medial hypertrophy to vessel size and are summarised as:

- A ratio of medial thickness to vessel lumen (Larabee WF 1949, Morlock CG 1939), external diameter (distance between diametrically opposite points on the external elastic lamina{EEL}) (Goodale F 1954), or as a percentage of these values (Heath D 1958, Wagenvoort CA 1965, Hicken P 1965, Hasleton PS 1968, Grotjohan HP 1993)
- Medial thickness measured at 4 points around the circumference, one in each quadrant defined by perpendicular lines used to measure diameter in both long and short axis. The percentage wall thickness is then calculated by the formula

percentage wall thickness = (2 x medial thickness x 100) / external diameter (Morrell NW 1995)

All these studies however have been criticised on the basis that vessel diameter and wall thickness are affected by many confounding factors including vasoconstriction during surgery due to either manual handling of the specimen, intraoperative single lung ventilation/regional hypoxia, post-mortem collapse (Fernie JM 1985a), lung inflation during fixation, and tissue fixation with paraffin wax or glycol methacrylate (Fernie JM 1985b). Morphometric analysis of wall thickness is also affected by a tangentially cut vessel (Barbera JA 1994). This has been corrected for in several ways in the literature. Morrell NW 1995 measured the distance between opposite external elastic laminae perpendicularly in the long and short axis, and averaged the two values to give a mean arterial diameter. Barbera 1994, however excluded all vessels with a long axis diameter : short axis diameter of greater than 3.

The measurement of the internal elastic lamina (IEL) is an extension of the above techniques for quantifying vessel wall thickness. It has been demonstrated to show excellent repeatability, and correlates well with the square root of the medial area (Fernie JM 1985a).

The purpose of this study was to objectively quantify intimal and medial hypertrophy, and assess any relationships between the pathological features and morphometry.

Methods

All samples from the previously graded 20 patients were reviewed and only those with serial sections stained with both H&E and EVG were identified. Measurements were taken only from the EVG stained slides, from which the corresponding H&E serial section had been graded. Only vessels with intact internal and external elastic laminae (EEL), and a single complete internal elastic lamina (IEL) were examined, to allow accurate measurement of vessel wall compartments. Vessels that appeared to have been tangentially sectioned on the H&E slide were also excluded from the study.

Morphometric analysis of the arterial walls was performed using Qwin v3 image analysis system (Leica Systems, US). After appropriate calibration for the objective lens used, the luminal surface of the endothelium, the IEL and EEL were traced and the distances recorded. The area encompassed by each line was also recorded. The vessel diameter in the long and short axis and the corresponding medial thickness in these four quadrants was also measured.

These measurements were then compared between vessels whose medial hypertrophy had been graded as 0, 1 or 2 by the grading system described in Chapter two.

Results

Only five patients were found to have serial sections resulting in 117 vessels being analysed. The area occupied by the vessel and its individual components (i.e. lumen, intima and media) are compared between the grades of medial hypertrophy in table 3.1. The median vessel diameter (calculated by circumference of EEL/π) was 717μm (range 267μm – 2457μm).

Table 3.1

Table showing vessel areas depending upon grade of medial hypertrophy. Data presented as median (range)

Variable	Grade 0	Grade 1	Grade 2	p-value
Lumen area (μm^2)	47307 (13141 - 473893)	88058 (6704 – 954033)	91908 (17210 – 584367)	0.32
Intimal area (μm^2)	15266 (6349 - 126976)	29228 (2741 – 172496)	37443 (9350 – 124592)	0.04
Medial area (μm^2)	31650 (13471 - 133999)	59844 (8298 – 258693)	100824 (29482 – 447185)	<0.001
Vessel wall area [intima+media] (μm^2)	47281 (20982 – 260974)	98594 (11039 – 374812)	145721 (43096 – 532944)	<0.001
Vessel area [lumen+intima +media](μm^2)	100743 (36133 - 571711)	184159 (23896 -1179588)	273506 (68532 -1096297)	0.02

There was no significant difference in luminal area between the three grades. However significant differences were present, when the intimal area, medial area, vessel wall area, and total vessel area are compared with the grades of medial hypertrophy. Significant correlations were demonstrated between the grade of medial hypertrophy and all components of the vessel and its wall except luminal area. The strongest

correlation was between medial area and grade of medial hypertrophy ($r=0.49$, $p<0.001$).

The relative contribution of the media to the overall wall thickness according to grade of medial hypertrophy is summarised in figure 3.1. With increasing grade of remodelling, the media occupies a greater proportion of the vessel wall ($r=0.36$, $p<0.001$). A similar picture was found when the mean medial thickness was calculated from measurements at the four quadrants, as per Morrel N 1995 (figure 3.2). A significant correlation was found between grade of medial hypertrophy and mean medial thickness ($r=0.60$, $p<0.001$)

In order to index the medial area to vessel size (larger vessels are more likely to have thicker walls), many researchers have used either external diameter or length of IEL as a ratio to the area under investigation (Fernie JM 1985a). In this study we used the ratio of medial area to length of IEL. As shown in figure 3.3, with increasing severity of hypertrophy, the media occupied significantly more area. Again a significant correlation was found between grade of medial hypertrophy and medial area to IEL length ($r=0.58$, $p<0.001$). The medial area to lumen circumference ratio was also significantly correlated with medial hypertrophy ($r=0.58$, $p<0.001$) media score($r=0.47$, $p<0.001$) and overall severity of vessel remodelling ($r=0.56$, $p<0.001$).

Figure 3.1

Box and whisker plot demonstrating increasing percentage of the vessel wall area (intima and media) occupied by the media with increasing grade of hypertrophy. Thick black line represents median and whiskers represent range; red box represents interquartile range.

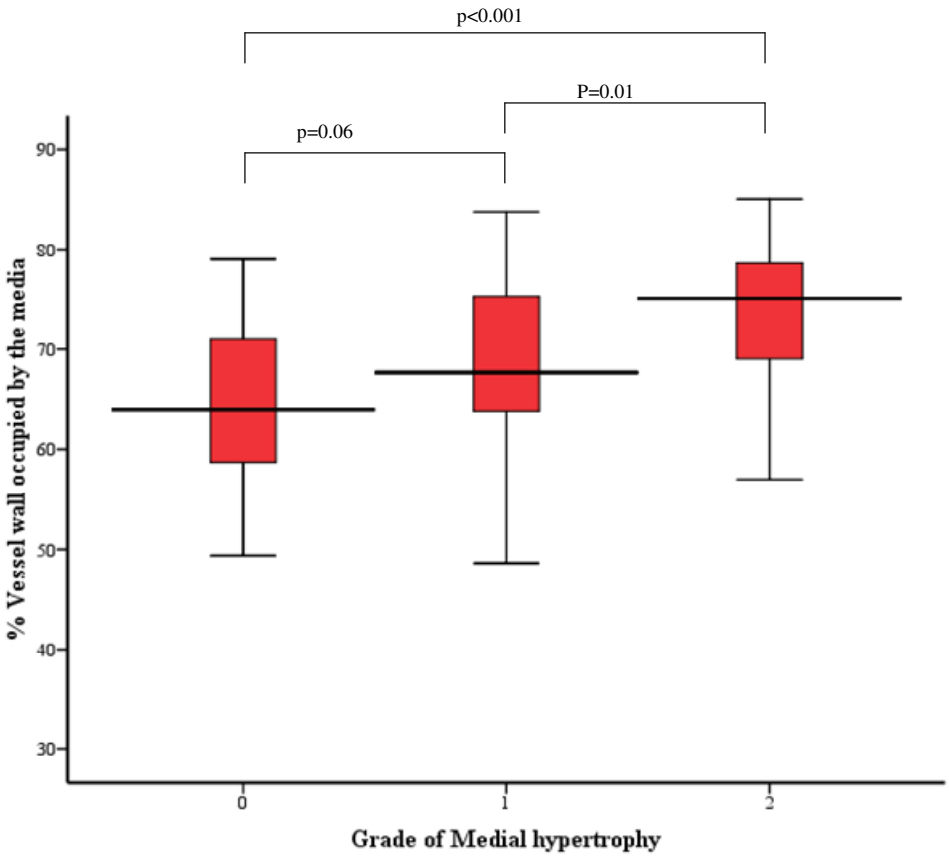


Figure 3.2

Box and whisker plot demonstrating increasing medial thickness with increasing grade of hypertrophy. Thick black line represents median and whiskers represent range; red box represents interquartile range.

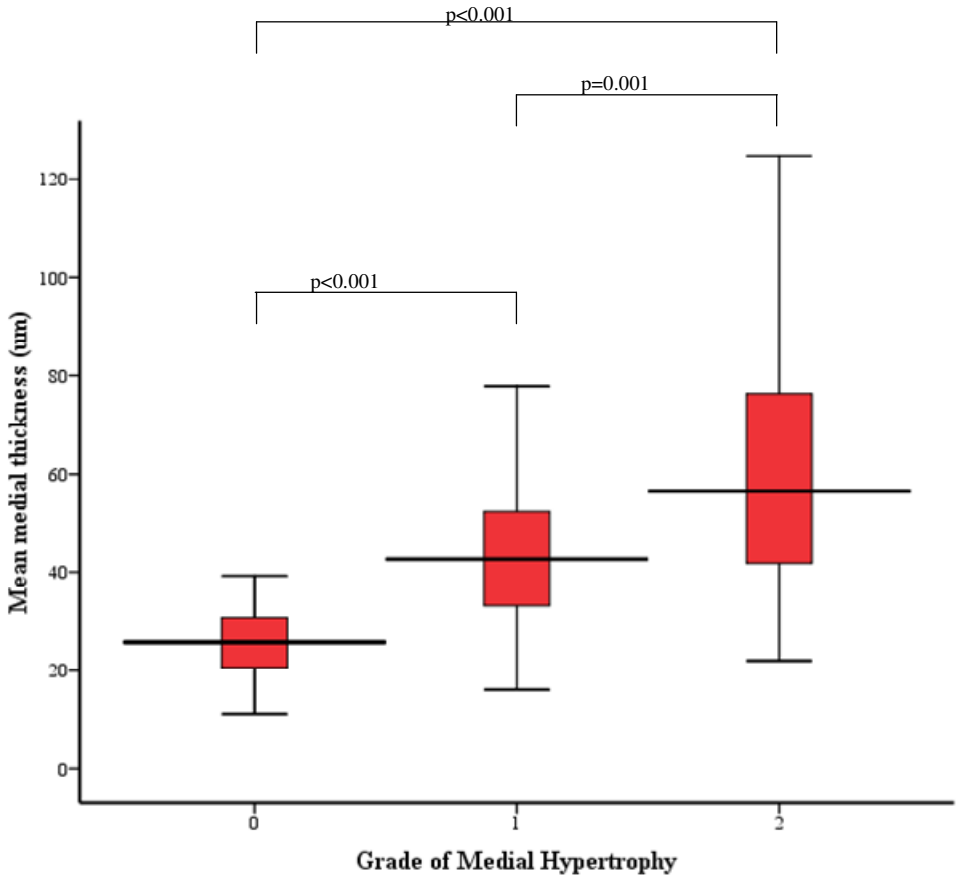
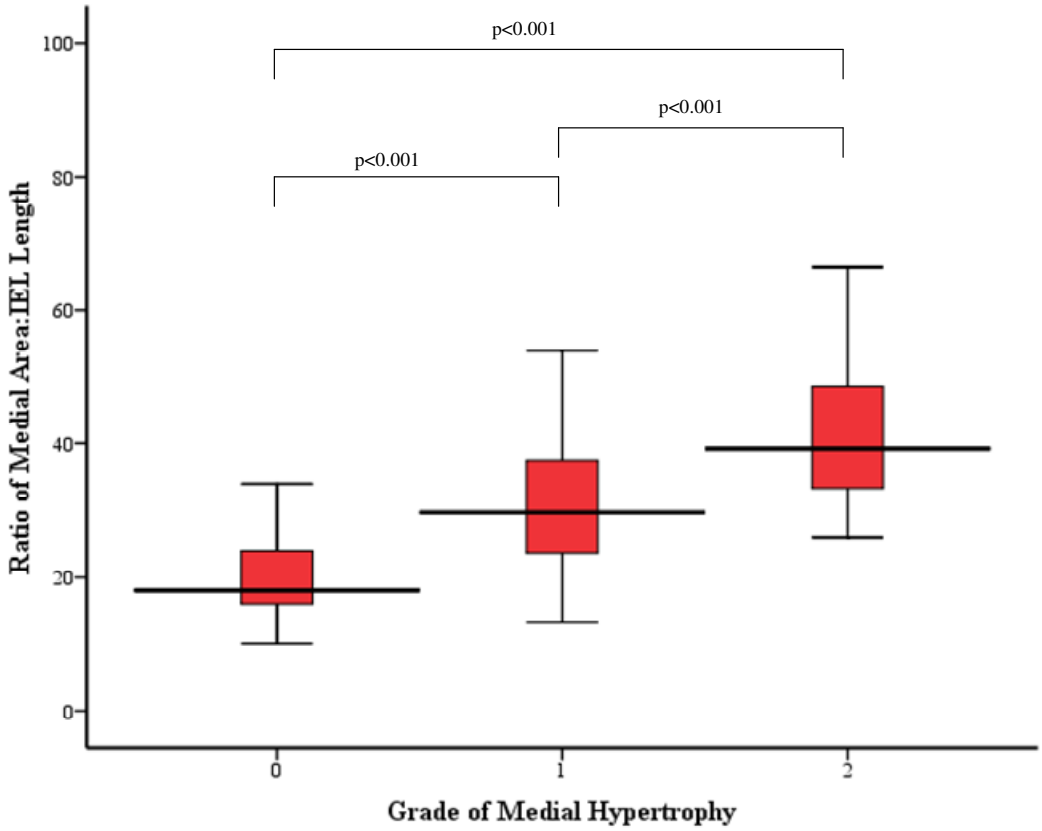


