Histopathology of diffuse lung parenchyma epithelial metaplasia in COPD

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COPD is a chronic, multifactorial lung disease with multi-organ, systemic presentation that affects approximately 20% of smokers. Diffuse parenchymal epithelial metaplasia is seen in COPD patients and although rarely described, is usually considered to be Lambertosis, a reparative extension of airway epithelium.

Cellular phenotype and pulmonary distribution of diffuse metaplasia was examined histologically and the cells characterised as either rounded and uniform, large and irregular resembling macrophages or squamous and flattened. The lesions were found to be localised to the external adventitia of the airways and vessels, sub pleural zone, hilar regions, ectopic fibrotic deposits and the external facet of alveolar epithelium. This distribution is not consistent with Lambertosis.

This repair process hypothesis was evaluated by cellular markers consistent to known airway progenitors. No expression of CK5/6 basal nor CC10 Clara cell was detected. Surfactant presence suggested a type II phenotype indicating an alveolar origin. These findings further refute Lambertosis. The metaplastic population was further characterised using epithelial and progenitor markers. These cells displayed occasional multipotent, progenitor markers possibly originating from bone marrow or resident stem cells (CD34/CD133).

The cytokeratin profile consistent with simple epithelium suggested new cell colonisation and mixed cytokeratin phenotype indicated a failure to mature. In addition to this evidence of repair response, metaplastic cells were found on expanded mesenchyme. This remodelled, scarred tissue further indicates damage and repair. Mutation detection of EGFR L858R by PCR did not show molecular changes indicative of early change in some adenocarcinoma, suggesting these cells do not progress to pre-neoplastic.

From this, we can conclude that diffuse metaplasia in COPD peripheral lung is not consistent with Lambertosis. Overall, characteristics including surfactant and mucin presence, suggest a primitive epithelial phenotype, potentially a result of damage and aberrant repair.

Acknowledgements

I would like to thank Martyn Foster and Howard Pringle for their unwavering support as supervisors throughout the years. Thanks go also to Sarah Bolton who has been on hand day to day to help with queries, all the ladies in the lab, (Elaine, Jamie, Sylv, Hina, Sue, Pav, Gina and Isabel) who have helped maintain my sense of humour and been extremely supportive of my continued absence from the work rota! Thanks go also to people from other labs, Angie and Linda who put up with me experimenting in their lab at Leicester, Tom Fehniger who provided information about mucin staining protocols. Simon Brocklehurst and Allison Bigley at Alderley Park who shared my enthusiasm for and helped me produce all the EM pictures. Thank-you to fellow students Paul Vaughan, Guisy Sirico, Melinda Carpenter and Laura Mackay who have shared stresses and ideas. Most of all thanks to my future husband Nick who has waited patiently whilst I found my feet with this research lark.

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Abbreviations

AAH Atypical adenomatous hyperplasia

ARDS Adult respiratory distress syndrome

BAC Bronchioloalveolar carcinoma

BMP Bone morphogenic protein

BO Barrett's oesophagus

BOS Bronchiolitis obliterans syndrome

BPD Bronchopulmonary dysplasia

CA IX Carbonic anhydrase IX

CCAM Congenital cystic adenomatoid malformation

CC10 Clara cell 10kDa

CD Cluster of differentiation

CDC6 Cell division cycle 6

CEA Cancer embryonic antigen

CF Cystic fibrosis

CFTR Transmembrane conductance regulator

CGRP Calcitonin gene-related peptide

CK Cytokeratin

COPD Chronic obstructive pulmonary disease

CXCR4 CXC chemokine receptor 4

DNA Deoxyribonucleic acid

EGF Epidermal growth factor receptor

EGFR Epidermal growth factor receptor

EM Electron microscopy

EMA Epithelial membrane antigen

EVG Elastic van Gieson

FBN1 Fibrillins-1

FEV_1 Forced expiratory volume (in 1 second)

FGF Fibroblast derived growth factor

FISH Fluorescent in situ hybridisation

FOX Forkhead box

FVC Forced vital capacity

GCH Goblet cell hyperplasia

GI Gastrointestinal

GSTPi Glutathione s-transferase factor-1

H&E Haematoxylin and Eosin

HGF Hepatocyte growth factor

HIF Hypoxia inducible factor

HSC Haematopoietic stem cell

Ig Immunoglobulin

IHC Immunohistochemistry

KGF Keratinocyte growth factor

IL Interleukin

IPF Idiopathic Pulmonary Fibrosis

LVRS Lung volume reduction surgery

NSCLC Non small cell lung carcinoma

NSE Neuron-specific enolase

MMP Matrix metalloproteinase

PAS Periodic acid Schiff's

PCR Polymerase chain reaction

PDGF Platelet derived growth factor

PGE2 Prostoglandin E2

SCLC Small cell lung carcinoma

SDF Stromal derived factor

SSEA Stage specific embryonic antigen

TGF Transforming growth factor

TIMP Tissue inhibitor of metalloproteinase

TNF Tumour necrosis factor

TPA Tissue polypeptide antigen

TPS Tissue polypeptide-specific antigen

TTF Thyroid transcription factor

VAT Video-assisted thorascopic

VEGF Vascular endothelial growth factor

VHL Von Hippel Lindau

WHO World Health Organisation

Chapter 1. Introduction

COPD is a condition that involves all the compartments of the lung. Inflammation is indisputably the key phenotype observed in COPD. Much of the COPD research has focused on this and the subsequent damage affecting the lung. There has been little work performed to examine how the lung responds to this inflammation and damage. The main objective of this work was to examine the diffuse metaplastic lesion observed in the parenchyma of COPD patients. This phenomenon has been noted previously (Leslie 2005), however it is often overlooked or assumed to be type II cell hyperplasia. Prior observations suggest that the cells are an extension of damaged airway epithelium spreading into the lung surrounding the airways. This is often referred to as peribronchiolar metaplasia (Fukuoka *et al.* 2005). It should be noted that the lesion occurs on many adventitial surfaces, sub pleura, vascular adventitia and fibrotic regions, not just surrounding airway adventitia. The exact role, origin and significance of these ectopic cells have not been previously investigated in depth.

Epithelial metaplasia is the development of epithelial cells that differ from the normal population of the location in question. Epithelial metaplasia is seen within normal repair processes and in disease, not just within the lung but throughout the body (Leslie 2005, Puchelle *et al.* 2006). The focus of this investigation was around the ectopic epithelial cells within COPD parenchyma and all locations were considered. The overall aim of the work was to consider the epithelial cells in a more general epithelial response context, as well as considering this specific lung disease. With this in mind lung development and adult structure are described in this chapter to provide insights into lesion pathogenesis. This information gives an understanding of what is normal and abnormal within the lung.

1.1 Lung development

The main focus of this lung development section is the development of lung epithelium and epithelial phenotypes with consideration of matrix and connective fibres that influence development.

The lung forms from the primitive foregut. It begins as a longitudinal groove along the pharnyx growing into the larnygeotracheal tube. The tube is lined with endoderm, flattened cells that develop into columnar cells, usually contributing to muscular

structures. As a result of this origin, early developmental behaviour of the lung and gut may be similar. Initial budding and branching occurs during the embryonic stage (0-7 weeks). Human lung development takes place throughout gestation and continues until around 2 years of age (Demayo *et al.* 2002).

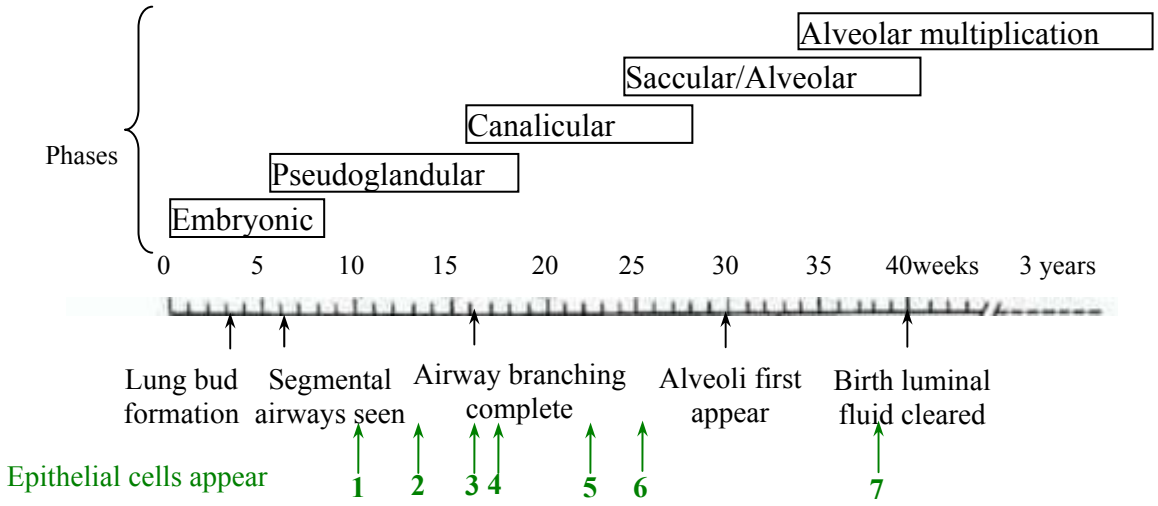


Figure 1.1 Development phases & evolution of cell phenotypes
A schematic demonstrating timeline of development of the key epithelial phenotypes through lung development. 1 Dense Core Granule cell, 2 Ciliated/ Goblet cells, 3 Intra Epithelial Lymphocyte, 4 Basal cells, 5 Type II cells, 6 Clara cells, 7 Increase in Dense Core Granule cell. Diagram adapted from Jeffrey (1998).

The mesenchyme and fibroblasts influence the formation and maintenance of the lung (Barsky 2003). The lung initially coats all surfaces with a pluripotent default epithelium that later become a number of different epithelial phenotypes. The timing of the appearance of these mature phenotypes is shown in Figure 1.1 above. There are a number of released and positional cues that control phenotypic development. Fibronectin is an important matrix component during development, playing a structural and migratory mediating role (Sinkin *et al.* 1998, Torikata *et al.* 1985). During early development the lamina densa of airway basement membrane forms. It is composed of collagen IV, laminins, and entactin/nidogen. These are augmented later with proteoglycans but this basic structure is maintained and persists.

1.2.1 Pseudoglandular phase (5-17 weeks):

Further branching occurs with up to 21 generations of the respiratory tree, up to terminal bronchioles are formed. Airway walls differentiate into adult structures showing cartilage, submucosal glands, bronchial smooth muscle and several epithelial cell types. Tissue has a glandular appearance at this stage, (Figure 1.2A).

Neuroendocrine cells mature first at 8 weeks gestation. Immunohistochemistry (IHC) for chromogranin or electron microscopy (EM) identifying cells with long basement processes but no glycogen production or microvilli identifies foetal neuroendocrine cells. Epithelial cells present on a thin basement membrane at around week 10 are undifferentiated with an ovoid nucleus in the apical or basal part of the cell. Initial undifferentiated epithelium mainly expresses simple cytokeratins (CK8, CK18 and CK19) with only weak expression of CK7 (Broers *et al.* 1989).

Cytokeratins are a family of skeletal proteins that are found exclusively in epithelial cells. These proteins occur in different pairings in different epithelial cells. During development surfactant D can be detected at around 10-20 weeks of gestation and airways are lined with surfactant staining. As gestation continues staining weakens and disappears, being absent first in proximal airways, then peripheral airways until it is confined to the parenchyma. This suggests foetal airway epithelial cells produce surfactants (Stahlman *et al.* 2002). Primitive mouse epithelium has been shown to co-express surfactants A and C, Clara cell protein (CC10) and calcitonin gene-related peptide (cGRP) (Wuenschell *et al.* 1996). These are markers of type II cells, Clara cells and neuroendocrine cells. Other work in mice suggests that ciliated, non-ciliated (Clara), goblet, type II, and type I cells all share a common lineage (Perl *et al.* 2002). Looking at early foetal epithelial cells using EM the apical surface had small microvilli. The cells contain mature and immature glycogen, numerous mitochondria and sometimes centrioles (Gaillard *et al.* 1989). Neuroepithelial bodies are found by 13-16 weeks of gestation and cGRP is only detected late in gestation and postnatally.

With regard to the extracellular matrix there are also a number of changes occurring. Tenascin-C is strongly present around chondrocytes and underneath pseudo-stratified epithelia, especially where branching occurs. Laminin α1 is restricted to the first trimester (0-12 weeks) and is mainly on epithelial and some mesenchymal cells. Laminins α2 and α4 are associated with mesenchymal cells and laminin α3 and α5 are associated with epithelial cells. Laminin α1, α2 and α3 are all in airway basement membrane in early development. Laminin α4 is seen around airway smooth muscle cells and later lining the vasculature (Nguyen, Senior 2006).

1.2.2 Canalicular phase (13-25 weeks):

Bronchi and bronchioli lumen enlarge and tissue is highly vascularised, whilst sacs begin to develop and type I and II pneumocytes start appearing. These changes are

summarised in Figure 1.2B. Angiogenesis occurs forming a dense capillary network, stimulating the epithelium to become type I cells. The terminal bronchioles branch to form several orders of respiratory bronchioles. The mesenchyme differentiates, producing chondrocytes, fibroblasts and myoblasts. Blood vessels begin to develop in this stage, following the airways as a template. Bronchial smooth muscle cells move around the vessels to form arteries.