Figure 3.3

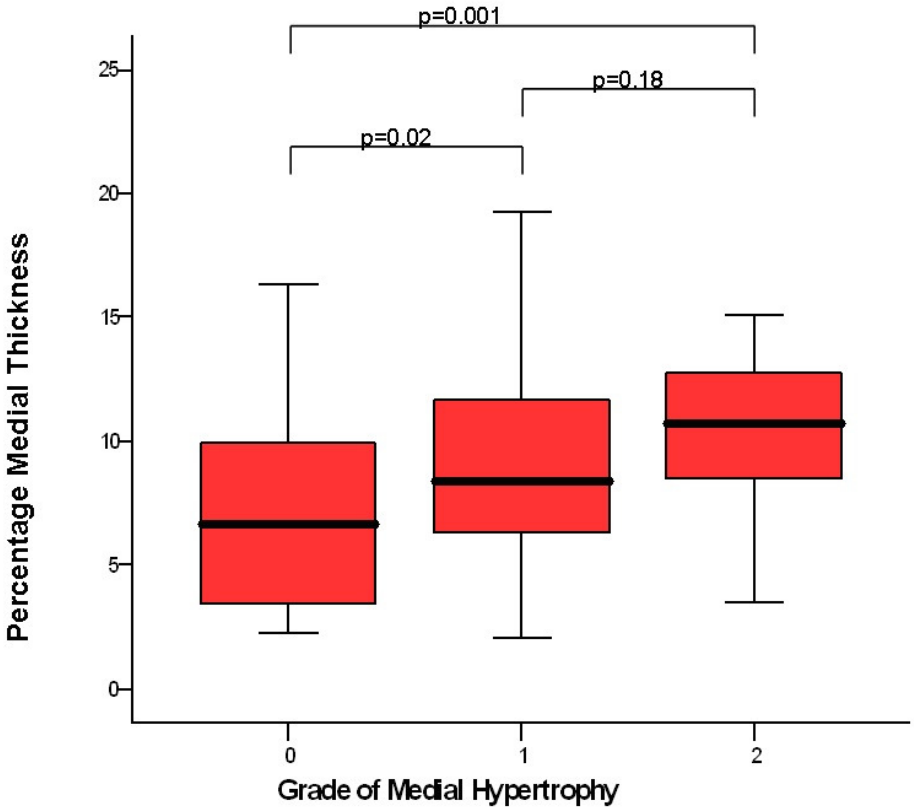
Box and whisker plot demonstrating increasing ratio of medial area to IEL length, with grade of medial hypertrophy. Thick black line represents median and whiskers represent range; red box represents interquartile range.



In humans, the normal medial thickness of muscular pulmonary arteries has been described as between 2% and 7% of the diameter (Heath D 1959). We calculated the mean medial thickness of the pulmonary arterioles expressed as a percentage of the vessel diameter. This is shown in figure 3.4, where it can be seen that the group without hypertrophy have a normal medial thickness, which is significantly less than those vessels with partial or circumferential hypertrophy.

Figure 3.4

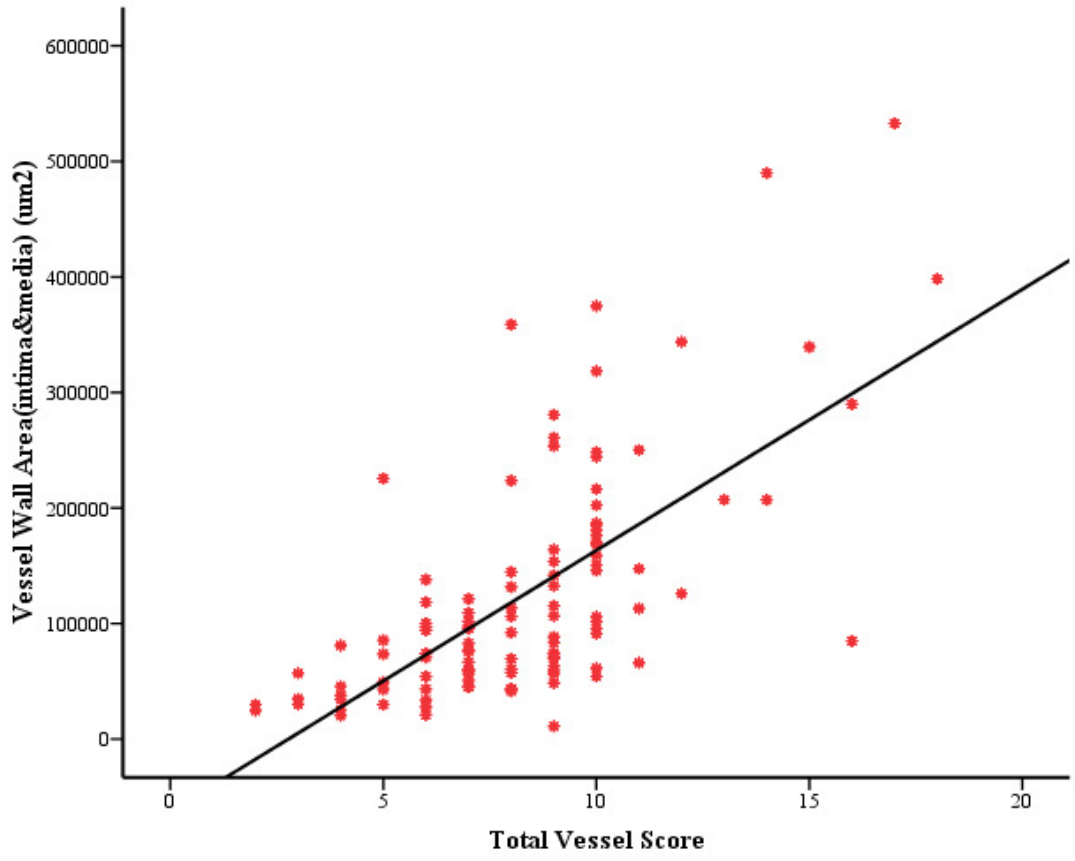
Box and whisker plot demonstrating increasing percentage of medial thickness with grade of medial hypertrophy. Thick black line represents median and whiskers represent range; red box represents interquartile range.



When grading the vessels we hypothesised that the severely remodelled vessels were larger. Therefore we investigated the relationship between remodelling severity and vessel size. The area encompassed by the EEL (lumen, intima & media), vessel wall area (intima & media), lumen area and EEL circumference were all markers of arteriolar size. Significant correlations were found between total vessel score and all of the above parameters ($r=0.50$, $p<0.001$, $r=0.67$, $p<0.001$, $r=0.33$, $p<0.001$, and $r=0.47$, $p<0.001$) respectively. An example is shown in figure 3.5.

Figure 3.5

Scatterplot demonstrating the correlation between severity of remodelling and vessel wall area ($r=0.67$, $p<0.001$)



Discussion

Morphometry was used in this study to investigate one of the commonest features of the grading system – medial hypertrophy. We have demonstrated significant positive correlations between medial thickness, medial proportion of the wall area, and severity of remodelling. We have also correlated vessel size with remodelling severity. By demonstrating these correlations, not only have we shown that the media becomes thicker and involve a greater percentage of the vessel wall when hypertrophy is present, but also validated this component of the visual grading system. The presence of such florid medial hypertrophy would seem to directly contradict some of the published literature (Santos 2002), which suggests that intimal hypertrophy is the dominant feature of remodelling. A significant positive correlation was found between intimal and medial area ($r=0.71$, $p>0.001$), suggesting that intimal and medial hypertrophy may co-exist in our cohort of patients. Given that Santos's study (2002) was in patients with mild COPD, one could hypothesise that medial hypertrophy is a marker of COPD severity.

We have shown that with increasing severity of medial hypertrophy, the media occupies a greater proportion of the vessel wall. The fact that the lumen area is not significantly different between the three grades, and there was no correlation between lumen area and grade of medial hypertrophy, implies that this is not simply due to larger vessels being measured.

The correlation between severity of remodelling, and markers of arteriolar size is interesting. It would suggest that the most severely remodelled vessels are larger in terms of lumen, and have thicker walls which are occupied by a greater proportion of media than less severely remodelled vessels. The reasons for this are unclear. We

hypothesise that the vessel wall oedema associated with inflammation in severe remodelling is a contributing factor. However we cannot completely exclude the possibility that features such as medial sclerosis, SMC apoptosis, matrix and SMC re-orientation primarily affect larger vessels, while smaller arterioles develop only hypertrophy. This could be a reflection of the number of smooth muscle cells present within the media.

Another interesting question is does the vessel wall expand into the lumen or outwards into adventitia? A significant correlation was found between lumen area and vessel wall area ($r=0.72$, $p<0.001$), implying that as the lumen enlarges, the wall becomes thicker. This would be expected as a normal finding in any artery/arteriole. There was no significant difference in lumen area between the three grades of both intimal and medial hypertrophy. This would suggest that with increasing hypertrophy the vessel wall expands outwards towards the adventitia rather than expanding into the lumen.

This study has a number of limitations. This is a pathological study, with the attendant surgery and tissue processing prior to examination. There are numerous factors that could influence measurements. The regional hypoxia of single lung ventilation during the surgery as well as the manipulation could all cause vasoconstriction prior to formalin fixation. During the processing of the tissue, there is a well recognised differential shrinkage factor between muscle and elastic tissue. Embedding the specimen in paraffin wax has also been shown to cause more tissue shrinkage than glycol methacrylate (GMA), although previous work at Astrazeneca has shown that the rank order of differential shrinkage is maintained. However, because all samples were exposed to the same surgical conditions and uniformity of processing, then any inter-patient tissue shrinkage should be constant.

Vasoconstriction or tissue shrinkage is visible on slides stained with EVG, as the 'crinkled' or crenellated internal and external elastic laminae. By magnifying each vessel as much as possible prior to measurement, we were able to trace every tortuosity of the elastic laminae, to give an accurate measurement of the *in-vivo* length. All *in-vivo* vessels have tone or state of contraction, which cannot be accounted for in pathological specimens, however the IEL has been shown to be affected least by tissue processing (Fernie JM et al 1985a) and can be used as a reliable measurement of *in-vivo* length.

Finally, all vessels with multiple elastic laminae or complete destruction of the usual wall architecture were excluded from this study, as they could bias the measurement of the intimal and medial compartments. Despite excluding one entire remodelling phenotype containing arterioles from the severest end of the remodelling spectrum, we have still demonstrated that with increasing severity of remodelling, the vessel wall expands outwards and the media occupies a greater proportion of the vessel wall.

Chapter Four

Image Analysis using:

Density Thresholding to investigate matrix alterations

Introduction

Elastin is a highly cross-linked connective tissue protein found predominantly in the parenchyma where with collagen it forms the architectural skeleton of the lung (Dunsmore SE 2008). Elastin is also found as parallel and perpendicular bundles in both the mucosa and submucosa of airways (Wright R 1961). Within the pulmonary arterioles elastin is found as concentric sheets mainly comprising the internal and external elastic laminae (Dunsmore SE 2008). Elastin is essential for the biomechanical properties of the lung – namely providing the recoil to restore the lung to its original shape and volume at the end of inspiration. The radial "pull" of the elastic fibres within the lung parenchyma are thought to be important for the maintenance of airway (Brantigan O 1959), and extra-alveolar arteriolar patency (Permutt S 1997).

Disorders of elastin homeostasis have been reported in many conditions. Increased synthesis of elastin fibres within the intima of peripheral pulmonary arterioles is characteristically described in pulmonary hypertension (Keeley FW 1995, Pietra GG 2004). Elastin has been described within the intima as an age-related finding (Wilkinson M 1988), and also been shown to be deposited within the lung parenchyma as a result of chronic inflammation in pulmonary fibrosis (Davidson JM 1990). Emphysema has been well characterised by excessive or uncontrolled elastin fibre degradation within the lung parenchyma (Sharafkhaneh A 2008). This is due to the imbalance between neutrophil elastases (Stockley R 1994), matrix metalloproteinases 9 & 12 (Ohnishi K 1998) produced by alveolar macrophages (Finkelstain R 1995), and the inhibitory capacity of Tissue Inhibitors of MetalloProteinases(TIMPS). The antiprotease alpha1-antitrypsin also plays a role (Travis J et al 1983, Janoff A 1985, Davidson JM 1990). This destruction of elastic tissue results in the characteristic

reduction in lung compliance, enlargement of the alveolar bed, and the classical pathophysiology that is well described.