Mucin 4 is the earliest mucin identified in foetal lung. By 12.5 weeks the predominant mucin is mucin 1. High mucin levels are seen in upper airways of fetal lung and lower airways of adult lung. Ciliated cells develop primitive cilia initially, followed by a second ciliogenesis phase to produce adult cilia. From around 13 weeks, ciliated cells mature, near the trachea first and spreading down the respiratory tree (Gaillard *et al.* 1989). Cytokeratins detected in ciliated cells are CK5, CK7, CK8, CK18 and CK19 (Broers *et al.* 1989). Secretory cells are observed at around week 14/15. Differentiation of secretory cells also starts in the trachea, opposite cartilage. Fully mature goblet cells are rare in fetal lung. Clara cells, detected using CC10, have been observed at around 15 weeks of gestation, initially clusters of 3-5 cells usually at branch points. Mucin 2 is only observed in low levels after 18 weeks. The number of secretory cells peaks at around 19 weeks and then declines, being replaced by ciliated cells.

It is thought that sac structure is determined by elastic fibre position. Without a fibre network the lung develops an emphysematous phenotype (Wendel *et al.* 2000). Tenascin-C remains strong underneath simple and branching epithelium. Bateman, Turner-Warwick and Adelmann-Grill (1981) have examined the distribution of collagens I, II, III and IV, in samples at 16-24 weeks of gestation. The cartilage plates within the bronchial wall stained positive for both type I and type II collagen, with type II collagen confined to cartilage. Type I collagen was in all bronchus layers except the mucosa. Type III collagen was not associated with the cartilage but in all other locations weak type III co-localises with type I collagen.

1.2.3 Terminal sac period/Alveolar stage:

By 24 weeks Clara cells make up 11.2% of the bronchioles (Barth, Wolf & Ramaswamy 1994). In central airways Clara cells are less common, observed at around 5.4%. Surfactant A positive cells are seen in the airways by 29 weeks, increasing in number until 39 weeks (Endo & Oka 1991). Non-ciliated surfactant producing cells act as progenitors for ciliated epithelium and Clara cells (Evans, Cabral-Anderson &

Freeman 1978). Tenascin-C staining becomes weaker around airways throughout the terminal sac period. Strong expression is seen in the intima of veins. Intimal and chondrocyte staining lessens as 40 weeks of development approaches. Lung parenchyma appearance during this phase is summarised in Figure 1.2C.

1.2.4 Alveolar Period:

Muscle cells migrate from bronchial smooth muscle to form around the arteries. In later development, fibroblasts form arterial mesenchyme becoming a smooth muscle phenotype. Veins have smooth muscle derived from fibroblasts as opposed to migrated bronchial muscle (Hislop 2002). Alveolarization starts proximally to the bronchioles, beginning as out growing pouches from bronchioles (Plopper *et al.* 1992). At around 37 weeks bronchioles, alveolar bed and mucus glands show CK8, CK18 and CK19 expression. All bronchiolar cells except the basal layer are CK7 positive, as are alveolar cells and mucus glands. CK14 cells are now found in large airway basal cells and within mucus gland basal cells arising from columnar epithelium. These are the last cell type to be seen. Scattered alveolar cells are positive for CK13 and CK4. The cytokeratin profile of the newborn is similar to that seen in late fetal development (Broers *et al.* 1989). Thibeault *et al.* (2003) showed that parenchymal collagen increased throughout gestation and by 30 weeks there was a fine network of collagen throughout the lung. The structure of this network dictates final lung structure, damage to this network damages the whole lung. Alveolar networks are comprised of collagens I and III and are involved in branching (Kaarteenaho-Wiik *et al.* 2004). Collagen IV is also significant as this can be produced by epithelial cells and facilitate cell migration (Legrand *et al.* 1999). The main parenchymal collagens are collagens I, III and IV. A study in mice looked at embryonic stem cell development into airway epithelium. It showed that within eight days of culture stem cells differentiated into cells Clara cell specific protein producing cells if cultured on type I collagen, but not on type IV or VI (Coraux *et al.* 2005). This suggests that the presence of collagen I is important for airway development. This final alveolar phase of development continues until around 2 years of age. Figure 1.2 shows an overview of the three main stages of development.

The lung is a complex organ that can experience damage even *in utero*. This may constitute a failure or fault in development or damage due to infection, trauma or overexpression of signals as mentioned above with transforming growth factor β

(TGFβ). These lung insults can result in diseases that permanently alter lung structure or have lasting functional consequences.

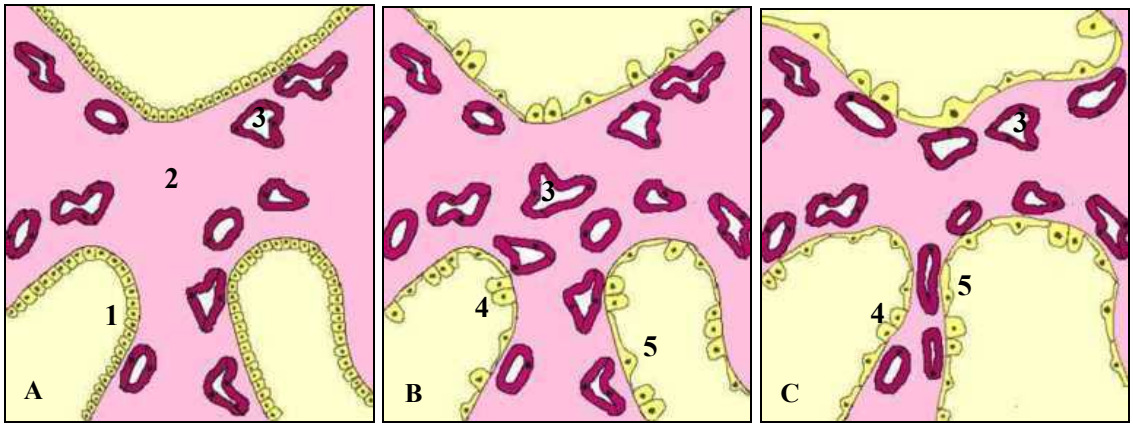


Figure 1.2 Overview of development phases
Adapted from diagrams by Burri *et al*. (1984).- 1 cubic type II pneumocytes, 2 mesenchyme, 3 capilliary, 4 Type II pneumocyte, 5 type I pneumocyte. This shows lung during the pseudoglandular phase, at this stage the lungs resemble a gland. Epithelium is a cubic primitive pluripotent phenotype, uniform throughout the lungs. 16 generations of the airways are formed. This progresses 20 generations by the end of the pseudoglandular period. At the end of this phase type II cells are distinguishable from cubic epithelium. These are the first to develop. 1.2B shows the lung during the canalicular phase, during this phase the canaliculi branch out from the terminal bronchioli. This is the respiratory part of the lung, the parenchyma. There are changes occurring within the epithelial cells and mesenchyme. Type I and Type II cells are both identifiable. The capillaries are proliferating to prepare the lung for gaseous exchange. 1.2C shows the lung during the sacculation phase, the matrix is important in this phase and it helps determine the phenotype of overlying epithelium. The alveolar sacs continue to form at this point the septae walls are quite thick, thinning will allow gaseous exchange by birth.

Developmental lung diseases will be discussed further in section 1.5.2 that looks at bronchopulmonary dysplasia, a failure to complete development thought to be triggered in part by infection. Another example is pulmonary sequestration: the development of an entirely separate mini lung lobe that has no communication with the main lung. This may remain asymptomatic or harbour infection causing lung wide impact.

1.2 The general structure and function of the adult lung

The lungs are found within the chest cavity, surrounded by pleura, a protective sheath made up of mesothelial cells. The main function of the lung is gaseous exchange. The lung begins with the trachea and branches up to 23 times, giving rise to around 300 million alveoli. This increases surface area and takes air for gaseous exchange to the alveolar bed that comprises of type I pneumocytes and capillaries. Adult lung capacity is 4-6 litres. This varies according to the size and gender of the person. Smoking status and altitude also influence capacity. Only around half a litre is breathed in and when the individual is breathing normally.

Figure 1.3 shows respiratory tree structure. The lower lobe with airways of approximately 5mm and smaller represent the samples available for this study.

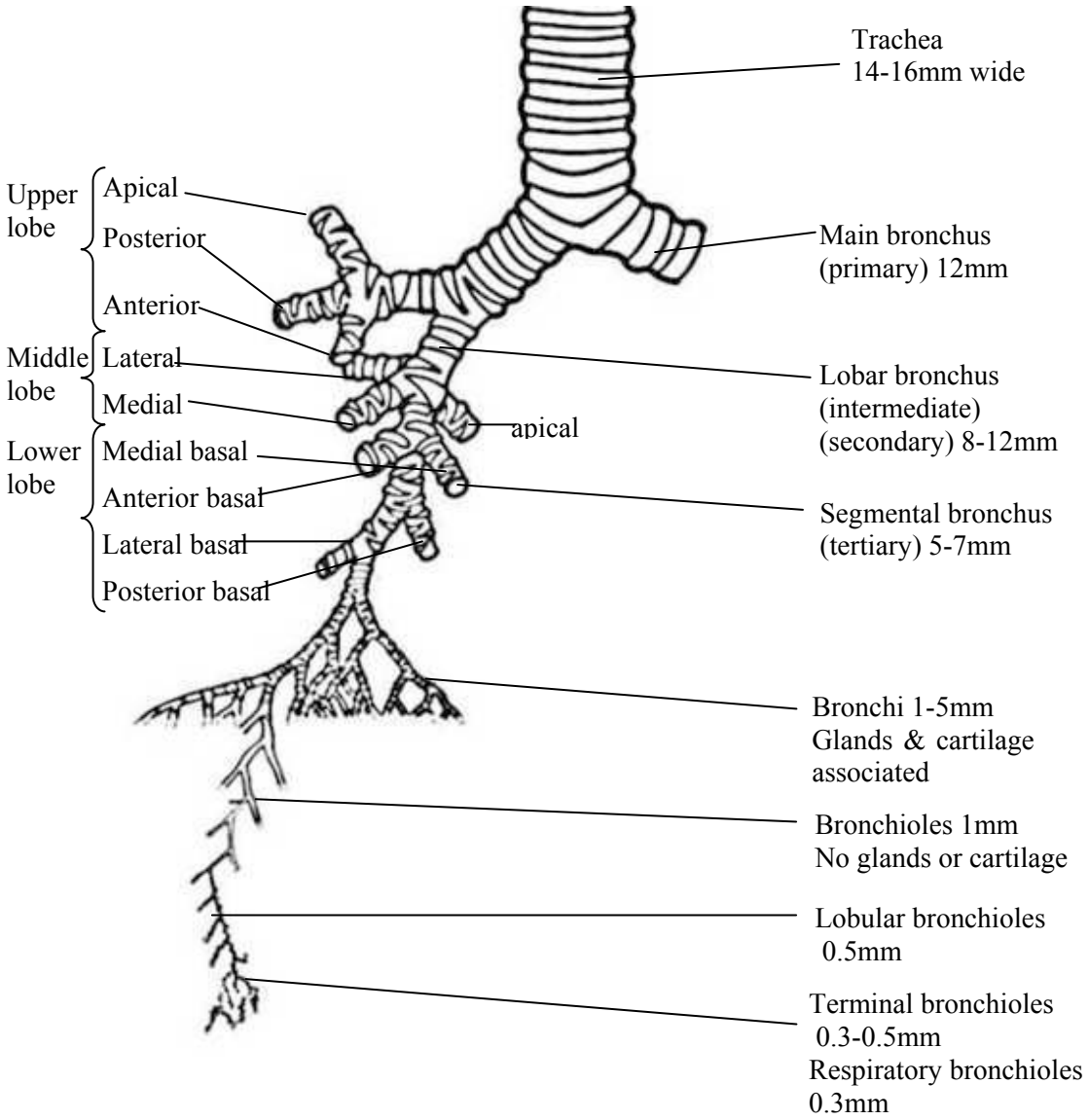


Figure 1.3 Architecture of the lung

Airway branches within the normal adult lung. The areas of lung that will be available for this thesis were mainly from the lower lobe. Diagram adapted from Young & Heath (2000).

Figure 1.4 demonstrates some of the overall structures and basic lung cell types. In the bronchial epithelium shown in Figure 1.4A the small round basal cells sit directly on basement membrane and a range of epithelial cells contribute to the upper layers. The figure shows ciliated cells in the luminal epithelial layer. The sub mucosa of this airway is a little oedematous, so is not entirely normal. Cell debris and a mucus plug within the airway lumen in Figure 1.4C are also not seen in normal healthy lung. Figure 1.4 B shows mucus glands associated with larger airways. Figure 1.4D shows alveolar bed, the small vessels within the sample are a little distended and remodelled, this is not considered normal. Figures 1.4E and F show vessels within the lung parenchyma and

pleura, these are a little oedematous. These lungs are from older individuals that died from causes outside the lung, however age and environmental damage still cause changes. Some cells types are difficult to identify from H&E.

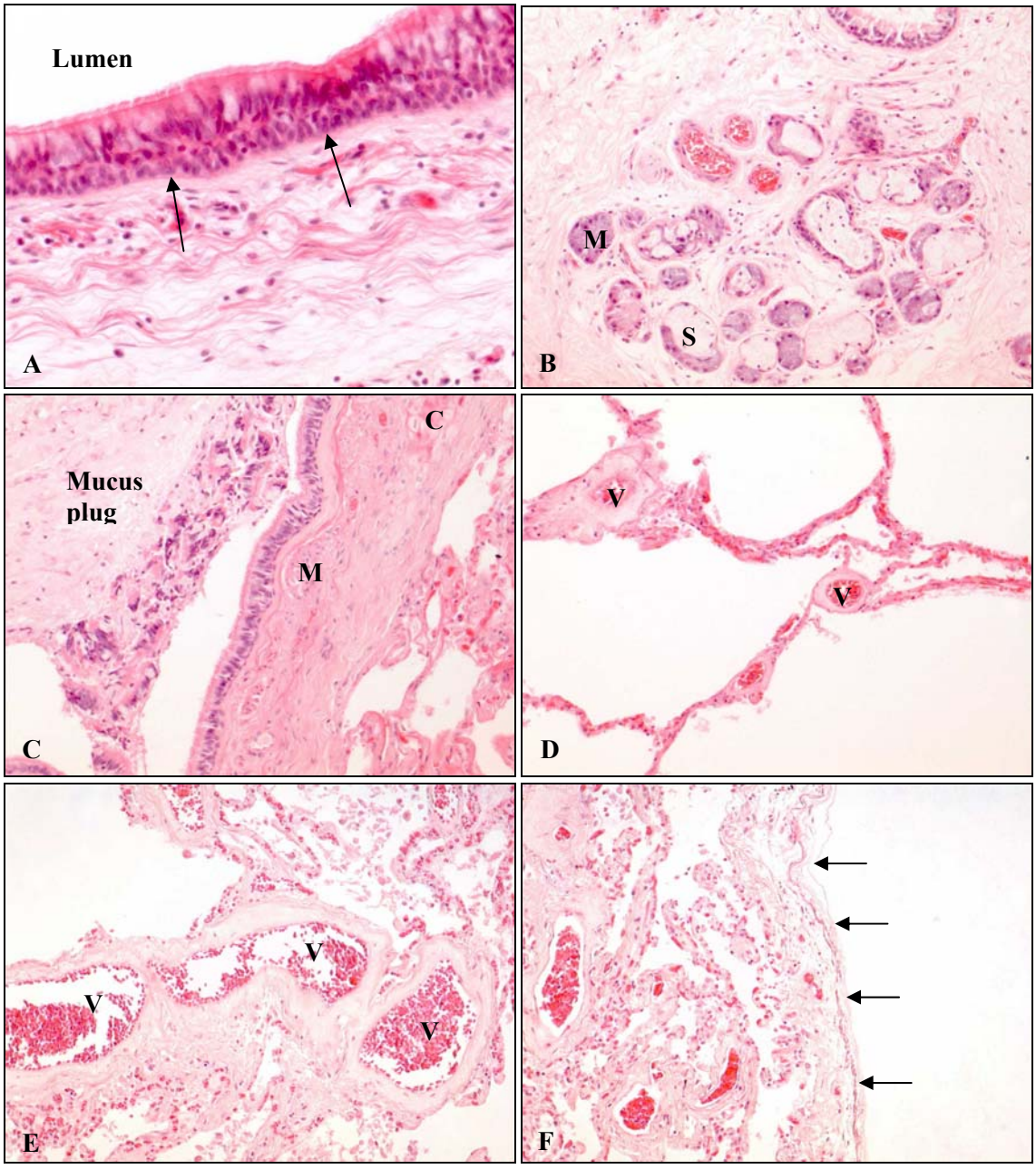


Figure 1.4 Examples of normal lung histology

Autopsy tissue from individuals who died from causes outside the lungs; represent relatively normal.
Figure 1.4A) x20 objective. Bronchial epithelium. Arrows indicate basement membrane surround.
Figure 1.4B) x10 objective. Glands usually associated with the upper airways. There is a mixture of pale serous (S) and purple mucus (M) glands within this group of glands.
Figure 1.4C) x10 objective. Large airways from lower in the respiratory tree than (A). The airway basement membrane pictured here is very thickened and not normal. Muscle blocks underlie the airway and at the top of the picture a small area of cartilage. The epithelium has visible basal and ciliated cells.
Figure 1.4D) x10 objective. Relatively normal alveolar bed. The alveolar septae are very thin pieces of tissue.
Figure 1.4E) x10 objective. Vessels within relatively normal lung (V).
Figure 1.4F) x10 objective. Pleura within relatively normal lung (arrows).

There are around 40 different cell types found within the lung, including a range of specialist epithelial cells. Figure 1.5 illustrates many of the key cell phenotypes. Epithelial phenotypes found within the lung are discussed in section 4.1.

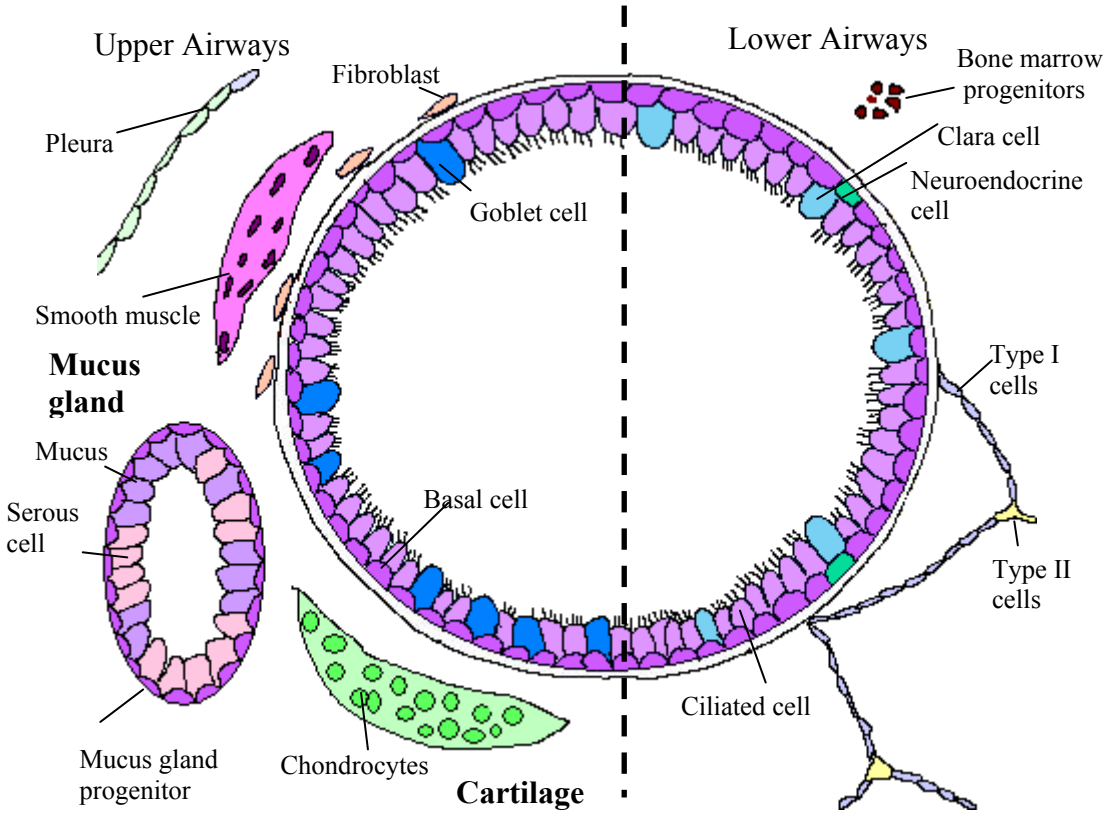


Figure 1.5 Diagram to show the cell types in & around the airways
The epithelial cells and associated cells in the upper and lower airways and out into the alveolar bed. The left of the diagram shows the upper airways structure and main cell types and the right hand side shows the lower airways.

1.3 Repair processes

Lung physiology leads to constant exposure to inhaled particles, bacteria and viruses. This results in repeated cycles of damage and repair of the lung mucosal barrier. To allow comparison of normal and abnormal repair, it is necessary to understand the normal lung repair phenotype. Lung tissue has a lower baseline turnover than tissues such as the skin and gut. Lung proliferation has been demonstrated to be less than 1% of epithelial cells under normal baseline conditions (Ayers & Jeffery 1988), this will include some normal cell replacement and some repair. Gut has a baseline proliferation of between 8% and almost 18% depending upon where in the GI tract measurements are

taken (Potten *et al.* 1992). Proliferation will increase in all these organs as a result of damage.

The epithelial response to injury can be summarised into a five-step process. (1) Remaining epithelial cells migrate to cover bare basement membrane, (2) epithelial, usually basal cells, proliferate, replacing damaged or lost cells and may become squamous or mucus cell metaplasia. (3) After proliferation, overpopulated areas of epithelium discard cells though apoptosis to reach normal levels. (4) Once cell numbers are re-established maturation to normal cell phenotypes occurs. (5) Finally, rat studies examining ozone exposure have shown that epithelial cells develop a memory of chronic exposure conditions responding more quickly if exposed again. These steps are summarised in Figure 1.6 allowing rapid response to minimise injury (Jeffery 1996, Tesfaigzi 2003).

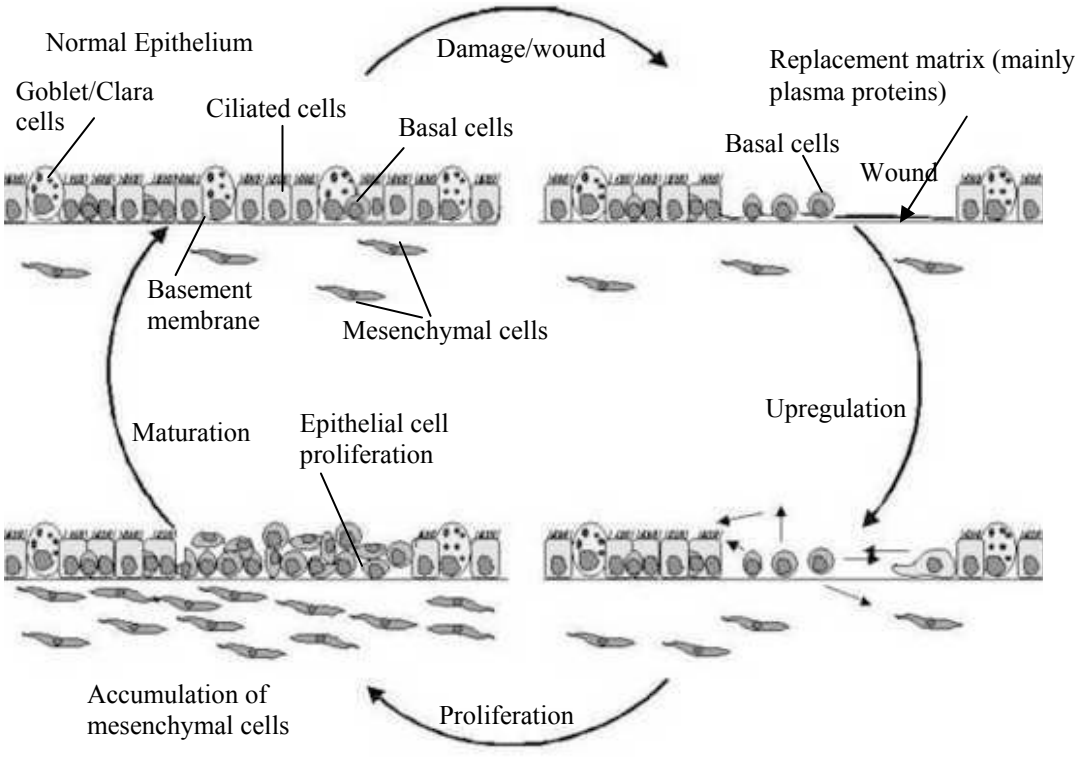


Figure 1.6 Epithelial repair processes

The cycle of normal airway repair, damage occurs, cell mediators are released and the basal cells move to cover the denuded area of basement membrane. Cell mediators are released (small arrows). Epithelial cells and underlying fibroblasts release messages that act as autocrine or exocrine mediators. Cells proliferate and mature into differentiated phenotypes, apoptosis may occur to clear any excess cells. Diagram adapted from Rennard (1999).

Previous work has shown that insult may cause mucosal phenotype change including, but not limited to, serous cells to mucus cells, ciliated cells to secretory cells and

secretory cells to squamous cells. These changes are reversible and allow the epithelial regeneration. Phenotype changes may lead to goblet cell hyperplasia and a range of metaplasia including squamous and non-squamous and all of the various phenotypes of mucus cells. In COPD basal and mucus cell hyperplasia, mucus cell proliferation and squamous cell metaplasia have been observed and are considered as aspects of the repair process. In COPD there is not a complete resolution to normal epithelium, probably as a consequence of the continuous and incomplete mucosal repair cycles (Puchelle *et al.* 2006).

Specialist lung defence mechanisms include clearance via the muco-cilary escalator. The surface cilia of airway columnar cells beat, moving mucus and entrapped debris up the respiratory tract to be expelled. Cigarette smoke and air pollutants may significantly increase mucus secretions, an initial response to injury. Soluble particles are cleared into the blood stream through the airway mucosal surface or via pinocytotic ducts in mucosal cells.

Epithelial cells are linked via tight and intermediate junctions to form one continuous barrier. These tight junctions control epithelial permeability, allowing secretion or absorption of fluids and molecules.