No data exists regarding elastin degradation or deposition within the arteriolar walls in severe COPD, let alone non-hypertensive COPD. Preliminary work has suggested that in the severe COPD population, elastin is degraded in the media and deposited in the intima (chapter 1).

Most studies of elastin remodelling, especially within airways (Bousquet J 1996) have been compromised by a subjective assessment of fibre degradation. In this study using density threshold techniques we sought to objectively quantify the elastin component of the extracellular matrix.

Methods

Using the grading system described in Chapter Two, we were able to stratify all 441 arterioles based upon the severity of their medial remodelling. From this we identified three groups, each comprising twenty vessels. The first group consisted of those arterioles with the lowest media score, the second those with the highest media score and the third comprised those arterioles with the median score. The severity of remodelling in these three groups was compared using a one-way ANOVA and is shown in table 4.1.

All slides containing these arterioles were then stained with Elastic van Gieson in a single batch to minimise differences between shading and intensity of staining.

Table 4.1

Demonstrating the significant differences in grading between the three groups. Data presented as median (range)

Severity of remodelling	Mild	Moderate	Severe	p-value
Media Score	2 (0 - 3)	5 (5 - 5)	10 (8 - 11)	<0.001
Total Vessel Score	5.5 (3 - 8)	9 (9 - 11)	16 (11 - 19)	<0.001

As previously described in Patients and Methods section, for each image the Leica Qwin image analysis software automatically calculates a hue, saturation and intensity (HSI) score for optimal colour visualisation. When these HSI scores are altered, all pixels above these thresholds are highlighted by the computer (see Patients and Methods).

Every EVG stained arteriole was visualised using the Qwin Image Analysis Software using constant illumination. The Hue, Saturation and Intensity were altered until the computer had highlighted all the black elastic tissue, and only the black elastic tissue. The new HSI scores were documented.

Results

When viewed using light microscopy, mildly remodelled arterioles showed a little elastin deposition within the intima. The fibres within the media remained homogenous in terms of length, density and corkscrew appearance. Both the internal and external elastic laminae were easily identified (figure 4.1).

Moderately remodelled vessels showed increased deposition of elastin within the intima in a lattice or net-like pattern, and loss of regularity of the medial fibres. The external elastic lamina was easily identified, although the internal elastic lamina was not always readily apparent (figure 4.2).

In arterioles with severe remodelling multiple elastic laminae were present throughout the vessel wall, with fragmented fibrils distributed evenly throughout. The usual corkscrew appearance of the fibres was lost (figure 4.3).

Figure 4.1. EVG x20 objective magnification. A mildly remodelled arteriole, where both the IEL and EEL are clearly visible. The medial elastic fibres have a normal appearance, while there is evidence of elastin deposition within the intima, as shown by the deeper purple colour.

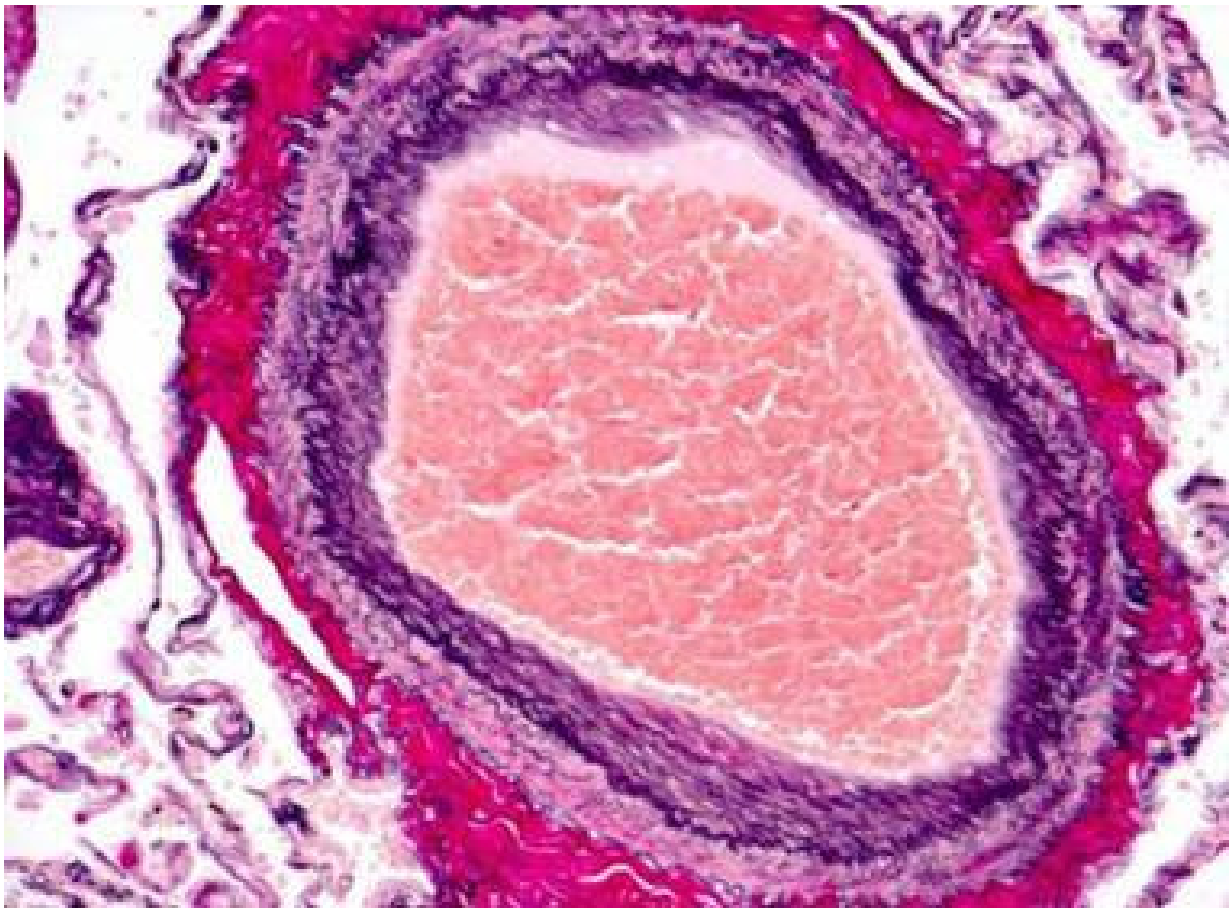


Figure 4.2. EVG x40 objective magnification. A moderately remodelled arteriole, showing increased elastin deposition within the intima. The medial fibres are longer and thicker, with less regularity.

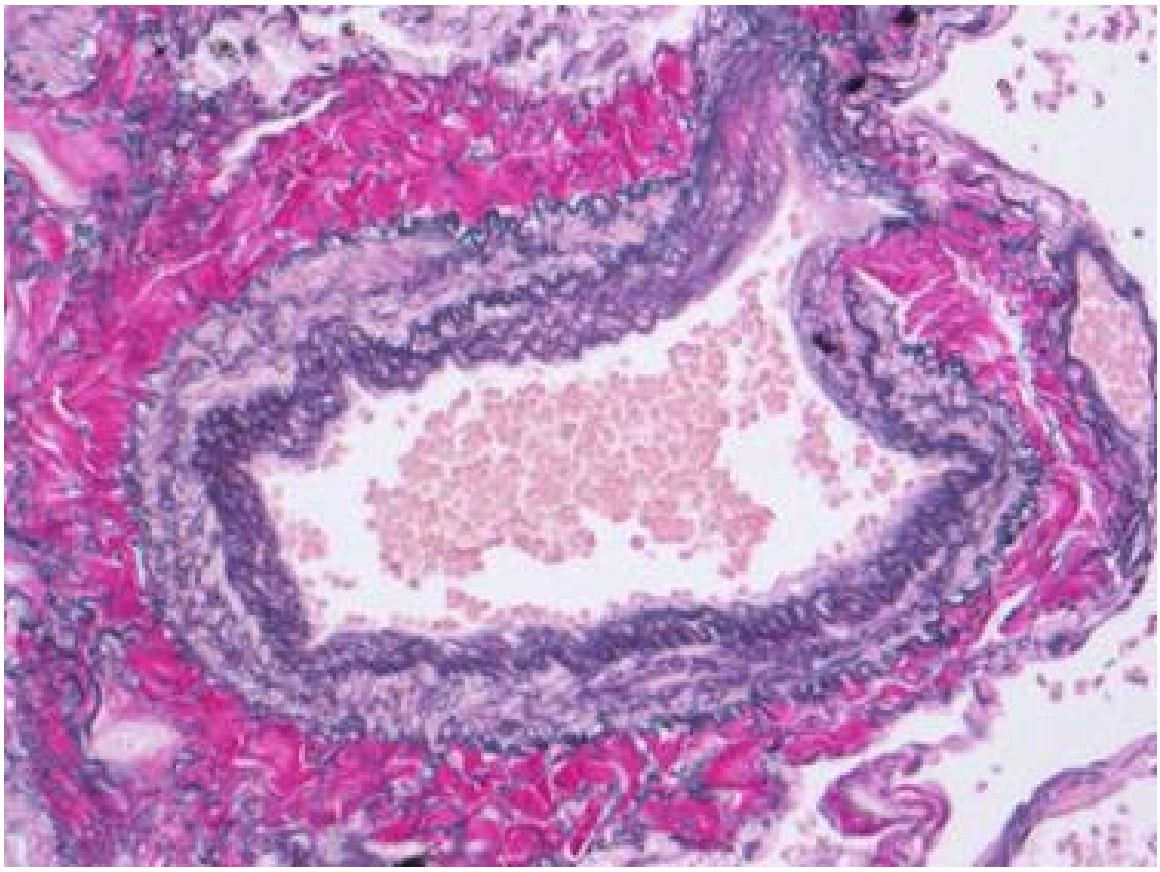
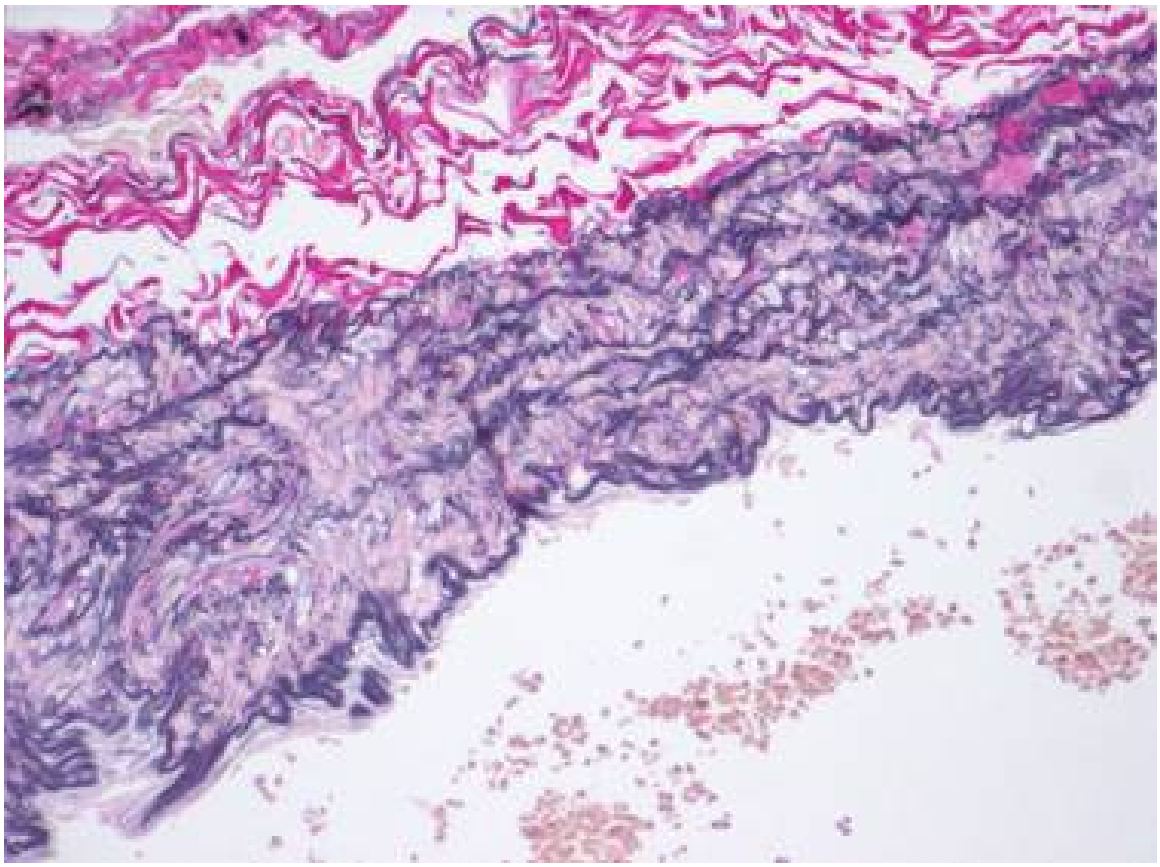


Figure 4.3. EVG x40 objective magnification. A severely remodelled arteriole demonstrating multiple elastic laminae throughout the vessel wall. The fibrils appear fragmented, with focal areas of destruction.