In order to understand the function of some repair processes it is helpful to have information about some lung diseases and the damage occurring in these disease processes. Tight junctions become leaky if damaged by smoke or allergen exposure. This breach may allow bacterial agents and released factors to attack the submucosa. It takes several days for the barrier to recover and in chronic lung disease, such as COPD the barrier may not be as effective after repeat infections. The transmembrane conductance regulator (CFTR) forms part of the control of ion and water content of the periciliary lining fluid that allows cilia to beat. If CFTR is impaired, mucus clearance suffers and mucus load increases. CFTR distribution may be altered in COPD sufferers by the squamous metaplasia and mucus hyperplasia commonly seen after exacerbation (Puchelle & Zahm 1996).

Cell and organelle movement during repair utilizes cytoskeletal proteins. The major signalling pathway involves trefoil factor family proteins and other mediators. Actins are needed for cells to extend lamellipodia during migration to wounded sites. Actin interaction is necessary for cell movement and has been shown to disrupted by virulence

factors, for example from *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These observations demonstrated that infection can disrupt repair (Puchelle *et al.* 2006). Matrix metalloproteinase 9 (MMP9) is necessary for type IV collagen degradation, allowing the cell to detach and move forward. Other MMPs are also important during the repair process. During injury macrophages, fibroblasts, endothelial cells and surface epithelial cells produce fibronectin, to act as a chemoattractant and an adhesive substrate for reparative epithelial cells.

Growth factors modulate all repair processes, including epithelial-mesenchymal interactions required for restoration of normal alveolar architecture after lung injury. Platelets, macrophages, fibroblasts, mesenchymal cells and epithelial cells all secrete growth factors. TGF-β_1 is involved in matrix distribution control, IL-13 can increase mucus gene expression, IL-4 may contribute to mucus cell metaplasia in COPD and IL-9 stimulates goblet cell hyperplasia, whereas the inhibition of EGFR tyrosine kinase halts the re-epitheialization process. Keratinocyte and hepatocyte growth factor may stimulate extracellular matrix synthesis (Puchelle *et al.* 2006, Jeffery 1996).

The lung has a resident population of macrophages within the interstitium to remove particles and any cell debris. During injury resident macrophages are capable of dividing. Aside from resident macrophages bone marrow also produces macrophages. These bone marrow macrophages enrich the resident population (Radzun, Parwaresch & Kreipe 1983). Other inflammatory cells are present or recruited to the lung during injury. These cells can cause tissue destruction by the release of enzymes and cells migration invading through epithelial barriers and vessels.

Alongside epithelial cell replacement and repair, there are also adaptations and repair of basement membrane and extracellular matrix. Airway associated fibroblasts are located outside the basement membrane in the sub mucosa. These contribute to repair by producing matrix, growth factors and cytokines. One very specific example of normal adaptation and repair is the production of relaxin during pregnancy. Relaxin is a peptide hormone from the insulin-like family. It stimulates fibroblasts in the reproductive tract to produce collagens I and III and is associated with growth and remodelling (Masterson *et al.* 2004).

Epithelial cells have several receptors that connect and interact with matrix protein. Epithelial receptors include collagen-laminin receptor, that binds α2, α3 and α6 chains,

fibronectin receptor and vitronectin receptor, which links with αv chains, and finally α6 β4 receptor. These integrin receptors act as adhesion molecules. The non-integrin receptors in epithelium are laminin receptor and membrane proteoglycans. The presence of these adhesion molecules suggests that collagen, fibronectin, vitronectin and laminin are all deposited underlying the epithelium (Montefort & Holgate 1991). Indeed epithelial repair involves deposition of laminins, collagens I and IV and fibronectin. Deposition is mediated by MMP-7 and MMP-9 as well as TIMP-1 and IL-8. These are key repair mediators however, prolonged or over-expression can lead to unwanted remodelling. Repair can lead to replacement of respiratory tract parenchyma with connective tissue comprising of collagen III and V, hyaluronic acid and fibronectin.

Inhibition of EGFR tyrosine kinase completely inhibits the re-epitheialization process. Epithelial migration is also mediated by mitogen peptides such as insulin, insulin-like growth factors, hepatocyte growth factor, calcitonin gene-related peptides and the antimicrobial peptide cathelicidin LL-37 (Puchelle *et al.* 2006).

Repair involves numerous growth factors and mediators that must be appropriately released to generate repair and then attenuated once repair is complete. Repair includes metaplastic cell deposition and then maturation. The aim of repair processes is to maintain lung homeostasis and it is crucial that these mechanisms are switched on and off under the appropriate conditions. This ensures that metaplasia is a controlled beneficial transitional part of repair. This is not always the case as metaplasia may also be seen in disease.

1.4 COPD

COPD is the disease affecting the cohort studied here. The GOLD (Global Initiative for Chronic Obstructive Lung Disease) definition of COPD is characterised by "airflow limitation that is not fully reversible. Airflow limitation is usually associated with an abnormal inflammatory response of the lung to noxious particles or gases". Contributing conditions include chronic bronchitis, bronchiolitis, emphysema and bronchiectasis, although chronic bronchitis and emphysema define the main clinical phenotype. These disease overlaps are represented in Figure 1.7.

There may be several other complications, for example airway hyper-responsiveness, an elevated immune response, asthma and other lung conditions (Mannino 2002).

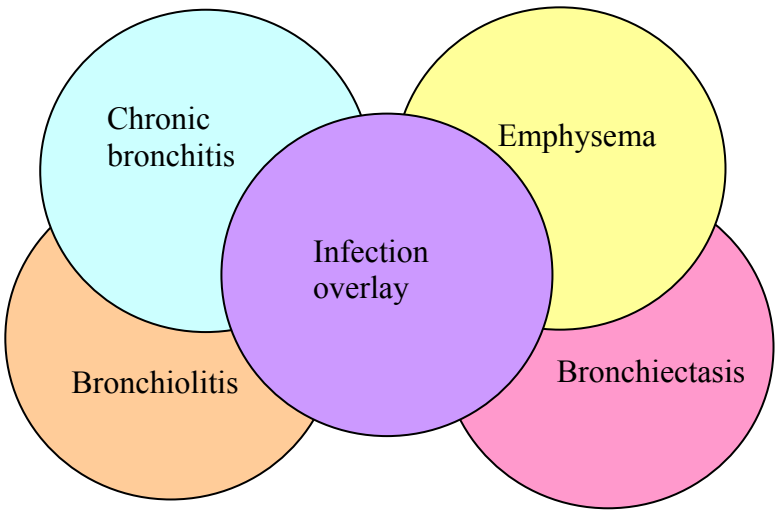


Figure 1.7 Venn diagram demonstrating the phenotypes of COPD
Venn diagram with the contributing conditions to COPD, demonstrating the overlapping phenotypes that may occur. Infection can affect the lung at any time and so can interact with any of the other conditions. Diagram designed by M Foster, personal communication.

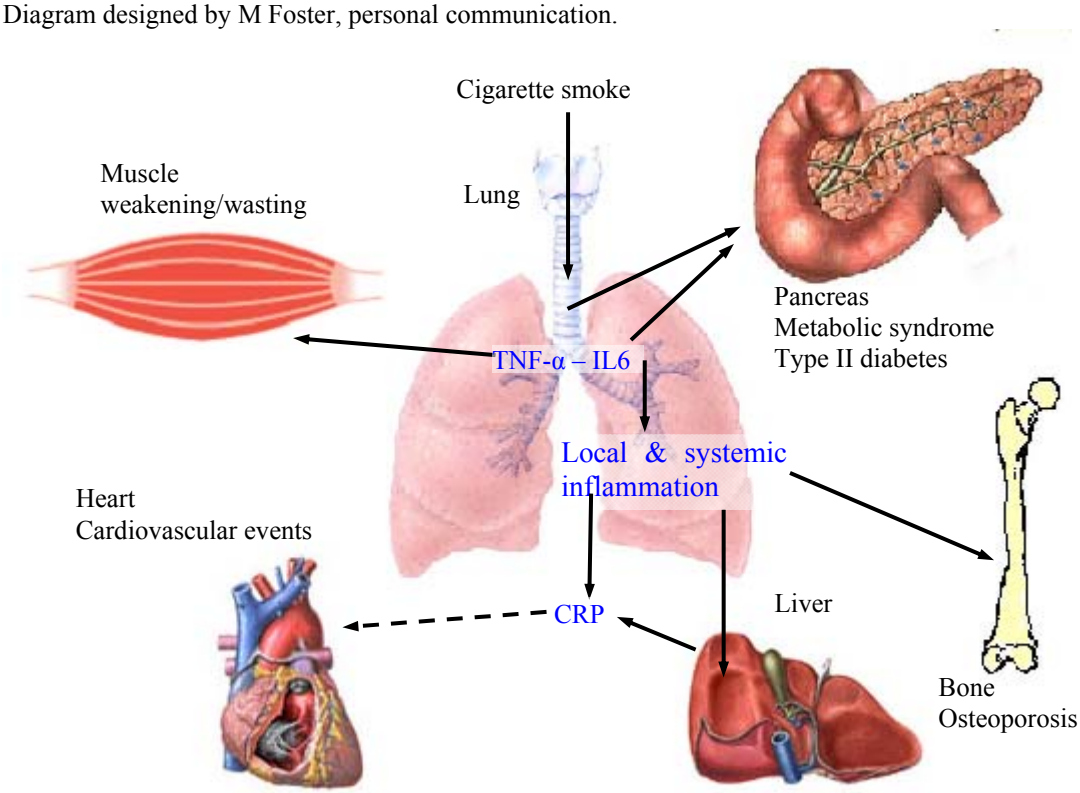


Figure 1.8 diagram of systemic effects
The range of systemic effects that COPD patients may suffer from, inflammation causes the liver to produce CRP that may lead to cardiovascular events. The bones may be weakened by osteoporosis. There is muscle weakening and possible fibre type alterations. The individual may develop type II diabetes. Diagram adapted from Fabbri *et al.* (2008).

There are extra pulmonary changes that occur with COPD patients, summarised in Figure 1.8. The effects of these contribute to lung changes. These may include muscle wasting or weakening. Other effects include cardiovascular events due to high levels of C-reactive protein (CRP), metabolic changes such as type II diabetes and osteoporosis of the bones. COPD is not a disease that is a decline in lung function; it is a failure of

integration of parts of the lung. The pathology does not associate in a linear manner to FEV i.e. some areas are fibrotic but the FEV of the individual is not too impaired. This may be due to the regional nature of the fibrosis.

1.4.1 Epidemiology of COPD

COPD is predicted to be the third leading cause of death worldwide by 2020 (Chapman *et al.* 2006). In 2000 it was the fifth leading cause of death in the United Kingdom, COPD is estimated to have affected 32 million individuals in the United States alone (Kleinschmidt 2001). COPD cost the NHS £486 million in 1998 (Britton 2003). It is a leading cause of death that it is still increasing in numbers (Barnes 2000). The main cause of COPD is smoking, illustrated by COPD prevalence and smoking patterns. In 2000 5.4% of male and 3.2% of female deaths in the United Kingdom were directly attributable to COPD and a further 4% had COPD as a contributory factor on the death certificate (Calverley & Bellamy 2000). In 2009 Van Durme reviewed the male to female ratio. There are still more male COPD sufferers however, female numbers are increasing. This may reflect increases in female smokers from 1940's onwards (van Durme *et al.* 2009). It has been shown that all individuals who smoke have bronchiolitis to a greater or lesser degree (Fraig *et al.* 2002). Smokers demonstrated a decline in FEV_1 and decreased lung capacity, however, this could be improved with smoking cessation in healthy smokers but not in COPD sufferers (Pride *et al.* 1980). Lokke *et al.* (2006) suggested that only around 25% of smokers progressed to COPD in the cohort of over 8000 smokers studied. Approximately 11% of smokers developed lung cancer, whilst around 90% of all lung cancer sufferers were smokers, indicating that smoking is the major risk factor for the development of lung cancer (Decker 2002).

1.4.2 Clinical diagnosis and symptoms of COPD

The clinical grouping applied to COPD patients is the GOLD criteria, which assigns a grade according to lung function (Rodriguez-Roisin 2009). The forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) are the main criteria.

Stage I. Mild: $FEV_1/FVC < 0.70$ and $FEV_1 \geq 80\%$ predicted

Stage II. Moderate: $FEV_1/FVC < 0.70$ and $50\% \leq FEV_1 < 80\%$ predicted

Stage III. Severe: $FEV_1/FVC < 0.70$ and $30\% \leq FEV_1 < 50\%$ predicted

Stage IV: Very severe: $FEV_1/FVC < 0.70$ and $FEV_1<30\%$ predicted or $FEV_1<50\%$ predicted plus chronic respiratory failure

There are several conditions contributing to COPD briefly outlined below. Chronic bronchitis is common it affects the upper respiratory tree. It is clinically diagnosed as presence of a productive cough for more than 3 months over a two-year period and is often linked to smoking history. Chronic bronchitis increases susceptibility to infection as the altered mucus phenotype compromises the muco-cilary elevators.

The other main COPD-related disorder of the upper airways is bronchiectasis. This is a permanent, abnormal dilatation of the cartilaginous airways. Diagnostic criteria for bronchiectasis include a non-tapering bronchus with an internal diameter 110% or greater than the adjacent pulmonary artery and the presence of visible bronchi within 1cm of the costal pleural surface or adjacent to the mediastinal pleural surface (Patel *et al.* 2004). Constant dilatation leaves airways vulnerable to infection, which often causes sputum overproduction.

Bronchiolitis, the classical 'smokers airway', although similar to chronic bronchitis affects small airways, those of 2mm diameter and less (Ryu, Myers & Swensen 2003). It is progressive and non-reversible, often diagnosed late when lung function is very impaired. The following criteria can be used to define bronchiolitis FEV_1 of <80% predicted and FEV_1/FVC ratio <70% (Saetta *et al.* 2001). The patient has a history of chronic progressive symptoms such as cough, wheezing, breathlessness and repeat infection. It is classed as an airway obstruction that does not respond to treatment and the individual often smokes.

Another peripheral contributory disease is emphysema. There are two main types, centrilobular emphysema that is more central and pan acinar emphysema that is more distal. It is permanent, abnormal enlargement of any part of the lung gas-exchanging structures, accompanied by destruction of the respiratory alveolar tissue (Cherniak 1991). Loss of elasticity makes breathing hard and chronic inflammation is thought to contribute to chronic cycles of alveolar wall damage. Several groups have shown that apoptosis plays an important role in the progression of emphysema (Demedts *et al.* 2006, Imai *et al.* 2005, Yokohori, Aoshiba & Nagai 2004). Apoptosis is important during repair to maintain normal cell population numbers (Henson, Vandivier & Douglas 2006). There is also speculation that apoptotic cells send out repair signals,

such as VEGF and hepatocyte growth factor contributing to the altered repair phenotype of COPD (Henson, Vandivier & Douglas 2006).

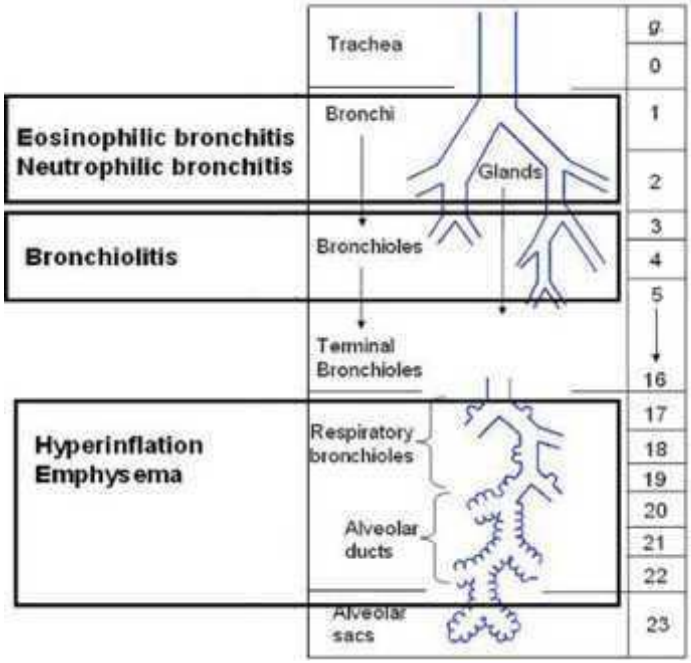


Figure 1.9 Lung involvement in diseases contributing to COPD
Boxes representing lung compartments in which the diseases are pathology relevant. Eosinophilic bronchitis and neutrophilic bronchitis both affect the upper respiratory tract, the bronchi in the first two generations of the airways. The right hand column shows the airway generations, 0-23. Bronchiolitis affects the bronchioles airway generations 3-5 finally emphysema, hyperinflation and gas trapping affect the peripheral lung, the respiratory bronchioles, these are at airway generations 17-22. Diagram adapted from Parent *et al*. (1992).

Much of the pathological aetiology of COPD is linked to smoking, susceptibility to infection and repeat damage and repair cycles with an overt inflammatory phenotype. COPD patients show a gradual decline of lung capacity as damage progresses. Lung capacity can drop to 40% at which point there is a significant exercise limitation that can be disabling (Wise 1997). Small airways become narrowed by muscle hyperplasia and blocked by cell debris and mucus. Air often becomes trapped in the periphery causing lung hyperinflation. Figure 1.9 summarises most affected regions of the lung.

1.4.3 Risk Factors for the development of COPD

There are a wide range of physical, environmental and familial factors that can contribute to COPD pathogenesis and development. Smoking is the greatest risk factor for COPD. All smokers have some degree of bronchiolitis but causative mechanisms are still unclear. In non-smokers and particularly children, bronchiolitis usually results from bacterial or viral infection (Leung, Kellner & Davies 2005).

It has been estimated that there are nearly five thousand different chemicals in cigarette smoke, many of which could contribute to damage and provocation of the airways (Green 1996). Pharmacologically active chemicals include nicotine and acrolein. The numerous chemical interactions contributing to damage may include the formation of free radicals and DNA adducts. Nicotine and tar levels in midstream cigarette smoke vary depending on the filter, as much as 30mg of tar may be inhaled from one cigarette (Jeffery 1996). It is also known that cigarette smoke includes both direct and indirect carcinogens. Inhaled smoke contains around thirteen thousand million particles per cm^3, ranging from 0.2 m to 1.0 m in diameter adding a physical assault aspect to epithelial damage (Jeffery 1996). The most peripheral airways have a diameter of around 0.5mm and so small, smoke particles, less than 1 micron thick can penetrate the entire airway tree (McBride 1992). Bronchiolar branching in humans and primates is asymmetrical causing airflow turbulence at bifurcations, enhances particle impaction and exacerbates inflammation.

Living in low socio-economic conditions seems to be a contributing factor to COPD development. This may be the result of poor nutrition, low birth weights or a history of childhood respiratory infections. Exposure to indoor pollution or use of biomass cooking fuels can also produce COPD-like symptoms (Calverley & Walker 2003).

Viral infections are strongly linked to COPD exacerbations and work by Hogg (2001) has suggested that latent adenovirus infections (including Respiratory Syncytial Virus) may determine the severity of bronchiolitis. When smoking damages the lung lining, the virus becomes activated by a smoking-related compromised immune defence system.

The presence of B cell foci within COPD lung samples lends support to the idea of viruses contributing to COPD development, however this feature is not diagnostic since lymphoid hyperplasia is also a feature of bacterial infections. COPD patients have been shown to exhibit both viral and bacterial infections during exacerbation (De Serres *et al.* 2009). It has also been shown that individuals with adenovirus infections no longer respond to steroids for asthma treatment. This suggests the involvement of different or additional inflammatory pathways that may explain the transition of some individuals from the bronchiolitis seen in all smokers to the COPD phenotype.

1.4.4 Genetic susceptibility to COPD

There are a number of genetic susceptibilities that have been identified in COPD. Plymoth *et al.* (2007) examined the lavage fluid of COPD patients and were able to demonstrate that smokers exhibit a different protein expression profile to non smokers. This may suggest genetic alterations are affecting protein expression levels.

Less than one percent of COPD sufferers have α_1-antitrypsin deficiency (Barnes 2000), however 80% of those with the worst form of deficiency will develop COPD. α_1-antitrypsin deficiency is an inherited genetic disorder linked to a 90% reduction in liver production of α_1-antitrypsin. This enzyme protects against neutrophil elastase (Calverley, Walker 2003, Barnes 2003, Barnes, Hansel 2004). Individuals with α_1-antitrypsin deficiency tend to exhibit a much more aggressive histological phenotype of emphysema and need treatment at a much younger age than most COPD sufferers.

Data from epidemiological studies in a Taiwanese population, who have a genetic predisposition for increased TNFα production, are 10 times more likely to develop COPD or chronic bronchitis. In this cohort, a 5' end polymorphism of TNFα gene, TNF2. Individuals with one or two copies of this allele showed a greater risk of developing chronic bronchitis (Huang, Su & Chang 1997). Another altered gene in COPD is a genetic polymorphism in microsomal epoxide hydrolase. This enzyme is involved in metabolising epoxides, found in cigarette smoke and charred meat. Disease development risk increases 4 fold when an individual smokes (Barnes 2000, Molfino 2007). Mixed findings have been analyzed, these suggested that EPHX1 polymorphisms and different rate phenotypes contribute to COPD susceptibility (Hu *et al.* 2008). British individuals with the same predisposition do not exhibit a greater risk, which suggests confounding factors in the Taiwanese population such as, differences in air pollution and particle inhalation at work and in the environment (Barnes 2000).

Ning *et al.* (2008) have examined a large patient cohort in China to look for polymorphisms in aquaporin 5. The group found a single nucleotide polymorphism in intron 3 at +2254 caused an A>G change. This decreased the risk of COPD development. Aquaporin 5 is a water channel found in type I cells which helps control fluid balance within the lung parenchyma and is important for fluid clearance during infection.

Takabatake *et al.* (2009) have investigated a polymorphism the ATPase cell division cycle 6 (CDC6). It is associated with cellular activity regulating the initiation of DNA replication and is involved in proliferation and apoptosis homeostasis. The presence of haplotype SNP5G/SNP6A (Ile)/SNP7G/SNP8T is thought to lead to lung function decline in smokers and even quit smokers. These alterations may affect lung apoptosis.

Sadeghnejad *et al.* (2009) examined a large Dutch cohort for the presence of polymorphisms within ADAM33. Of 25 mutations examined, 5 were found to significantly associate with FEV_1/FVC ratio <70% and ppFEV_1< 75%, which are criteria for COPD diagnosis. The 3 single nucleotide polymorphisms that remained significant after correction were Q-1, S1 and V4. Previous investigations suggest that ADAM33 may be involved with growth factor release or activation. This may influence proliferation rates affecting the same mechanisms as altered CDC6 enzyme.

In a study of Taiwan Chinese patients, polymorphisms in the p53 and p21 gene families were studied in 'healthy' smokers and COPD patients. It was found that COPD patients had significantly higher occurrence of certain alleles for both p53 Proline allele (codon 72) and p21 Arginine allele (codon 31). Both p53 and p21 are involved in cell cycle regulation suggesting that dysregulation of cell proliferation and apoptosis in COPD may reflect this geneotype. Additionally p53 polymorphism has been associated with some cancers, although p21 polymorphism has not (Lee *et al.* 2006).

1.4.5 Pathology of COPD

Pathological changes observed within chronic bronchitis include mucus gland hypertrophy and hyperplasia, goblet cell hypertrophy and hyperplasia with the production of altered sulphated viscous mucus. This goblet cell hyperplasia may extend into more peripheral airways compared to the goblet cells observed in normal tissue (Glynn & Michaels 1960). Some groups have found that goblet cell hyperplasia is a characteristic of COPD, meaning increased numbers of goblet cells (Ma *et al.* 1999). Other work examining mucins in COPD found that mucin proportions were altered but, the number of goblet cells was not significantly different between normals, smokers and COPD patients. Mucin 4, 5B and 5AC were on epithelial cilia, cytoplasm and goblet cells; mucin 2 was found only on cilia. Mucin 6 was uniformly present in all epithelial cells in cytoplasm and cilia. Goblet cells usually produce mainly mucin 5AC not mucin 5B which may be what causes the mucus imbalance (Caramori *et al.* 2004). Caramori *et al* suggest that mucin 4 and mucin 6 expression is the same in COPD and normal

individuals. There is conflicting data on the number of goblet cells present in the COPD lung, but it is clear that the mucus profile is altered. Mucus ducts are also dilated down to 2-4mm. An example of COPD goblet cell hyperplasia is shown in Figure 1.11A.

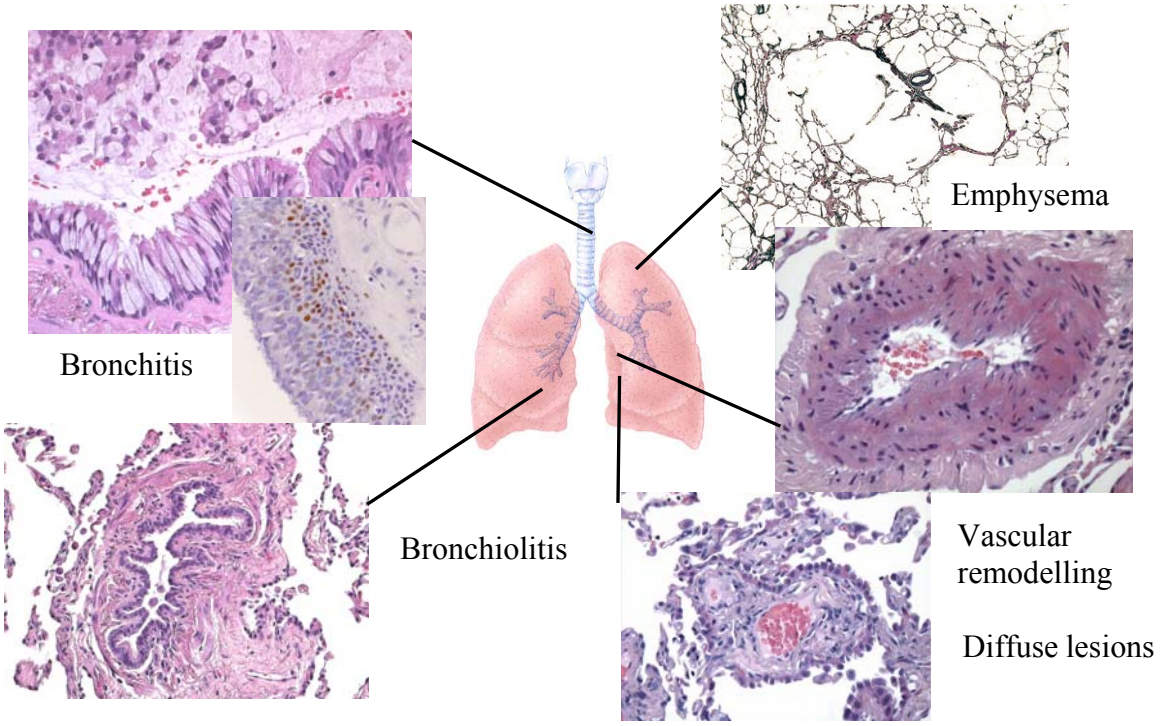


Figure 1.10 Spectrum of histopathology in the lung
The range of pathology occurring in the different lung compartments in COPD. Diagram adapted from work by M Foster, personal communication.