The Hue Saturation and Intensity scores are demonstrated in Table 4.2 and Figure 4.4. There were no differences in hue or intensity scores between the three groups. The saturation score however was significantly lower in the severely remodelled group than the mildly remodelled group. There were also significant correlations between saturation score, the medial remodelling score (r=-0.34, p=0.01), and the total vessel score (r=-0.39, p=0.004).

Table 4.2

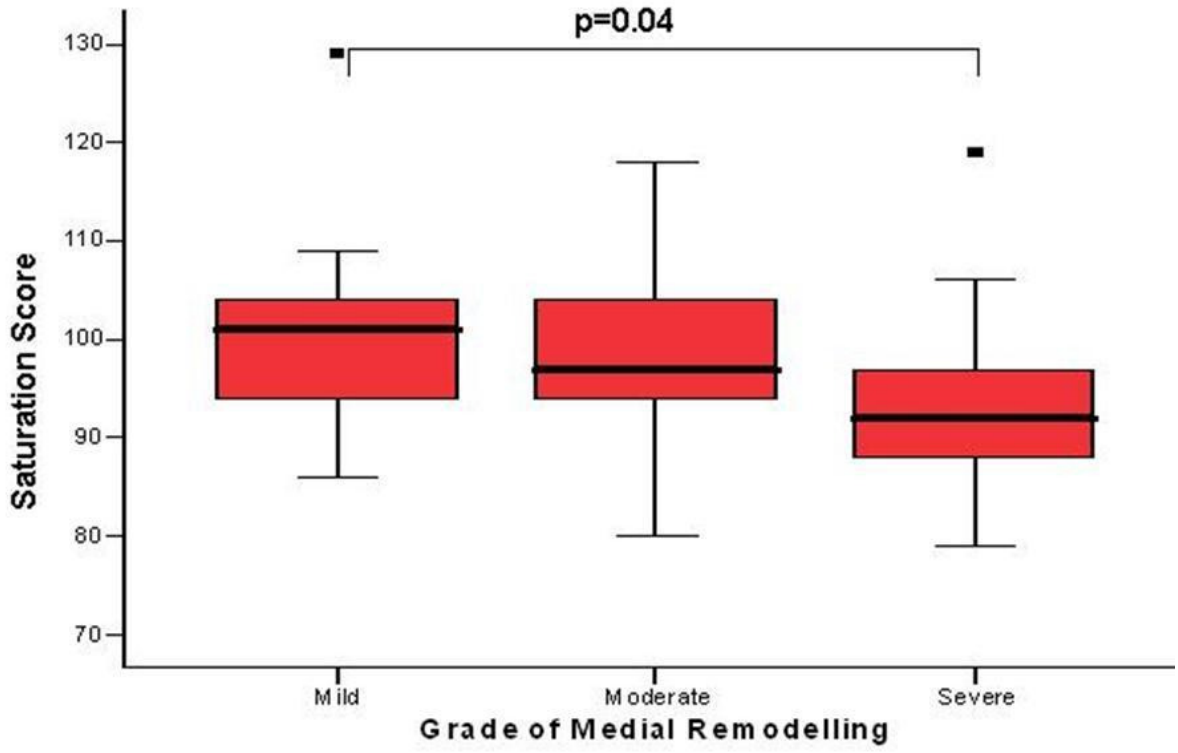
Values expressed as median (range)

	Mild	Moderate	Severe
Hue	199 (182 - 220)	202 (188 - 223)	207.5 (186 - 225)
Saturation	101 (86 - 129) *	97 (80 - 118)	92 (79 - 119)*
Intensity	183 (167 - 213)	191 (173 - 208)	194.5 (174 - 216)

* $p<0.05$

Figure 4.4

Box and whisker plot demonstrating a significant difference in saturation scores between mild medial and severe medial remodelling. The thick black line represents median and whiskers represent range; red box represents interquartile range.



Discussion

Density thresholding is a technique that allows interpretation of the intensity of staining, or can objectively quantify subtle differences in colour and shade (hue and saturation). Using the elastin stain EVG, we were subjectively able to document changes to the elastin fibres with increasing severity of remodelling. There are no other studies that have done this in COPD, although elastin deposition and matrix changes are well documented in PAH (Jeffrey TK 2001).

There is a well recognised variability to EVG staining which depends upon many factors, not least the time stained with potassium permanganate and method of fixation. This variability was evident in our cohort of twenty LVRS patients, and thus standardised staining was required for this study. This uniformity of staining was confirmed by the measurement of Hue, which showed there was no significant difference between the three groups. When viewing the slides, and measuring the HSI, the illumination of the light microscope had to be constant. This entailed standardising the brightness and taking all measurements in as few sessions as possible. This uniformity of illumination was also confirmed by there being no significant differences between the three groups' intensity scores. The difference in saturation scores shows that in severely remodelled arterioles, the elastic staining is less intense and paler than in mildly remodelled vessels. This can be interpreted as due to the elastin fibres being less dense, or more fragmented due to increased elastolysis with increasing severity of remodelling. Oedema of the vessel wall secondary to inflammation could however be a confounding factor. If significant oedema was present, it would affect the staining and therefore the hue scores. Alternatively the higher scores in the mild group could be as result of excessive elastin deposition within the intima as seen in Figure 4.1.

In summary we have demonstrated in a small study, that elastin homeostasis within the arteriolar wall is disordered in severe COPD and that a correlation exists between elastolysis and severity of remodelling.

Chapter 5

Investigating post-inflammatory hypoxic remodelling

Introduction

Acute hypoxia (low alveolar $P0_2$) is well known to cause pulmonary arteriolar vasoconstriction. This evolutionary response is thought to divert blood to the most ventilated regions of the lungs, for optimal gas exchange. The vasoconstriction mostly affects arterioles less than 1mm in diameter (Al Tinawi 1994), and pre-capillary vessels (Pak O 2007), resulting in a concomitant rise in pulmonary arterial pressure.

When the hypoxia is sustained, multiple effects are noted. Adventitial fibroblasts proliferate, migrate into the media, and differentiate into smooth muscle cells (Stenmark KR 2002). Endothelial cells become hypertrophied, produce more elastin, laminin and fibronectin (Botto L 2006). Endothelial cells also produce more vasoconstrictive, pro-proliferative factors (endothelin(ET)-1 and angiotensin II), and reduce the production of vasodilatory, anti-proliferative mediators (nitric oxide and prostaglandin-I2) (Pak O 2007). Medial hypertrophy affects the more proximal SMC (Meyrick B 1980). Deposition of extracellular matrix proteins predominantly collagen and elastin (Stenmark KR 2006) also occurs. This results in thickening of the intima, media, and peripheral muscularisation. This effect was noted by Heath D (1981, 1990) in humans living at altitude and thus chronically hypoxaemic.

Despite the above findings – mainly from animal models and cell cultures, hypoxaemia (low arterial $P0_2$), is a late clinical finding in patients with COPD, implying that hypoxia (regional or global) alone cannot fully explain the remodelling seen, especially in the earlier stages of the disease. These hypoxic disease models also do not fully explain the inflammatory components of COPD development.

It is well established that cigarette smoking results in a chronic inflammation of the peripheral airways (Hogg JC 1993). The number of inflammatory infiltrates within

these airways has been inversely correlated with FEV_1 (Saetta 1998, Peinado VI 1999), and correlated with the extent of pulmonary vascular remodelling in patients with mild COPD (Barbera JA 1994, Peinado VI 1999).

The relationship between inflammation and regional alveolar hypoxia in the development of pulmonary arteriolar remodelling remains unclear. Given this uncertainty we hypothesised that the arteriolar changes in our emphysema cohort are as a result of regional hypoxia secondary to post-inflammatory remodelling.

There have been no studies demonstrating cellular hypoxia within the pulmonary arteriolar walls.

Methods

Using the data obtained from the grading of bronchovascular pairs (chapter 2), we were able to identify arterioles within the LVRS cohort that best demonstrated the following features:

- End-stage remodelling with significant sclerosis affecting both media and intima with or without disruption of the IEL
- Medial sclerosis with minimal intimal pathology
- Intimal sclerosis with minimal medial pathology
- Vasculitis only with minimal pathological changes

Four arterioles that best demonstrated each feature were identified from the grading database and confirmed by reviewing the H&E slides. The intimal, medial and total scores for these arterioles are shown in Table 5.1.

Table 5.1

	Intima Score	Media Score	Total Score
End-stage	8 (8 – 8)	10.5 (9 - 13)	18.5 (17-21)
Medial Sclerosis	2 (1 - 4)	6 (5 - 9)	8 (6-13)
Intimal Sclerosis	3.5 (3 – 5)	0.5 (0 - 5)	4 (3-10)
Vasculitis	3 (1 - 3)	1 (1 - 2)	4 (2-5)

The arterioles were also categorised as normal, mild, moderate or severe, according to the score achieved from the grading. See Table 5.2

Table 5.2

Total Vessel Score	Grade
0 - 4	Normal – 0
5 – 8	Mild - 1
9 – 12	Moderate – 2
> 13	Severe - 3

Serial sections were then cut, and these slides were then stained using immunohistochemistry to the following panel of antibodies:

- Carbonic Anhydrase IX (CA IX). A marker of hypoxia
- Von Hippel Lindau protein (VHL). A marker of normoxia
- Collagen I and III
- Vascular Endothelial Growth Factor (VEGF)
- Ki 67. A marker of cellular proliferation
- Caspase 3. A marker of cellular apoptosis

All experiments were performed within a single batch. An assessment of the number of cells stained or the amount of matrix staining was then quantified by a visual analogue scale which comprised the following grades:

Table 5.3

Grades	0	1	2	3
Cellular interpretation	No staining	Occasional cell stained	Approximately 50% of all cells stained	Every cell stained
Matrix interpretation	No staining	<33% of area stained	33%-66% of area stained	>66% of area stained

This visual analogue scale was then applied to the endothelium, intima, IEL, medial SMC, medial matrix, EEL and adventitia for each vessel.

Correlations and differences between the severity of remodelling and the patterns of staining were assessed.

Results

Carbonic Anhydrase IX

Weak cytoplasmic staining was noted within all the cells of the bronchial epithelium. There was no staining deep to the basement membrane, nor was the majority of the parenchyma or pneumocytes stained. Only a sub-pleural zone of parenchyma was highlighted. In mildly remodelled arterioles there was no staining noted in normal endothelial cells; more intense staining being noted when endothelial fibrillation was present (see figure 5.1). Regardless of remodelling severity, the cytoplasm of most intimal and medial SMC was highlighted with CA IX. This tended to localise closer to the IEL than uniformly throughout the intimal and medial compartments. The IEL and EEL themselves did not stain, but fibroblasts within the adventitia were occasionally stained. The scores achieved for CA IX staining are summarised in table 5.4.

Von Hippel Lindau (VHL)

As with CA IX, the variability of staining quality across the panel of slides was low. The cytoplasm of the bronchial epithelial cells stained intensely positive. No submucosal staining was evident. Within the parenchyma, both type I and II pneumocytes were stained positive. Intense staining was occasionally seen in the normal endothelial cells of mildly remodelled vessels, although generally there was no endothelial staining (see figure 5.1). The intimal SMC's were stained more frequently than the endothelium, although there was no obvious pattern seen. Regardless of severity, the majority of medial SMC stained positive, although this was subjectively less intense than the staining of the normal endothelium. The adventitia rarely stained positive. The scores achieved for VHL staining are summarised in table 5.4.