In bronchiectasis at a cellular level there is often inflammation with chronic microbial colonisation, extensive epithelial loss (Figure 1.11C) or in regions where the mucosa is intact, abnormal epithelial distribution with either multiple layers of columnar cells or squamous areas (Figure 1.11B) (Corrin 2006). Pathological changes within the small airways in COPD include chronic inflammation (Figure 1.11D) and small airway remodeling. Changes are seen particularly within terminal bronchioles, including epithelial metaplasia, increased airway smooth muscle, goblet cell hyperplasia and submucosal gland hypertrophy. The presence of changes appears to be disease linked.

Within bronchi and bronchioles remodelling leads to increased deposition of collagen III and IV, fibronectin and laminin (Kranenburg *et al.* 2006, Parameswaran *et al.* 2006). The extracellular matrix is in part regulated by airway smooth muscle, which produces matrix and matrix metalloproteinases to mediate matrix deposition or degradation. This shows that epithelial cells are influences by numerous cells and released mediators. Altered muscle attributable to lung remodelling may influence matrix deposition that in

turn manipulates the epithelial phenotype. The COPD lung also exhibits vascular remodelling as either a primary or secondary event (Figure 1.11E). This remodelling increases the amounts of muscle and collagen within the lung (Santos *et al.* 2002).

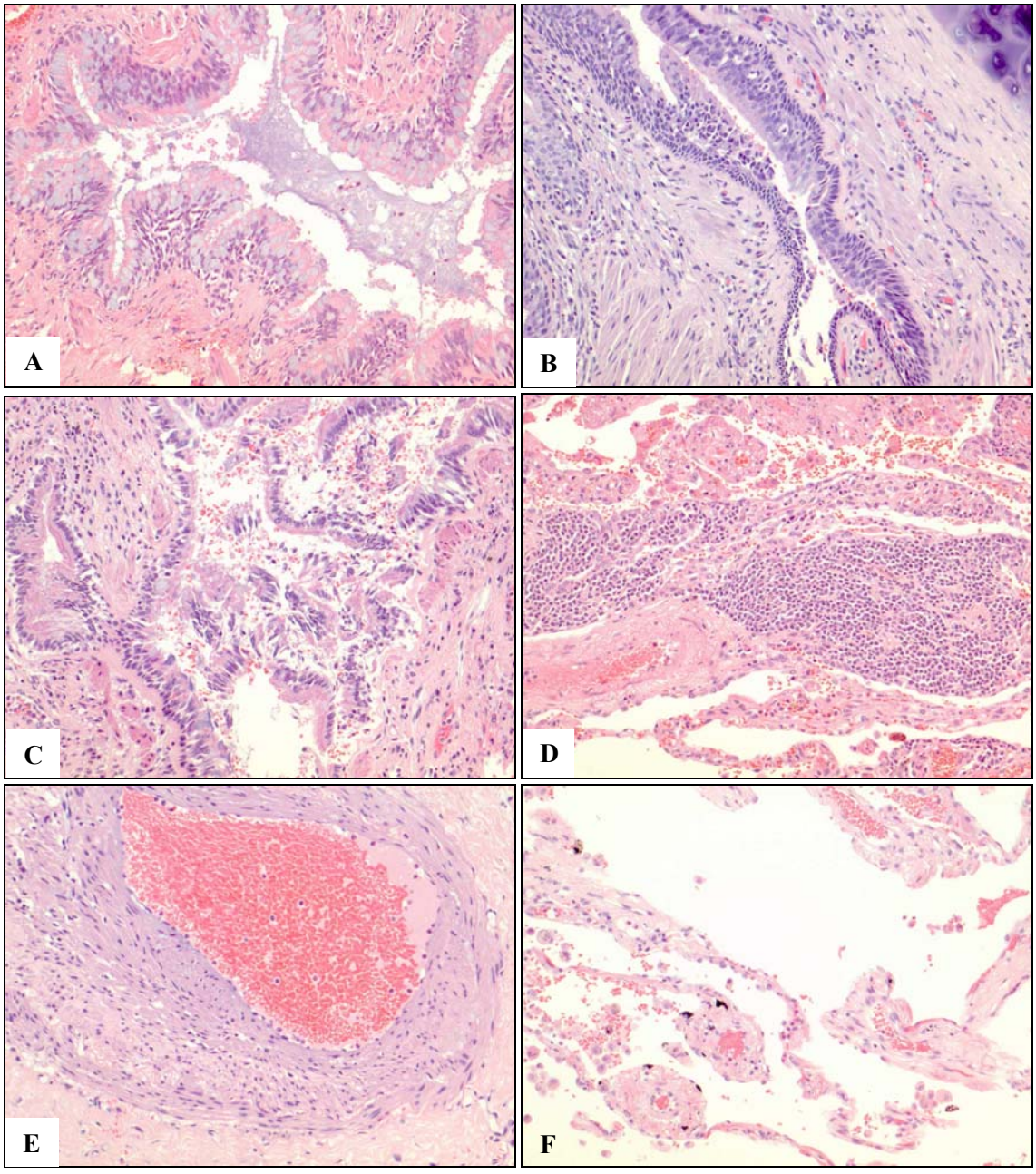


Figure 1.11 Example pathology of COPD
Some pathological changes associated with COPD. These examples are from the study cohort.
Figure 1.11A) Airway with goblet cell hyperplasia and mucus in the airway lumen.
Figure 1.11B) Larger airway with squamous epithelium present.
Figure 1.11C) Obliterative bronchiolitis, thick membrane, airway lumen contains mucus & epithelium.
Figure 1.11D) Parenchymal lymphocytic aggregate, there is some association with nearby vasculature. Both acute and chronic inflammation may be observed in COPD.
Figure 1.11E) Remodelled vessel with inflammatory infiltrates, thickened altered media and adventitia.
Figure 1.11F) Parenchyma where alveolar attachments have been lost. Emphysematous change.

In the peripheral lung, emphysema is the main cause of damage. Emphysema is defined by permanent enlargement of airspaces distal to the terminal bronchioles, caused by the

destruction of alveolar tissue. An example of this tissue loss may be seen in Figure 1.11F. This leads to loss of connectivity in affected areas. There is a loss extra cellular matrix thought by some to be caused by enzymes like neutrophil elastase.

Lymphoid expansion is a feature of the COPD lung. Chilosi *et al.* (1993) looked at the upregulation of tenascin in lymphoid tissues and showed that the presence of tenascin is associated with a mobile T cell population. Tenascin may be acting as a chemoattractant for inflammatory cells. Aberrant tenascin expression may be one aspect contributing to the recruitment and aggregation of T cells in the COPD lung.

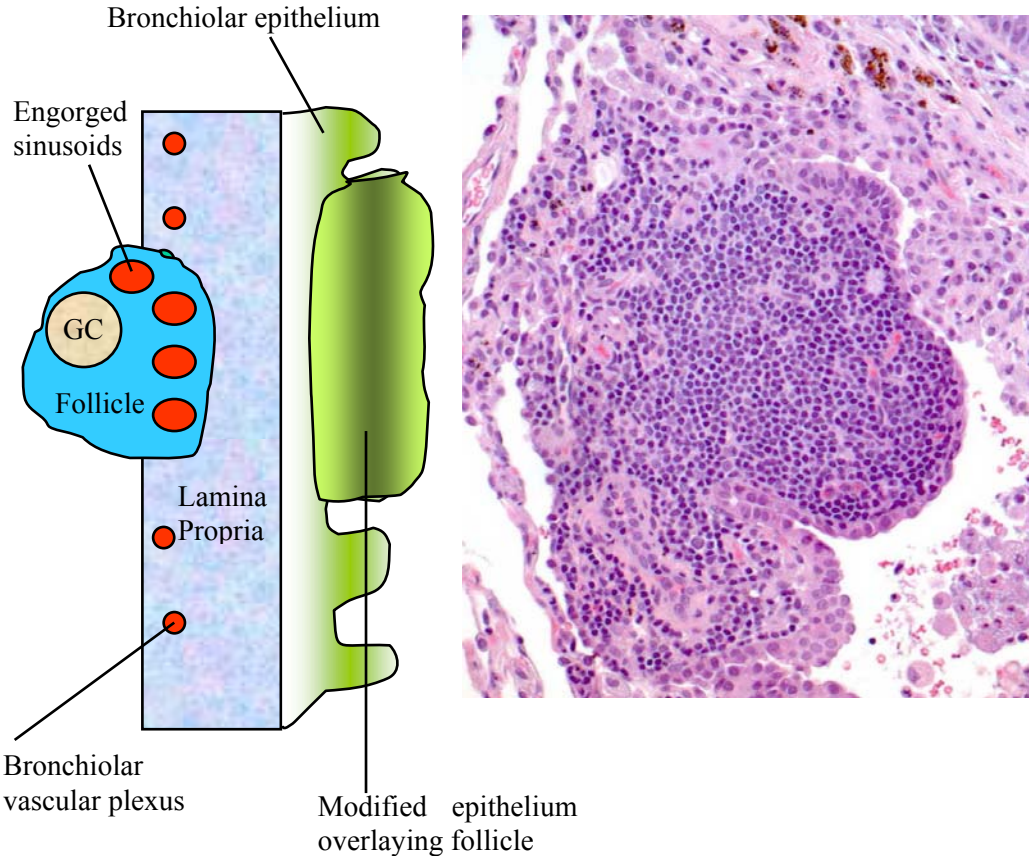


Figure 1.12 Lymphoid Expansion in COPD
Lymphoid expansion and influence in the COPD lung. The lymphocytes shown here are adjacent to an airway. Adapted from diagram by M Foster personal communication.

Araya *et al.* (2007) looked at gene changes occurring in airway squamous metaplastic cells harvested from COPD patients. IL-1β elicited the highest upregulation showing a 13.4 fold increase in expression. Many identified genes are induced by TGFβ expression in epithelial cells. There were also a number of integrins involved with signal transduction to other cell types including fibroblasts. Consideration of molecules identified by Araya may add understanding to mechanisms via which the metaplasia

occurs, for example CK6B, CK14 and involucrin were identified and considered to be markers of squamous epithelium. Figure 1.10 summarises the heterogeneity of COPD pathology.

1.4.6 The metaplastic lesion of COPD

Diffuse metaplasia is often observed in small strands or clusters within the periphery of the COPD lung, however it is rarely mentioned in scientific literature. A peripheral epithelial response, usually noted as peribronchiolar has been described in small airways disease. Fukuoka *et al.* (2005) examined several cases of interstitial lung disease with extensive peribronchiolar metaplasia. Airways were assessed for the following features; bronchiolar fibrosis, inflammatory infiltrate, submucosal granulation tissue causing constrictive change, mucus plugging, dilation, asthmatic change defined as eosinophillic infiltrate, basement membrane thickening and goblet cell hyperplasia. The peribronchiolar metaplasia appeared to spread from the airway to line the alveolar bed. The surrounding cells were cuboidal to columnar and ciliated cells. There were not usually goblet cells present. The percentage of bronchioles affected ranged from 37% to 100% of bronchioles seen. Fukuoka suggested that these lining cells may be the result of non-specific reaction to airway fibrosis. Rice & Nicholson (2009) examined a range of small airways diseases suggesting that "peribronchiolar metaplasia and fibrosis is a characteristic pattern of damage in and around small airways that is generally non-specific and probably represents a 'tombstone' marking...damage". This non-specific damage and inflammation response fits with COPD pathology and perhaps explains why the lesion is observed here too. Peribronchiolar metaplasia referred to as Lambertosis (defined in section 1.5.1) has been mentioned in association with COPD by Leslie (2005). Inflammation has been widely investigated in COPD but the response by the lung and consequence of the inflammation has perhaps been a little overlooked. The metaplasia response may be a post-inflammatory, post-damage attempt at repair.

Figure 1.13 shows the pathological changes that occur in acute injury, smokers and COPD sufferers. Many of the changes seen in COPD can be seen initially triggered by acute damage. The arrows represent how some of changes may progress from one condition to another, some arrows represent hypotheses as opposed to known mechanisms. This may indicate areas of lung response that are initiated as a repair attempt and develop into inappropriate remodelling or fibrosis.

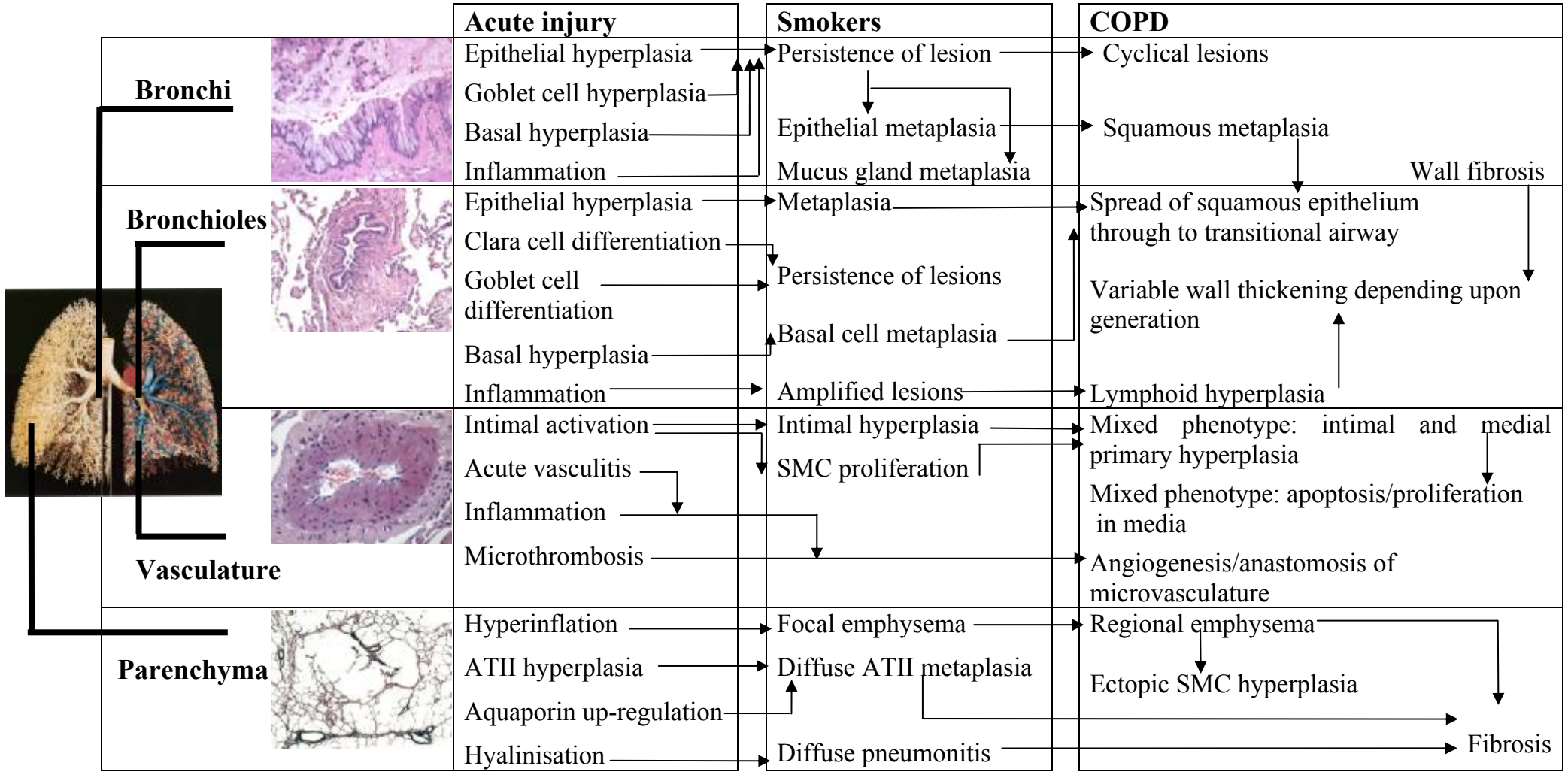


Figure 1.13 Typology of key lung remodelling phenotypes from histopathology

The changes observed in acute injury, smokers and COPD. The arrows represent the possible pathways and mechanisms in which damage and changes can perpetuate and increase.

1.5 Lung metaplasia

Metaplasia is the term used to describe cell transdifferentiation and the development of an altered cell phenotype replacing the normal cell population (Slack & Tosh 2001). There are three types of metaplasia. (1) Direct metaplasia occurs when differentiated cells change to another state of differentiation. The resident cell alters phenotype reacting to damage or stress stimuli. The term cell transdifferentiation can be considered synonymous with the term metaplasia when used in this context. (2) Indirect metaplasia where infiltrating cells are of an altered differentiation. Cells are lost or damaged and the new replacement cells are of an altered phenotype. (3) Stem cell metaplasia is a metaplasia that develops via a pluripotent stem cell, often more prolonged than direct metaplasia.

It has been mentioned that metaplasia can occur as part of lung homeostasis and repair in section 1.3. Metaplasia has also been mentioned as one of the changes that occurs in COPD. There is airway epithelial metaplasia that is well reported and studied (Araya *et al.* 2007, Kim *et al.* 2008). The parenchymal metaplastic lesions have not been formally characterised in the literature. To further understand the lesions occurring in COPD, it is helpful to have an overview of normal resolving metaplasia, the influence of smoking and other lung disease that features metaplasia.

Park *et al.* (2006) have demonstrated that ciliated epithelium in mice can transdifferentiate and migrate to areas of damage to maintain the airway surface. Here cells mature into a normal columnar phenotype once stress signals caused by the damaging process are removed.

Metaplasia can occur as part of a progression of repair, with epithelial hyperplasia preceding metaplasia. This is not always the case, metaplasia can occur directly as a result of injury with no transition. Mechanical damage, noncarcinogenic gases and possible carcinogens can all cause mucus cell hyperplasia. This hyperplasia leads to increased mucus production and secretion. Alongside these areas of hyperplasia, tessellate cells may be seen. Another form is stratified hyperplasia, consisting of epithelial cells forming between four and six layers. These cells are polygonal and cuboidal, with cells nearer the surface flattened, often with increased numbers of enlarged desmosomes. Metaplasia as a result of injury may occur after the mechanical

injury of intubation. When squamous cell metaplasia occurs, there is often no transition zone and altered cells sit adjacent to normal epithelium, although there may also be a slight tapering of metaplastic cells reaching under the normal cell layer.

Cigarette smoke has been shown to cause the replacement of mucus-secreting columnar cells with simple squamous epithelium. It has been known for over 50 years that cigarette smoke in animals can induce bronchial squamous metaplasia. Short-term smoking models in dogs have demonstrated reversible metaplasia, although repair and reversion to normal phenotype, took several months after the final insult. Tipton & Crocker (1964) suggest that the epithelial changes originate from the basal cell population. Lasnitzki (1958) showed that cigarette smoke fractions are capable of producing squamous metaplasia *in vitro* using human foetal tissue. Long-term smokers may develop a metaplasia that is not reversible even when patients stopped smoking and the stimulus was removed (Wright *et al.* 1983). Barth *et al.* (2000) propose that squamous metaplasia and basal cells share the same CK5 and CK6 profile. This suggests that basal cells are the progenitor for squamous metaplasia.

Squamous cell metaplasia often shows elevated proliferation rates compared to normal adult lung tissue (Leube & Rustad 1991). Work carried out by Solomon, Greenberg & Spjut (1990) has shown that 73% of lung tumour cases examined showed hyperplasia or metaplasia in the tissue surrounding the tumour. The progression from hyperplasia to metaplasia to dysplasia to carcinoma in the human airways has been shown previously (Auerbach *et al.* 1958).

Different lung compartments can give rise to different forms of metaplasia with diverse appearances and characteristics. Stockstill *et al.* (1995) used an ozone exposure rat model to induce changes within the alveolar bed. These changes included a proximal alveolar metaplasia. Type I cells appeared smaller and more frequent. The proximal alveolar regions had extensive cuboidal metaplasia. This had ciliated, Clara and other cuboidal cells. The unciliated epithelium differed from the terminal bronchioles. The presence of ciliated and Clara cells suggests a Clara cell progenitor but, the additional presence of unciliated cuboidal suggests a second progenitor or that there was an attenuation in maturation.

Berkheiser (1959) examined the presence of bronchiolar proliferations and metaplasia associated with bronchiectasis, pulmonary infarcts and anthracosis. Bronchiectasis is a

contributory condition for COPD. These proliferations included increased small airways or epithelial hyperplasia spreading into the parenchyma. The epithelial cells were single layer and largely uniform and cuboidal. The proliferations seen with anthracosis were much less structured with numerous variable cell shapes. The cells seen in association with healed infarcts were often multilayer with variable nuclei. This work confirms the presence of metaplastic cells in a number of lung diseases and suggests links with smoking and epithelial injury.

1.5.1 Lambertosis

As metaplasia can occur as part of a repair process and as part of disease there has been confusion over what proliferations are a repair process and what is potentially harmful disease progression. This diagnostic confusion has been addressed by Lambert (1955) who examined extensions of the airway epithelium through ducts into the surrounding parenchyma, these were named canals of Lambert or Lambertosis. Ducts of epithelial lined tissue run from the airways into the surrounding parenchyma.

These are considered to be part of normal lung structure that may overgrow if stimulated. This can potentially lead to mild metaplastic spread but is not considered to be disease. These ducts provide a direct link from the airways into the alveolar bed and are thought to allow movement of macrophages and other inflammatory cells into the parenchyma. Canals of Lambert may also provide collateral ventilation in the lung.

The phenomenon of Lambertosis was first noted in the lungs of miners who had pulmonary anthracosis. This may suggest that the lung has been exposed to particulate damage and the epithelial expansion is part of the repair process.

Although Lambertosis was first used to define ducts occurring in normal tissue, the term has subsequently been used to describe epithelial overgrowth leading to peribronchiolar metaplasia. Peribronchiolar metaplasia has been described in association with idiopathic pulmonary fibrosis, usual interstitial pneumonia as well as non-specific and desquamative interstitial pneumonia in varying frequencies (Cordier 2007, Haddad & Massaro 1968, Mark & Ruangchira-urai 2008). Cordier (2007) described the epithelial phenotype as “tall columnar to low cuboidal or flattened cells” and that it is not really a morphology pattern reflective of the airway epithelial phenotype but is usually adjacent. Flieder described Lambertosis as the “presence of ciliated epithelium along centrilobular alveolar septae” (Flieder & Koss 2004). The example of Lambertosis

shown by Flieder shows epithelial cells of the same morphology, as those observed in the airways, is more in keeping with the epithelium found in adjacent airways. With this diverse range of descriptions of Lambertosis, care is needed when assigning this term.

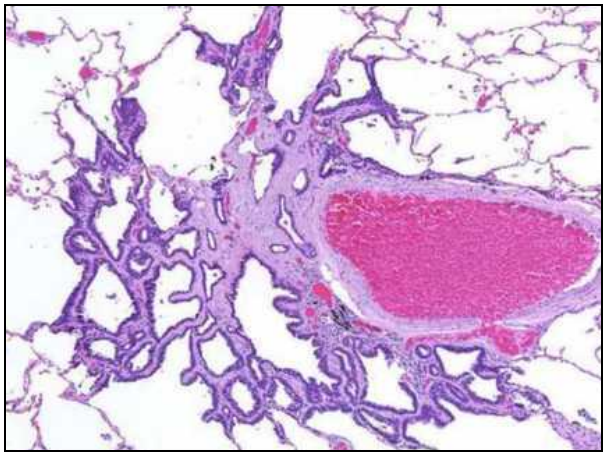


Figure 1.14 An example of Lambertosis
Lambertosis in human lung adjacent to a vessel. Picture reproduced with permission from D Flieder

Lambert has also described ducts in rabbits and cats. Rabbits demonstrated channels in the respiratory and terminal airways but not in upper airway. A rat model of Lambertosis was produced to examine epithelial morphology. This chronic model used repeat doses with corn oil and acetone for up to two years to mimic the repeat damage cycles of cigarette smoking. One of the main changes observed was bronchiolar metaplasia. PAS (periodic acid Schiff's) and Alcian Blue staining showed the cells were associated with mucus production and GSTPi immunohistochemistry indicating metabolic activity. This may suggest a goblet cell or mucus cell phenotype (Brix *et al.* 2004).

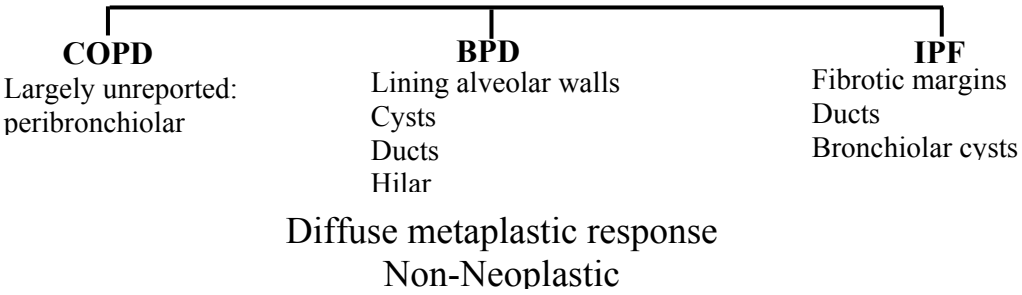


Figure 1.15 Locations of ectopic epithelium in lung disease
Areas that are colonised with epithelium in BPD and IPF. In BPD lung structures are not fully formed so most surfaces are coated with lining cells. In IPF there is some structural damage and cystic change. The lining cells are found on all fibrotic and cystic surfaces.