Figure 5.1. Demonstrating distribution of CA IX and VHL staining (objective magnification)

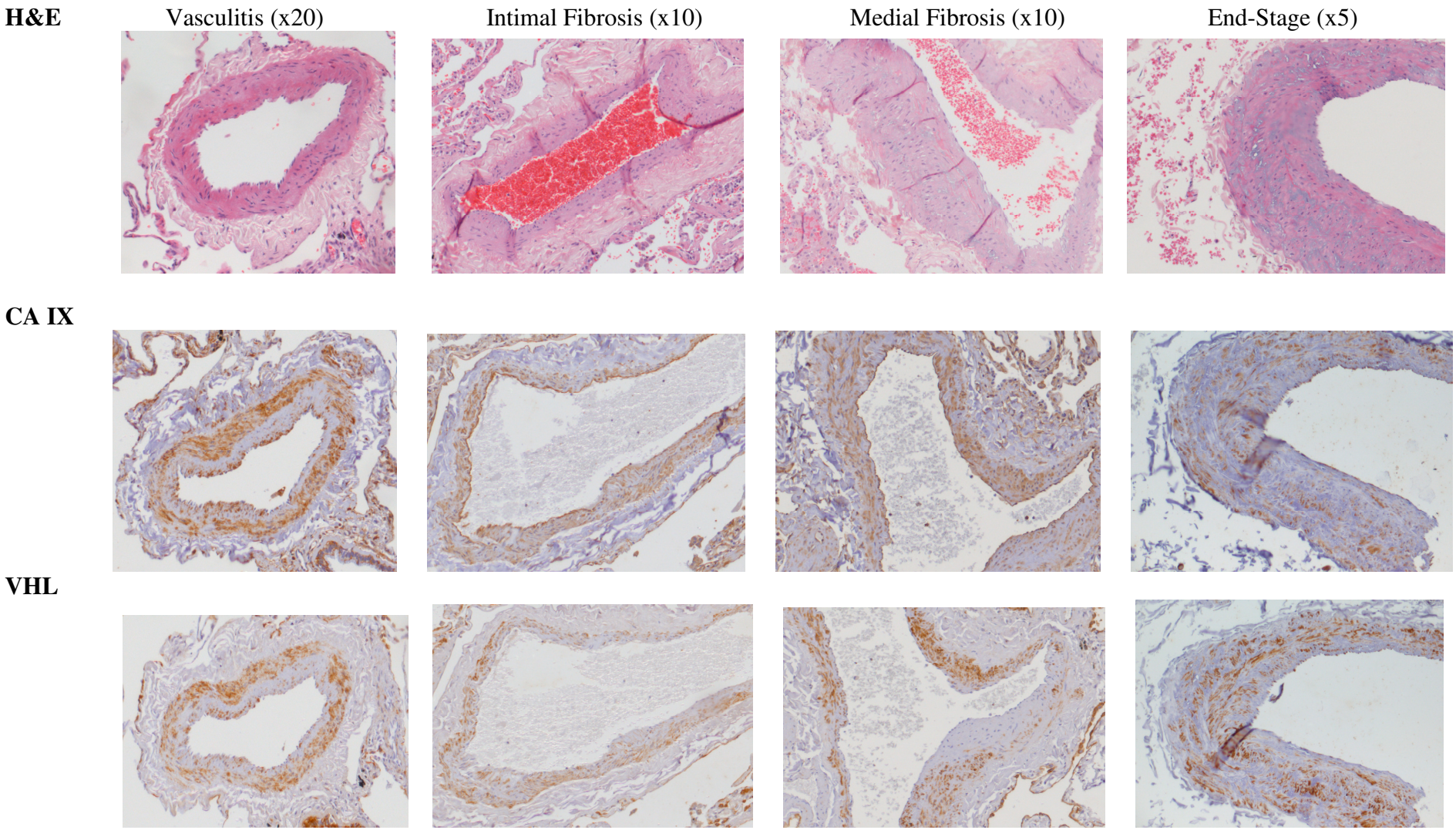


Table 5.4

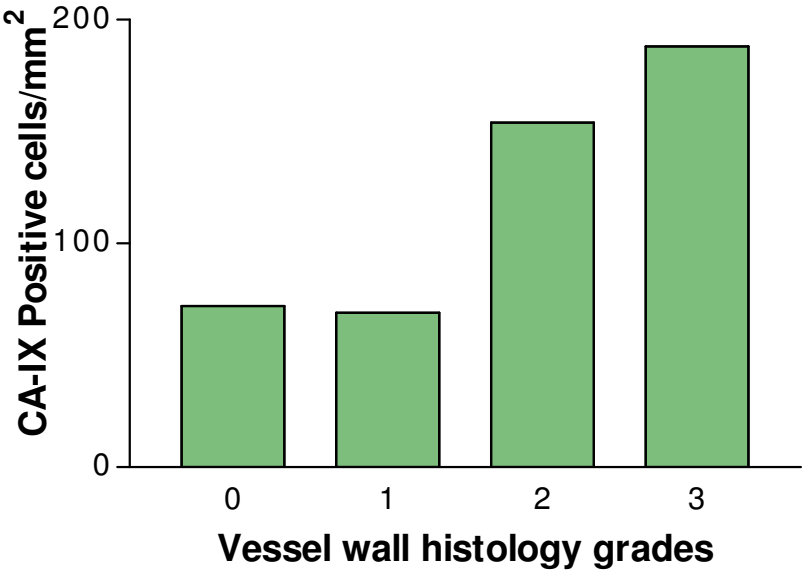
Demonstrating median (range) scores achieved for CA IX and VHL staining

		Endothelium	Intima	Media	Adventitia
CA IX	**End-stage**	3 (2-3)	2.5 (2-3)	3 (3-3)	2 (0-3)
	Medial Sclerosis	3 (3-3)	2.5 (0-3)	3 (3-3)	1 (0-2)
	Intimal Sclerosis	3 (2-3)	2 (0-3)	2.5 (2-3)	1 (0-3)
	Vasculitis	2 (2-2)	3 (2-3)	3 (3-3)	1 (0-2)
VHL	**End-stage**	1 (0-2)	2 (1-2)	3 (2-3)	0.5 (0-3)
	Medial Sclerosis	0 (0-0)	1 (0-2)	3 (3-3)	0 (0-0)
	Intimal Sclerosis	0 (0-1)	0 (0-2)	2.5 (2-3)	0 (0-0)
	Vasculitis	1 (0-1)	1 (1-1)	2 (1-3)	0 (0-1)

Regardless of the severity of remodelling, significantly more endothelial cells ($p=0.001$), intimal cells ($p=0.004$), and adventitial cells ($p=0.01$) were positive for CA IX than VHL. No significant difference was found between staining of the medial SMC.

In a separate study, using the same samples and point counting of all CA IX positive cells, Howard LS 2008, found that with increasing severity of remodelling, increased numbers of cells expressed CA IX (see figure 5.2). The severity of remodelling was significantly correlated with the number of CA IX positive cells per mm^2 ($r=0.82$, $p<0.001$).

Figure 5.2



Caspase 3

Overall there was little variability in staining with this marker of apoptosis. Intense staining was noted in the cytoplasm of hyperplastic bronchial epithelial cells and nuclei of type I pneumocytes. The endothelium tended to have one or two positive cells in most arterioles however the staining was washy/dilute in the mildly remodelled vessels (see figure 5.2). Most, if not all SMC appeared to be positive, both in the intima and media. Subjective assessment indicated more colour intensity, with cells appearing browner, in the medial SMC of arterioles of the re-orientation/apoptosis phenotype. In severely remodelled vessels with complete loss of structure every cell within the wall appeared positive. Adventitial fibroblasts were frequently positive. The scores achieved for caspase 3 staining are summarised in table 5.5.

Ki 67

The staining across the panel of slides with this proliferation marker was also consistent. The most intense staining was found in the nuclei of most hyperplastic bronchial epithelial cells, tissue macrophages and type II pneumocytes. Regardless of remodelling severity, only occasional endothelial cells and intimal SMC stained positive (see figure 5.2). More proliferative cells were noted within the medial SMC especially when matrix re-orientation and SMC apoptosis was present. When end-stage sclerosis was present, proliferating cells were seen randomly scattered throughout the arteriolar wall. Occasional adventitial fibroblasts were stained regardless of remodelling. The scores achieved for Ki-67 staining are summarised in table 5.5.

Figure 5.2. Demonstrating the distribution of Caspase 3 and Ki 67 staining (objective magnification)

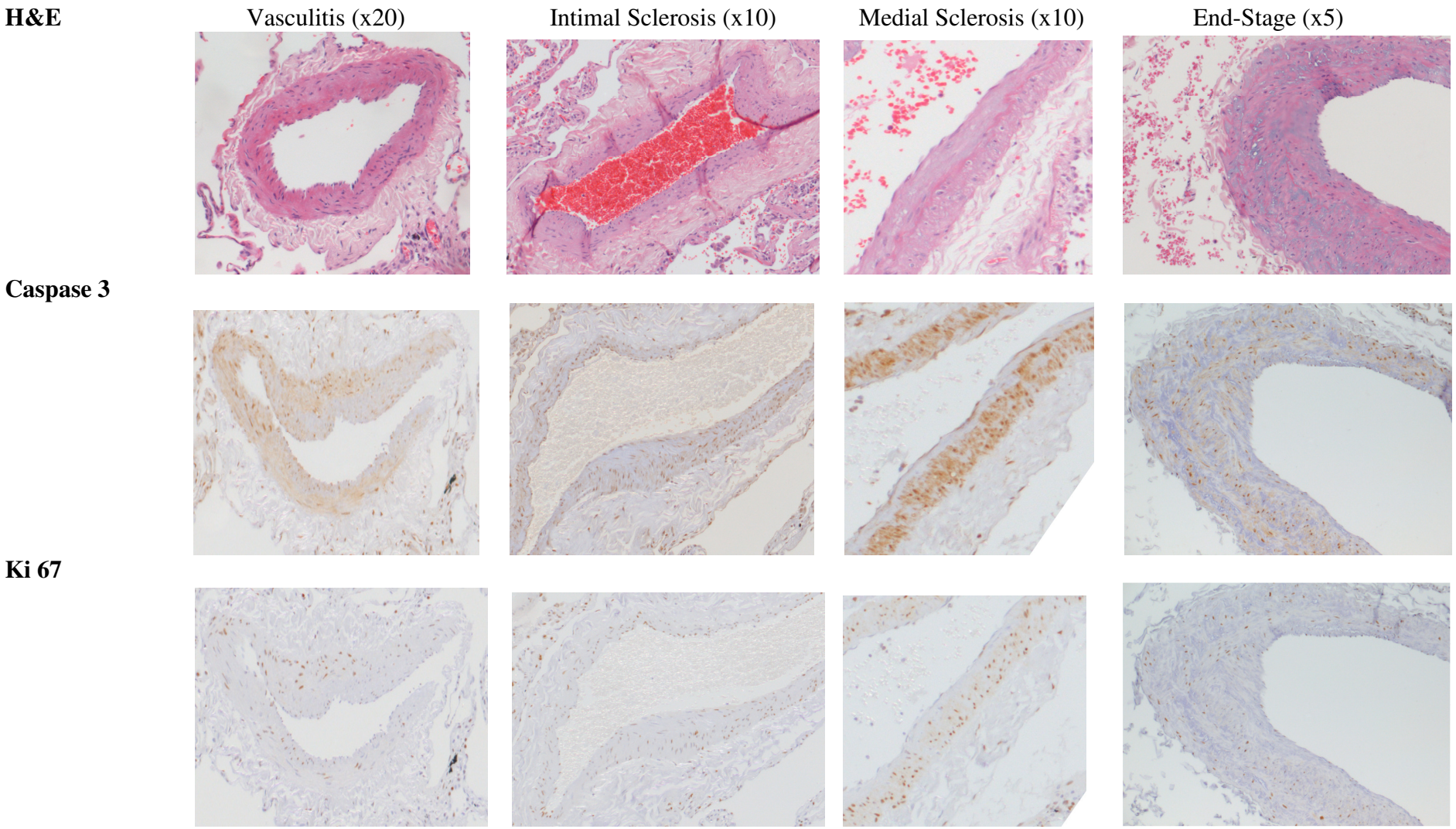


Table 5.5

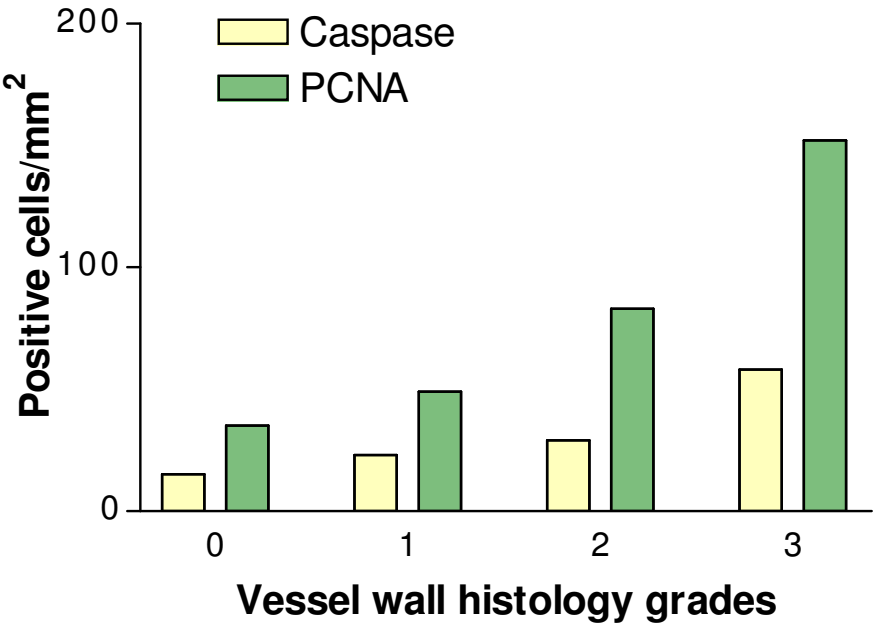
Demonstrating median (range) scores achieved for Caspase 3 and Ki 67

		Endothelium	Intima	Media	Adventitia
Caspase 3	**End-stage**	2 (2-2)	3 (2-3)	3 (3-3)	2 (1-3)
	Medial Sclerosis	1 (0-2)	0.5 (0-3)	2.5 (0-3)	1.5 (0-2)
	Intimal Sclerosis	2 (0-3)	1.5 (1-3)	3 (2-3)	2.5 (2-3)
	Vasculitis	1 (0-1)	2 (1-2)	3 (0-3)	2 (0-3)
Ki 67	**End-stage**	1 (1-2)	1 (0-2)	2 (1-3)	2 (1-3)
	Medial Sclerosis	1 (0-2)	0.5 (0-1)	1 (1-2)	1 (1-1)
	Intimal Sclerosis	2 (0-2)	0.5 (0-2)	0.5 (0-1)	1 (0-1)
	Vasculitis	1 (0-1)	0 (0-2)	0 (0-3)	1 (0-2)

Regardless of remodelling category, significantly more intimal ($p=0.01$) and medial ($p=0.005$) SMC stained positive for caspase 3 than Ki 67. Within the medial compartment, a significant correlation was found between number of proliferating cells stained and the medial ($r=0.62$, $p=0.01$) and total vessel scores ($r=0.58$, $p=0.02$).

Using a different marker of proliferation (proliferating cell nuclear antigen PCNA), again with the same samples and using point counting of all Caspase 3 and PCNA positive cells, Howard LS 2008 also demonstrated increasing expression of both apoptosis and proliferation with increasing severity of remodelling (see figure 5.3). Again significant correlations were found between the number of Caspase 3 positive cells and the number of PCNA positive cells ($r=0.75$, $p<0.001$), as well as severity of remodelling and caspase 3 ($r=0.78$, $p<0.001$) and PCNA ($r=0.93$, $p<0.001$)

Figure 5.3



Collagen I

Staining for collagen I was subjectively much less consistent than for other antibodies. Some slides were completely devoid of any staining, while in others the distribution of staining was patchy across the slide. When present collagen I was localised in the sub-pleural adventitia, interlobular septae, fibrotic parenchyma, and occasionally to the bronchiolar submucosa. Within the vasculature, collagen I was consistently found in the adventitia, and never stained the cellular components of the endothelium, intima media or the IEL and EEL. There was no staining of the arteriolar walls in the vasculitis group. When sclerosis was present, weakly positive patchy staining was seen within the corresponding intima or media (see figure 5.3). Interestingly there was little or no staining in vessels with end-stage sclerosis (see table 5.6). There was no correlation between remodelling severity and grade of matrix staining.

Collagen III

Staining for collagen III was more consistent than for collagen I, the reasons why are unclear. Collagen III was located within the sub-pleural adventitia, and the peri-bronchiolar adventitia. The bronchiolar basement membrane separating epithelial and smooth muscle cells was particularly emphasised. No staining was noted within interlobular septae or fibrotic parenchyma. Collagen III was highlighted most consistently within the adventitia and close to the IEL and EEL. Again very little staining was observed in vessels with vasculitis or end-stage remodelling. The most prominent collagen III staining was noted around the IEL and EEL with patchy matrix staining on either side in sclerotic vessels. In some arterioles with medial sclerosis, a latticework of staining was evident across the media connecting the IEL and EEL. When the usual wall structure is retained, there were however significant correlations

between collagen III deposition around the IEL (r=0.65, p=0.03) and the EEL (r=0.67, p=0.02) with the severity of remodelling as measured by the grading system.

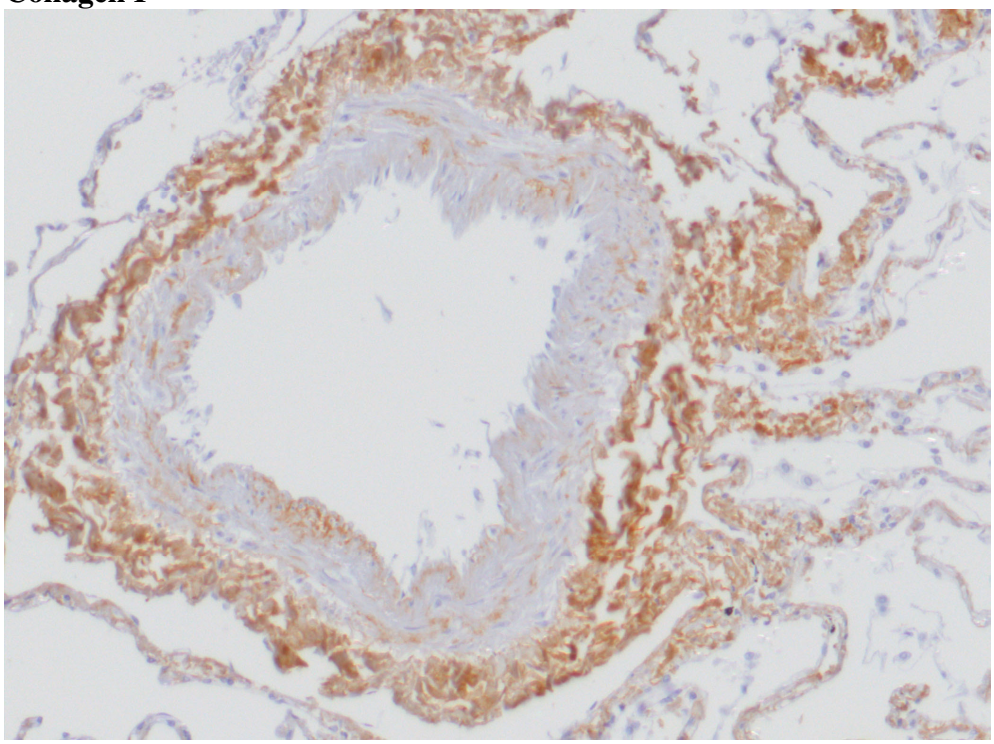
Table 5.6

Demonstrating median (range) scores achieved for Collagen I and III

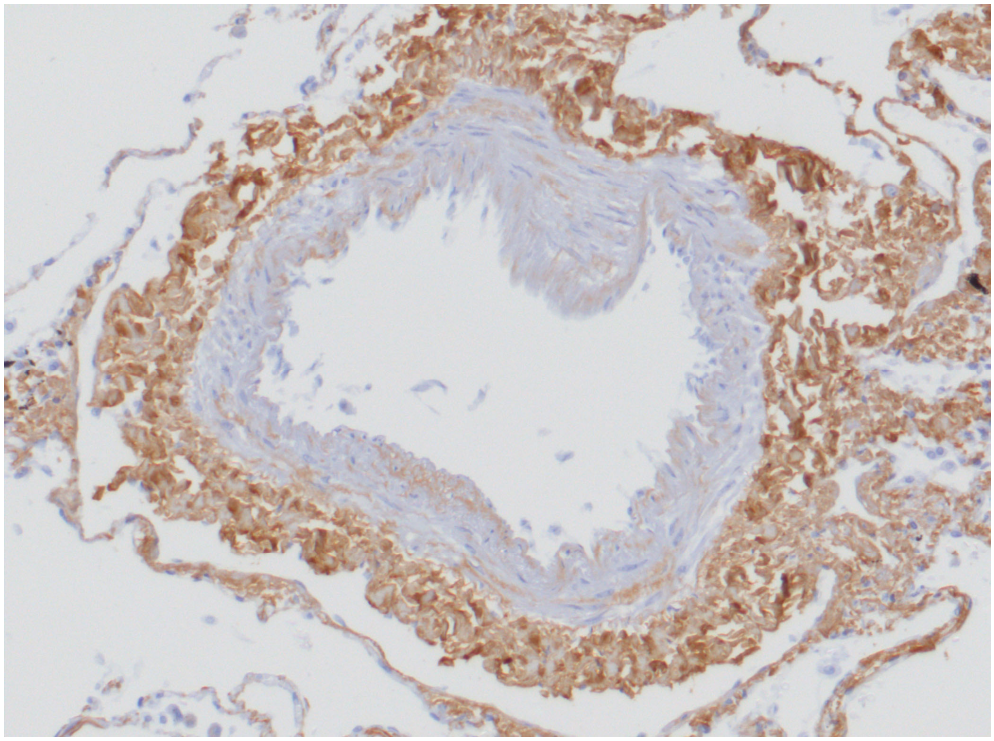
		Intima	IEL	Media	EEL	Adventitia
Collagen I	**End-stage**	0 (0-1)	0.5 (0-1)	1 (0-1)	0 (0-0)	3 (2-3)
	Medial Sclerosis	1 (0-2)	0 (0-0)	1.5 (0-2)	0 (0-0)	3 (3-3)
	Intimal Sclerosis	2 (1-3)	0 (0-0)	1.5 (0-2)	0 (0-0)	3 (2-3)
	Vasculitis	0 (0-1)	0 (0-0)	1 (0-1)	0 (0-0)	2 (0-3)
Collagen III	**End-stage**	1.5 (1-3)	1 (0-1)	1 (1-2)	1 (0-2)	3 (3-3)
	Medial Sclerosis	0.5 (0-2)	3 (1-3)	1.5 (1-3)	3 (3-3)	3 (3-3)
	Intimal Sclerosis	1 (0-3)	2.5 (0-3)	1.5 (1-3)	2.5 (0-3)	3 (3-3)
	Vasculitis	1 (0-1)	0 (0-0)	1 (0-2)	0 (0-1)	3 (3-3)

Figure 5.3. Demonstrating Collagen I and III distribution in an arteriole with both intimal and medial fibrosis (x20 objective magnification)

Collagen I



Collagen III



Vascular Endothelial Growth Factor (VEGF)

The staining across the panel was inconsistent with there being no staining on the majority of slides. When staining was present most cytoplasm of bronchial epithelial cells was positive. The submucosa and bronchial smooth muscle were never stained. Most endothelial cells were stained positive, whether activated or normal in appearance. Intimal and medial cells only tended to stain positively in the most severely remodelled arterioles, or in the re-orientated and apoptotic media.

Discussion

Regional hypoxia *in vivo* is a phenomenon that has been widely hypothesised but is not well studied in COPD. Much attention has been focussed upon the effects of hypoxia upon pulmonary artery SMC's in hypoxic culture, where both proliferative and apoptotic phenotypes have been demonstrated (Rabinovitch M 1979, Frid MG 1997a, Howard LS 2008). These results have always been correlated with remodelling seen in chronic hypoxaemia secondary to many conditions including cystic fibrosis and living at high altitude (Yu AY 1998). To our knowledge there is no literature investigating the *in vivo* distribution of regional hypoxic changes in the COPD population.

Our study shows that the endothelium and intima appear more affected by hypoxia regardless of severity of remodelling. This was demonstrated by significantly more CA IX positive stained cells than VHL positive cells. Vessel wall cells will receive oxygen from two sources – diffusion from blood within the lumen and more directly via the vasa vasorum. Blood within the pulmonary arterioles has a "venous" oxygen concentration, whilst the vasa vasorum are systemic vessels and therefore supply blood with a more "arterial" oxygen concentration. The intima is furthest from vasa vasorum and therefore most reliant upon the diffusion gradient and affected by hypoxia first. We hypothesise that this diffusion gradient is affected by several factors including medial hypertrophy (increased distance from vasa vasorum), and the increased deposition of matrix proteins such as collagen III would slow the diffusion of oxygen through the media. This would seem unlikely, however, as there was no correlation between CA IX and collagen III staining.

Most SMC's stained positive for both VHL and CA IX regardless of severity of remodelling. The LVRS patients are well known to be chronically hypoxaemic with the median $P0_2$ = 9.4Kpa in this population. This upregulates hypoxia-inducible genes, of which that encoding for CA IX is one of many so far documented. The VHL protein however targets the constitutively expressed hypoxia inducible factor 1α (HIF-1α) during normoxia resulting in its degradation. VHL is much more sensitive to acute changes in oxygen tension, and may be affected by the normoxia resulting from the intra-operative inspired oxygen.

Many studies have used the transcription factor hypoxia-inducible factor-1 alpha (HIF-1α) as a marker of hypoxia. During hypoxia, HIF-1α translocates to the nucleus where it then upregulates several inducible target genes including VEGF (cellular maintenance), endothelin-1 (vasoconstriction), erythropoietin (RBC production) and iNOS (vasodilation). Immunohistochemistry using HIF-1α has been extensively used to study angiogenesis in cancer (Zhong H 1999, Calzada MJ 2007). None of these studies used an isotype negative control. The lack of antibody specificity was demonstrated when we attempted to optimise HIF-1α using an isotype negative control, with both positive and negative slides staining equally. Despite multiple attempts to optimise the antibody staining by altering the pre-treatments and duration of application of primary antibody, the results were the same. We therefore used an antibody to Carbonic Anhydrase IX to demonstrate hypoxia and one to the Von Hippel Lindau protein to identify normoxia.

Our study does suffer several limitations related to the complexities of analysing regional and general hypoxia using the pathological examination of surgically resected tissue. Firstly there are clinical issues relating to the perioperative period. These chronically hypoxaemic patients are given supplemental oxygen intraoperatively to

maintain normoxia, while many surgical factors exist which will exacerbate hypoxic changes. The lung from which the specimen is resected is not ventilated and collapsed intraoperatively, the specimen will become even more hypoxic during the time taken from excision to formalin fixation. All these events may distort the histological data from the actual *in vivo* picture.

Secondly we used immunohistochemistry to determine the presence or absence of hypoxia. Hypoxia and HIF -1α is most commonly studied in cell culture and animal models using immunoelectrophoresis techniques, or in-situ micro-electrode measurement of tissue oxygen concentration. This approach would not have added to our study and would almost certainly have been impractical.

Thirdly *in vivo* markers of hypoxia are available (Pimonidazole. Cayman Chemicals Michigan, USA) for intravenous administration prior to tissue harvest. However the use of this product in our study would raise many ethical concerns.

Both Howard LS (2008) and Frid MG (2009) demonstrated two different phenotypic responses to hypoxia by SMC's in the distal pulmonary arterioles. These *in-vitro* studies have shown both SMC apoptosis and proliferation occur during prolonged hypoxia. We also identified ongoing apoptosis and proliferation, with admixing of the two processes such that identifiable clusters of cells were never seen. Both apoptosis and proliferation appear to be regulated by hypoxia and thus HIF-1α.

Regional arteriolar wall hypoxia is not due to medial and intimal hypertrophy alone. The deposition of collagens within the arteriolar wall in this study corroborates the findings from the tinctoral stains (chapter 1), where MSB and picrosirius red both demonstrated collagen within the medial compartment. Identifying vessels with intimal sclerosis and minimal medial pathology was difficult as there were very few such vessels. Vessels with medial sclerosis and minimal intimal pathology were much more

common, implying that in severe COPD, the media is primarily affected by inflammation or hypoxia.

Identification of collagen using immunohistochemistry is notoriously difficult to stain reliably. This is because the epitope has a low antigenicity, which was reflected in the variability of staining noted in this study. The lack of staining noted in the "end-stage" arterioles may however have a different explanation in that these are mature collagenous lesions. As collagen matures, it is well known that it becomes more anhydrous, resulting in closer packing of the helical structure, and thus further reducing the epitopes available to be stained. The deposition of extra cellular matrix proteins in COPD is well documented within the lung parenchyma and smaller airways (Bosken CH 1990, Hogg JC 2004), but there is little work investigating this phenomenon within the pulmonary vasculature.

The inconsistency of VEGF staining could be multifactorial, including technical issues such as antibody specificity, investigator error, antibody optimisation, or the pre-treatments used. However reduced expression of VEGF and its receptor KDR has been demonstrated in emphysema (Kasahara Y 2000). The absence of staining indicating reduced expression of VEGF could be accurate, but also could be due to problems with staining.

In summary, we have demonstrated that hypoxia is associated with more severely remodelled arterioles as judged by the grading system. In turn hypoxia also regulates the increased SMC apoptosis and proliferation demonstrated with increasing remodelling. Post inflammatory changes i.e. intimal and medial sclerosis were also correlated with severity of remodelling, and thus by implication to severity of hypoxia or extent of apoptosis / proliferation.

Summary of Thesis

Chronic Obstructive Pulmonary Disease (COPD), particularly emphysema, is a progressive, irreversible disease, is extremely common, and accounts for a major burden in healthcare.

Pulmonary arteriolar remodelling is well recognised in these patients and a spectrum of lesions described. Characterisation of the patient phenotype in these studies has been poor, and the lesions frequently ascribed to pulmonary hypertension (PAH). Although PAH often occurs in severe COPD, the association continues to be made despite several observations to the contrary. Remodelling is described in smokers without COPD, and some PAH vascular lesions are never seen in COPD. Lung Volume Reduction Surgery (LVRS) gives us the opportunity to study remodelling in the excised lung tissue of well-characterised patients with severe COPD without clinical PAH.

We identified three different remodelling phenotypes:-

- Fibrotic suggesting post inflammatory remodelling. Collagen deposition within the arteriolar wall was confirmed with both MSB and picrosirius red. The extent of collagen I and collagen III deposition with immunohistochemistry (IHC) was correlated with remodelling severity
- Medial cellular apoptosis / matrix re-orientation. This remodelling reaction has never been previously described *in-vivo*. Matrix re-orientation was confirmed with picrosirius red under polarised light, while apoptotic cells nested within a proliferating cell population was confirmed with caspase 3 and Ki 67 IHC. Increased expression of both proliferation and apoptosis was correlated with severity of remodelling

- Proliferative with complete destruction of the usual vessel wall structure. Again this phenotype has never been previously described. It is characterised by multiple fragmented elastic laminae, as well as elements of both the other phenotypes

Remodelling in severe non-hypertensive COPD was also characterised by cellular origin using desmin, vimentin and CD31 IHC. In contrast to PAH and previous work in mild COPD, the intimal and medial hypertrophy was exclusively mesenchymal, with CD31 expression being limited to the endothelium. This implies migration towards the intima of either medial SMC or adventitial myofibroblasts, or de-differentiation of in-situ intimal/endothelial cells.

A robust grading system encompassing all intimal and medial pathological features was developed and was validated by comparing with non-LVRS lung resections and also "worst vessels" upon each slide. Heterogeneity of remodelling was a key finding, such that relatively normal arterioles could be adjacent to the most severely remodelled vessels. This suggests localised responses to stimuli eg regional hypoxia. Cluster analysis and the attendant heatmap demonstrated two distinct clusters. Hypertrophy, inflammation, activated endothelium and apoptosis/reorientation affected most vessels suggesting a more generic remodelling response, whereas post-inflammatory remodelling, elastolysis and multiple elastic laminae predicted severity.

Morphometry objectively confirmed the assessment of medial hypertrophy as judged by the grading system. It also demonstrated that with increasing severity of remodelling, the media occupied a greater proportion of the vessel wall. A positive correlation was

also found between arteriolar size and remodelling severity, suggesting remodelling commenced proximally and spread distally, or that hypertrophy and vessel wall oedema were confounding factors. This study was only performed in arterioles with a single intact IEL, and therefore excluded the proliferative phenotype.

In a very small study we used density thresholding to investigate remodelling of elastic fibres. EVG stains suggested medial fibre degradation and intimal deposition. Total elastic content did not vary (Hue), although elastolysis and fibre fragmentation correlated with remodelling severity (Saturation).

Hypoxia of smooth muscle cells was demonstrated for the first time histologically in the severe COPD population using CA IX expression. The number of hypoxic cells was correlated with severity of remodelling. This further re-enforces the hypothesis of regional hypoxia driving remodelling. Apoptosis (caspase 3), proliferation (Ki-67 & PCNA) and post-inflammatory remodelling (collagen I & III), were all correlated with remodelling severity, and thus by implication hypoxia.

The originality of this work is focussed in two areas. Firstly the admixing of proliferation and apoptosis within the same vessel. This co-existence of phenotypes bears some resemblance to the pseudoglandular / canalicular phases of lung vascular development, when vasculogenesis is characterised by waves of apoptosis and proliferation. The second key feature is heterogeneity of remodelling, such that no single dominant phenotype was expressed in an arteriole. This was so extensive within a sample that intra-patient variation exceeded inter-patient variability. We were thus unable to identify particular patient remodelling phenotypes using the cluster analysis.

This heterogeneity and inability to separate patients based upon their remodelling phenotype, made correlating with post operative outcome difficult. However, we found that severity of medial remodelling was significantly correlated with percentage change in FEV_1 at 3 and 12 moths post op. The presence of inflammatory cells, and post-inflammatory remodelling were the main determinants of these correlations.
The presence of inflammatory cells was also correlated with post operative changes in BMI. This did not translate into a correlation between severity of intimal or medial remodelling and change in BMI.

In summary, we hypothesise that pulmonary arteriolar remodelling in COPD is primarily driven by regional hypoxia. This regional hypoxia stimulates both proliferation and apoptosis depending upon SMC phenotype. The post-inflammatory remodelling further enhances cellular hypoxia, resulting in a vicious cycle.

In conclusion, the pulmonary arteriolar remodelling in severe COPD is not morphologically contiguous with remodelling described in PAH. We hypothesise that cycles of regional hypoxia are contributory to an overall picture more akin to remodelling seen in pulmonary vascular development, albeit with impaired homeostatic mechanisms due to reduced VEGF expression.

Further work is clearly needed to elucidate the interactions between hypoxia and post-inflammatory remodelling and its role in severity of pulmonary vascular remodelling.

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Appendix I

Patient Information and Consent Form

Study Title:

Studies of immune cell function in inflammatory lung disease

Investigators:

Professor A Wardlaw and AstraZeneca Research and Development, Charnwood

PATIENT INFORMATION FORM

Thank you for thinking about taking part in this study. This leaflet explains why your doctor is doing the study, the benefits and possible discomforts of your participation and what we would like you to do during the study. If you are willing to take part you will be asked to sign this consent form and will be given a copy to keep.

INTRODUCTION

Lung disease causes pain, discomfort and can prevent sufferers from carrying out everyday activities. Whilst available treatments including steroids and other drugs may relieve symptoms, none provide a cure. Operations may help some people. More research is needed to find new treatments that can cure lung disease.

WHY IS THIS STUDY BEING DONE?

AstraZeneca Research and Development (R & D) at Charnwood, Loughborough are performing research studies to identify molecules that cause and exacerbate lung disease. It is not yet know what causes a disease such as Chronic Obstructive Pulmonary Disease (COPD) although it is thought that cigarette smoking is the principal cause. One approach to finding a cure is to identify changes in the lung that accompany COPD and other similar diseases, and then develop new drugs that can prevent these changes. We are looking for changes in tissues from the lungs of patients who have had different types of lung disease for different lengths of time.

WHAT ARE WE ASKING YOU TO DO?

Your surgeon has recommended having an operation should help you with your current condition. Some samples of lung tissue removed as a normal part of your operation would normally be discarded. If you are willing to take part in our research, your surgeon will provide us with some of the lung tissues that are removed as part of your operation. This will be a gift from you, and whilst it may help in the development of new treatments you will not be able to

benefit financially from its use. We will also record some information about your recent medical history, medicines taken and reason for the operation from your medical records.

DO I NEED TO TAKE PART?

NO. IT IS ENTIRELY VOLUNTARY. You can refuse to provide a sample at any time and you do not need to give reasons and your future treatment will not be affected.

You do not have to take part in this study to receive any particular medication or operation. During this study you will be told if any new findings occur that may affect you willingness to take part.

The way the operation is being done will not be affected in anyway by you agreeing to donate the lung tissue.

WHAT MIGHT I GAIN?

Taking part in this study means that you may possibly help suffers of lung disease in the future, as information about the changes that occur in the lung may be used to develop new treatments.

WHO WILL SEE ALL THE INFORMATION ABOUT ME?

All information resulting from you taking part in the study will be stored and analysed in a computer and will be treated confidentially. You will be identified in the computer by a number and only your doctor will be able to identify the number as belonging to you. The study records will not be made available in any form to anyone other than authorised representatives of the health authorities and AstraZeneca R & D Charnwood. In all instances, your confidentiality will be maintained, in accordance with the Data Protection Act or as local laws permit.

WHAT WILL HAPPEN TO THE SAMPLES THAT I HAVE DONATED?

Samples and associated medical data will be stored at AstraZeneca R & D Charnwood, Loughborough. The investigators will adopt procedures such that all coding information that could identify you on your samples and the associated medical data will be removed on or before 5 years after donation. All living cells derived from samples will be destroyed at or up to 6 months following sample donation. Blood samples may be stored for up to 5 years. Anonymised samples of lung tissue may be stored for up to 15 years. Lung tissues may be treated to develop methods for identifying key chemicals and cells involved in lung disease and stained to measure the amounts of molecules that may be important. These molecules may be extracted from the tissue to help us to identify them. It is hoped that these studies will help us to treat and diagnose illness in other patients in the future. However, the samples, which you donate will not themselves be used to treat or diagnose illness in other patients. Genetic testing will not be conducted on the samples or used by others, we would need to ask you again if that was something we should want to do in the future.

If you have any further questions about this study please do discuss them with:

Professor Wardlaw, Tel. No: 0116 256 3841

This document must be kept in the investigator's study file and retained for a minimum period of 15 years after completion of the study.

Initials

1. I have read and understood the patient information form on the above project, dated …… and have been given a copy to keep. I have had the opportunity to ask questions about the project and understand why the research is being done and any foreseeable risks involved. ……

2. **I agree to give a sample of tissue for research in the above project.** I understand how the sample will be collected and that giving the sample is voluntary. I am free to withdraw my approval for use of the sample at any time without giving any reason and without my medical treatment or legal rights being affected. ……

3. **I give permission for my medical records to be looked at** and Information taken from them to be treated in strict confidence by responsible people from Glenfield Hospital and AstraZeneca R & D Charnwood. ……

4. I understand that my doctor will be informed if any of the results of the tests done as part of the research are important for my health. ……

5. I understand that I will not benefit financially if this research leads to a new treatment or medical test. ……

6. I do know where to contact Professor Wardlaw, if I need to. ……

7. Do you agree to take part in this study? **YES/NO**

Signed: …… Date: ……

Name (Block capitals) ……

I, (Name of investigator, block letters) …… have explained the nature and purpose of the study to …… and believe that he/she understands what the study involves.

Signed: …… Date: ……

Letter to patient's General Practitioner.

Re: Research Study: Molecular and functional mechanisms of human lung disease
Investigators: Professor A Wardlaw and AstraZeneca R & D Charnwood

Dear Dr .....

Your patient ..... has been invited to participate in clinical study of molecular and functional mechanisms of inflammation in lung tissues. Through this study we hope to gain a greater understanding of the mechanisms which determine the severity and persistence of COPD and other fibrotic disease. In the longer term we hope to identify molecular targets and develop novel therapeutic approaches in the treatment of human lung diseases. The study will involve approximately xx patients in a year admitted for cardiothoracic surgery at Glenfield Hospital.

Your patient has been invited to allow us to use for histological, molecular biological and functional experiments lung tissues that have been removed during their recent operation. Participation in this study will not affect the type or amount of treatment that your patient will receive.

If you have any objections to your patient participating in this study, of if you have any further questions that you would like us to answer, please contact me.

Yours sincerely

Professor Wardlaw

Appendix II

Ingredients and Tinctoral Staining Protocols

Modified Gills Haematoxylin

Reagents:

De-ionised Water	1.475 litres
Magnesium Sulphate (heptahydrate)	29.5g
Sodium Hydrogen Carbonate (Sodium Bicarbonate)	5.16g
Bronopol BNPD(2 Bromo 2 Nitropropane 1,3 Diol)	1.48g
Aluminium Sulphate	70g
1,2 Ethanediol (Ethylene Glycol)	0.5 litres
80% Acetic Acid	25 ml
Sodium Iodate	0.4g
Haematoxylin	6g

Method:

Solution A

Heat 400 ml of water to 30°C in a beaker and add the Magnesium Sulphate, Sodium Hydrogen carbonate and Bronopol BNDP, ensuring that each chemical has dissolved before adding the next. If required, solution can be heated further but do not exceed 45°C.

Solution B

In a second beaker, heat 400 ml of water to 30°C and dissolve the Aluminium Sulphate whilst heating to 40-45°C and stirring.

Mix solution A and solution B together and add the 1,2,Ethanediol whilst mixing vigorously.

Make slurry from the haematoxylin and 45 ml of water; add this to the mixture followed by the Sodium Iodate and Acetic Acid. Finally add the remaining water (630ml) and stir the mixture well.

Haematoxylin and Eosin (H&E)

Reagents:

Modified Gill Haematoxylin – See protocol above

1% Eosin – 1g Eosin Y, 100ml Distilled Water and a spatula of Calcium chloride

Saturated Borax

Method:

1. Dewax slides in 99% Xylene and rehydrate through graded alcohols (Ethanol 95% x2, Industrial Methylated Spirits 70% x2, distilled water).
2. Stain in Gills Haematoxylin for approximately 8minutes
3. Rinse in running tap water
4. Treat slides with saturated Borax – 30-60 seconds
5. Rinse in distilled water
6. Stain in 1% eosin for 30-60 seconds
7. Quickly rinse in water
8. Dehydrate by reversing step 1, mount a coverslip and allow to dry

Results:

Briefly this stain colours nuclei dark blue/purple, cytoplasm is stained pink, while erythrocytes appear orange/red.

Miller's Elastic Van Gieson (EVG)

Reagents:

Acid Potassium Permanganate - 0.5% potassium permanganate in 3% sulphuric acid

1% Oxalic Acid

Miller's Stain (Miller P 1971)

Van Gieson - Saturated Picric Acid 50ml, 1% aqueous Acid Fuchsin 9ml, distilled water 50 ml

Method:

1. Dewax slides and rehydrate as described previously(99% xylene x2, 95% ethanol x2, 70% IMS x2, distilled water)
2. Acid potassium permanganate – 5 minutes
3. Rinse in distilled water
4. Decolourise in 1% Oxalic Acid – 2 minutes
5. Rinse in 95% ethanol
6. Place in Miller's stain in a closed coplin jar for 1-3 hours
7. Rinse in 95% ethanol
8. Rinse in distilled water
9. Stain with Van Gieson – 1 minute
10. Blot dry with tissue paper
11. Dehydrate by reversing step 1 as previously described, mount a coverslip and allow to dry

Results:

Briefly this stain colours elastic tissue black/deep purple, collagen appears red, and all other tissues are a pale yellow.

Picrosirius Red

Reagents:

Picro-Sirius Red **-** Sirius red 0.5g,

Saturated aqueous solution of picric acid 500g

Add a little solid picric acid to ensure saturation

16ml acidified water (45% glacial acetic acid).

Method

1. Dewax and rehydrate slides as previously described
2. Stain nuclei with Gill Hematoxylin for 10minutes
3. Wash sections in running tap water
4. Stain in picrosirius red for 1 hour
5. Wash in two changes of acidified water
6. Dehydrate in three changes of 100% Ethanol, then 99% xylene, mount a coverslip and allow to dry

Results:

In ordinary bright- field microscopy collagen is red on a yellow background. When examined with polarised light microscopy the larger, more mature and thus more dehydrated collagen fibres are bright yellow or orange. The less mature, thinner fibres including reticular fibres are green. Birefringence is highly specific for collagen.

Martius Scarlet Blue (MSB)

Reagents:

Martius Yellow – 0.5g Martius yellow, 2g phosphotungstic acid and 100ml 95% ethanol

Brilliant Crystal Scarlet – 1g Brilliant Crystal Scarlet, 2ml glacial acetic acid, 100ml distilled water

1% Phosphotungstic Acid – 1.0g phosphotungstic acid in 100ml distilled water

Methyl Blue – 0.5g Methyl blue, 1ml glacial acetic acid, 100ml distilled water

1% Glacial Acetic Acid – 1.0ml glacial acetic acid in 100ml distilled water

1% Celestine Blue – 1.0g Celestine blue in 100ml distilled water

95% Alcohol – 95ml IMS in 5ml distilled water

Method:

1. Dewax slides and rehydrate as previously described
2. Stain sections with Celestine blue for 5minutes
3. Drain and stain with Gills Haematoxylin for 5minutes
4. Rinse in distilled water
5. Rinse in Borax to maintain colour and check staining microscopically (erythrocytes will look blue but they will get differentiated in steps 7,9 and 12)
6. Rinse in 95% alcohol
7. Stain with 0.5% Martius yellow for 2mins (erythrocytes should be yellow)
8. Rinse in distilled water
9. Stain sections in 1% Brilliant Crystal Scarlet for up to 10mins (check microscopically after 5mins)
10. Treat sections with 1% Phosphotungstic acid to fix/differentiate the red stain for up to 5mins. Any longer and the red stain could be washed away
11. Drain (Do NOT wash in water, this will wash out the red)

12. Treat sections with 0.5% Methyl Blue for approximately 2-5mins (check microscopically that yellow and red colours remain)
13. Rinse in 1% acetic acid for up to 10mins (check microscopically)
14. Dehydrate slides, mount a coverslip and allow to dry

Results:

Nuclei	Black
Erythrocytes	Yellow
Fibrin	Red/Purple depending on age/maturity
Collagen/Connective tissue	Bright Blue/Purple depending on age/maturity
Smooth Muscle	Pink/Red

Appendix III

The standardised laboratory protocol used for immunohistochemistry of validated antibodies.

Method:

- Slides were rehydrated as previously described
- All sera and antibodies were made up in Bovine Serum Albumin in Phosphate Buffered Saline pH 7.4 with 0.05% Tween (a polysorbate surfactant).
- Washes between each step, except serum to primary, used Phosphate Buffered Saline pH 7.4 with 0.05% Tween. The wash between serum to primary used Phosphate Buffered Saline pH 7.4.

1. Antigen retrieval:

- Nothing
- 0.1% Trypsin in 0.1% Calcium Chloride pH 7.8 for 30 minutes at 37^{o}C
- Pressure cook at 15lb in Vector Antigen Unmasking fluid for 2 minutes
- Microwave in Vector Antigen Unmasking fluid to vigorous boil

Rinse

2. Quench endogenous peroxidases:

- 0.5% Hydrogen peroxide in methanol for 10 minutes

Rinse

3. Protein block:

- 20% Normal Serum –serum raised in the same animal species as the secondary antibody.

Rinse

4a. Primary Antibody:

- For 60 minutes at room temperature

4b. Negative Control:

- Relevant isotype and protein concentration or blocking peptide to primary antibody
- For same length of time as primary antibody.

Rinse

5. Biotinylated Secondary antibody:

- 30 minutes

Rinse

6a. Tyramide Signal Amplification (TSA) Kit:

- According to manufacturer's instructions
- Streptavidin Horseradish Peroxidase (SA-HRP), 30 minutes
- Biotinyl Tyramide, 10 minutes
- Streptavidin Horseradish Peroxidase (SA-HRP), 30 minutes

6b. 'Duet'

- According to manufacturer's instructions
- (Streptavidin AB/HRP), 30 minutes 1:100

Rinse

7. Diaminobenzidine tetrahydrochloride (DAB)

Appendix IV

Publications from this thesis (to date)

Papers:

Vaughan P, Oey IF, Steiner MC, Morgan MDL, Waller DA. A prospective analysis of the inter-relationship between Lung Volume Reduction Surgery and Body Mass Index. European Journal of Cardiothoracic Surgery 2007;32(6):839-842

Vaughan P, Oey IF, Nakas A, Martin-Ucar AE, Edwards JG, Waller DA. Is there a role for therapeutic lobectomy for emphysema? European Journal of Cardiothoracic Surgery 2007;31(3):486-90

Vaughan P, Waller DA. Surgical treatment of pulmonary emphysema. Surgery 2005;23(12):435-438

Abstracts Presented at Meetings:

American Thoracic Society May 2008.

Howard LS, Vaughan P, Crosby A, Southwood M, Morrell NW, Foster M. The Role of HIF-1α in Driving Apoptosis and Proliferation in Pulmonary Vascular Remodelling in Emphysema. American Journal of Respiratory and Critical Care Medicine, Apr 2008;177;A590

European Respiratory Society Sept 2006

Vaughan P, Pinnion K, Waller DA, Foster ML. A histopathological grading system for vascular remodelling in severe COPD lung resections. European Respiratory Journal 2006;28(50):803s

Sirico G, Pinnion K, **Vaughan P**, Waller DA, Foster ML. Histology of arteriolar wall remodelling in a lung volume reduction surgery (LVRS) patient cohort.
European Respiratory Journal 2006;28(50):803s

Vaughan P, Oey IF, Morgan MDL, Waller DA. Long term results of staged bilateral Video-Assisted Thoracoscopic(VAT) Lung Volume Reduction Surgery(LVRS) determined by patient preference.
European Respiratory Journal 2006;28(50):598s

British Thoracic Society 2006

Khalil MW, Oey IF, **Vaughan P**, Morgan MDL, Waller DA. Does the weight of lung resected influence the outcome of lung volume reduction surgery?

Thorax 2006;61 suppl II:74

Oey IF, **Vaughan P**, Morgan MDL, Waller DA. Spreading The Benefit Of Lung Volume Reduction Surgery – One Stage Bilateral Versus A Staged Unilateral Approach Determined By The Patient.

Thorax 2006;61 suppl II:72

Vaughan P, Pinnion K, Waller DA, Foster ML. Image analysis of elastin changes in pulmonary vascular remodelling in severe COPD shows loss of medial organisation.

Thorax 2006;61 suppl II:21

Vaughan P, Oey IF, Morgan MDL, Spyt TJ, Waller DA. Lung Volume Reduction Surgery (LVRS) for Emphysema – Lessons learnt from the first 100 cases in a single institution.

Thorax 2005;60 suppl II;106