The presence of Lambertosis in rat, rabbit, cat and man suggests that this is a generic mammalian repair response. Lambertosis has metaplastic lesion characteristics that can be considered to be a repair response. It is also important to examine other diseases

where metaplasia occurs as these may be neoplastic or non-neoplastic and can be initiated in infants as with BPD or later in adulthood as is usual for IPF. Figure 1.15 shows the location of altered wpithium in a range of conditions.

1.5.2 Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is a developmental lung condition considered to be abnormal arrest of development alongside abnormal repair of lung injury. BPD is often seen in premature births, such as 24 week gestation. Around 30% of those born with a birth weight of less than 1kg develop BPD (Jobe & Bancalari 2001). 24 weeks is the canalicular stage, completion of saccular development has not taken place. BPD may be linked to inappropriate or inopportune signalling as a result of injury. The trigger may be inflammation caused by antenatal infection, insufficient surfactant or oxygen toxicity from ventilation. Newborn BPD lungs show enlarged alveoli with simple structures that are fewer in number with emphysema-like changes. There are decreased malformed capillaries, variable fibrosis within the parenchyma and variable smooth muscle hyperplasia. The vasculature shows mild vascular lesions that include endothelial odema, medial thickening and elastic deposition. Finally the airway surround shows increased smooth muscle (Coalson 2003, Hislop *et al.* 1987).

The lungs of infants with BPD lack surfactant D. This reduces compliance and making breathing more difficult. It may also contribute to the damage or lack of repair in these lungs (Stahlman *et al.* 2002).

As BPD is a condition of preterm lungs that have not completed development and are still undergoing alveolarization the alveolar bed is lined with prominent type II cells. Stocker (1988) noted however, that changes in the alveoli include a type II hyperplasia along with type II hypertrophy. This does not represent normal development, indicating overgrowth of type II cells.

1.5.3 Idiopathic pulmonary fibrosis

Idiopathic pulmonary fibrosis (IPF) is a chronic progressive interstitial lung disease characterised by inflammation and parenchymal fibrosis. The epithelial proliferation external to and surrounding airway has been examined. Hinata *et al.* (2003) referred to the epithelial lining cells as a “cystic remodelling of air spaces lined with metaplastic bronchiolar epithelia or hyperplastic alveolar pneumocytes”. Fukuoka *et al.* (2005) described these cells as having an appearance similar to Lambertosis, suggesting the

proliferation seen is an extension through the canals of Lambert out into the surrounding tissue. Haddad & Massaro (1968) looked the parenchyma of cases of interstitial fibrosis, some with neoplastic change. The epithelial morphologies included cuboidal and columnar cells with a subset showing nuclear of cytoplasmic changes or stratification. Hinata *et al.* (2003) showed that lining cells were approximately 50% positive for CC10. Those with cuboidal morphology were a mixture of CK17 positive, CK14 positive and surfactant A positive cells. Some cells co-expressed CC10 and surfactant whereas others co-expressed CK17, CK14 and surfactant.

Iyonaga *et al.* (1997) also examined IPF lining cells, cuboidal cells were positive for CK7, CK8 and CK19. Flattened cells showed strong CK19 and co-expression of CK7 and CK8. Both flattened and cuboidal cells had some cells positive for CK17 and CK14. Two progenitor populations were proposed, those with CK17 and CK14 originating from airway basal cells and the areas without CK14 or CK17 present, believed to be derived from type II hyperplasia. Qunn *et al.* (2002) concluded that these cells were basal cell hyperplasia, squamous cells or metaplasia. Proliferating cell nuclear antigen (PCNA) staining, showed high proliferation, half the cells were positive for PCNA. The adjacent airways demonstrated about one third of cells as proliferating. IPF lining cells appear to be an active proliferative lesion that is in part derived from airway cells (Haddad & Massaro 1968). Phelps *et al.* (2004) examined the honeycomb lesion epithelial cells for the presence of surfactant. There was increased surfactant in IPF lungs when compared to normal and type II cell hyperplasia. The cells were a mix of positive and negative for surfactant. This further supports two epithelial populations within the IPF lung, hyperplastic type II cells and metaplastic airway epithelial cells. To conclude, several groups have examined the lining cells of IPF, which are generally accepted to originate from two populations. These progenitors may be type II cells and airway epithelial spread, the exact phenotypes are still unclear.

There are a number of other non-neoplastic diseases both within the lung and other organs of the body that have epithelial metaplasia. BPD has been mentioned as a foetal or developmental disorder, another phenomenon arising during development is pulmonary sequestration. This is a mini lung lobe that is not associated with the rest of the lung. Epithelial cells have been observed lining the alveolar ducts and saccular forms. These cells are usually ciliated and may produce mucin 1. The sequestered tissue may harbour infection and have inflammation and have fibrosis (Shaffrey *et al.* 1999,

Halkic *et al.* 1998). Barrett's oesophagus (BO) and Crohn's disease are both gastrointestinal conditions. Barrett's oesophagus is a pre-neoplastic lesion with intestinal epithelium in the oesophagus. Studies examining the cytokeratin and mucin profile confirm the intestinal phenotype. These cells are layers of metaplastic cells all at the same level of maturation. This may be due to acid reflux damaging the epithelium (van Baal *et al.* 2008, Couvelard *et al.* 2001). Crohn's disease is an autoimmune disease, with inflammation and acute and chronic epithelium. These cells show quite a high level of proliferation (Noffsinger, Unger & Fenoglio-Preiser 1998).

1.5.4 Diffuse metaplasia in neoplastic disease

Approximately one-tenth of all smokers are thought to develop lung cancer and for 90% of all lung cancer sufferers, smoking is the major cause of cancer development (Decker 2002). Lung cancers are broadly separated into two groups, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). NSCLC can be further classified as squamous cell lung carcinoma, adenocarcinoma or large cell lung carcinoma. There is a complex staging of tumours involved for all tumour types. This includes the presence of a diffuse metaplastic response, however the presence of this alone is not diagnostic of cancer (Sarantopoulos *et al.* 2004). Bronchioloalveolar carcinoma (BAC) is the name assigned to a subset of adenocarcinoma, often associated with smoking. There are two forms of bronchioloalveolar carcinoma, pure and papillary. The defining feature of BAC is a lepidic growth that colonises existing surfaces within the lung. Lepidic growth does not destroy the lung structure being coated with cells. The WHO guidelines define bronchioloalveolar carcinoma as "cylindrical tumour cells that grow along the walls of pre-existing alveolar walls without tissue destruction" (Kini, Greensheet & Purslow 2002).

The cytokeratin profile for NSCLC has been examined using CK5, CK7, CK8, CK20 and TTF-1 (type II cell marker). Squamous cell carcinoma stained positive for CK5 and CK8 with occasional CK7 and CK20. Adenocarcinomas were all CK7 and CK8 positive with very occasional staining for CK5 and CK20. Finally large cell carcinoma consistently showed CK8 staining, usually in association with CK7 and mixed staining for all other cytokeratins. All adenocarcinoma samples and around half large cell carcinomas also exhibited TTF-1 type II cell staining (Johansson 2004).

Some lepidic BAC cells are cuboidal with no striking features on H&E, however EM may demonstrate the presence of lamellae bodies (Churg 1988, Weydert & Cohen

2007). There are similarities in location, the lack of alteration to underlying tissue and possibly the ability to produce surfactant. These lepidic cells were found to be sporadically positive for CK7, mucin 1 and extensively positive for TTF-1, CEA (cancer embryonic antigen) and E-cadherin within mucinous and non-mucinous areas. These findings differ from work by other groups, TTF-1 is thought to be occasionally expressed in non-mucinous areas (Sato *et al.* 2006). The positive markers identified here may all be found on type II cells so there is no direct evidence to suggest that these cells develop from airway epithelium.

1.5.5 Comparison of Metaplastic responses

In BPD alveolar walls are lined with a single layer of epithelial cells and the morphology appears quite round so may be consistent with cuboidal phenotype. There does not appear to be a direct interaction with an airway (Coalson 2003). Mizoguchi *et al.* (2003) have shown using experimental models in mice that the epithelial phenotype changes as Crohn's disease progresses. A range of epithelial types and morphologies are present at one time. In Barrett's oesophagus epithelium is thought to have an intestinal phenotype, with a mix of cells present including productive and goblet cells (Flejou & Svrcek 2007). IPF shows a mixture flattened squamous appearing cells and more cuboidal cells; thought to represent two populations. Some groups think type II cells and epithelial cells comprise these populations, others suggest a more mesenchymal contribution.

The trigger for development of metaplastic cells is different in each case but there may be similar contributing factors. BO is linked to acid reflux which would cause repeat damage to the oesophagus and lead to chronic inflammation with cycles of damage and repair which may have analogues to the repeat insult cycles in COPD. Crohn's disease and idiopathic pulmonary fibrosis both have an element of autoimmune response that would cause continual assault to the epithelium. Chronic irritation and inflammation seems to be a recurring theme in the modulation of metaplastic conditions. The reversion to intestinal cells in BO may be a reversion to a more foetal reparative phenotype, although this remains to be established and is under-researched in the context of COPD.

1.6 Foetal development, cancer and COPD

There are many similarities in released mediators and mechanisms of change that can be seen in foetal development, cancer and COPD. It is pertinent to consider these mechanistic similarities to allow comparison of COPD pathogenesis to changes occurring in development and cancer. This will provide information about whether the lung is exhibiting a reversion to fetal lung behaviour or is harbouring a pre-neoplastic lesion.

During lung development growth factor concentration has been shown to be very important. Signal gradients form along developing structures, dictating the local environment and controlling neighbouring cells. Tyrosine kinase receptor mediated growth factor signalling by FGF, EGF, VEGF and PDGF promote division and differentiation. This may involve partial induction of the squamous phenotype (Stahlman, Orth & Gray 1989). Conversely serine-threonine kinase receptors, such as the TGFβ family including TGFβ1 and bone morphogenic protein 4 (BMP4) stop these events, usually by autocrine negative feedback loops mediated by prostanoids, notably PGE2. In normal adult lung factors probably compliment one another to maintain equilibrium. Disruption may occur in abnormal expression or activation leading to pathological change.

Growth factors are necessary for normal alveolar development and are implicated in disease states involving fibrosis and airspace structure. For example, fibroblast growth factor 10 (FGF10), which is released by mesenchymal cells, plays a critical role in determining the pattern of airway branching (Bellusci *et al.* 1997). BMP4 controls proliferation and differentiation in the emerging lung buds, the release of BMP4 is controlled by FGF10. FGF10 also controls cell differentiation in both the airway and alveolar compartments. The presence of FGF10 holds the cells in a dedifferentiated state. Nyeng *et al*'s (2008) transgenic mouse model that caused expression of FGF10 seemed to have a larger number of immature type II cells present and a complete absence of type I cells. The airway epithelial goblet cells were present extending to the alveolar bed and no Clara cells could be detected. The group concluded that the embryonic lungs had a goblet cell metaplasia originating from immature type II cells as opposed to the usual Clara cell origin of goblet cells. If there was ectopic expression within the adult lung, it may affect the phenotype of epithelial cells present.

Hedgehog signalling in the lung controls production of several growth mediators, BMPs, Wnt and FGF (Pepicelli, Lewis & McMahon 1998, Pongracz & Stockley 2006). It has been shown that ectopic production of BMP4 can lead to retarded epithelial differentiation. Sonic hedgehog is vital for normal lung branching and it is known that sonic hedgehog deficient humans do not survive. These morphogens produce very specific responses. Branching is drastically impaired in mouse mutants, the development of appropriate epithelial phenotypes was unaltered. The hedgehog pathway interacts with the Notch pathway that regulates cell death, migration, division and differentiation. Hedgehog genes have not been linked to the maintenance of developmental stem cells, however, hedgehog has been shown to be over expressed in NSCLC, sonic hedgehog has been shown in 97.5% of these tumours (Gialmanidis *et al.* 2009, Velcheti & Govindan 2007). This demonstrates the involvement of the developmental cassette in cancer pathogenesis. Also it has been shown that smoking activates hedgehog and Wnt mediators suggesting a role in smoking related disease such as COPD (Lemjabbar-Alaoui *et al.* 2006).

Increased TGFβ has been shown to inhibit proliferation or enhance apoptosis of lung epithelial cells possibly impeding septation during development. Mouse models with TGFβ blockade late in pregnancy have shown marked airspace enlargement with destructive changes, inflammation and increased matrix metalloproteinases, all characteristic of emphysema. This may show that early developmental changes in cytokine activity or matrix components could lead to disease in later life (Neptune *et al.* 2003). The TGFβ pathway seems to be closely associated with lung susceptibility. Interestingly TGFβ is often linked with asthma, which may explain many of the pathological similarities between asthma and COPD, including inflammation, thickened basement membrane and remodelling.

TGFβ on bronchial epithelial cells has the ability to promote squamous differentiation and interact with the proto-oncogene c-myc (Masui *et al.* 1986). Warburton *et al.* (2006) have studied null mutations in mice and found that the null mutation of *Smad3*, which is involved in the TGFβ pathway, leads to disorganised matrix deposition. Disorganised matrix appears to more readily activate matrix metalloproteinases and therefore exacerbates emphysematous changes. This mouse model showed that smoke accelerates this process as it also stimulates MMP production. Marfan syndrome, a dominantly inherited connective tissue disorder, is caused by a mutation in the fibrillin-1 gene

(FBN1). One sub group of sufferers demonstrates peripheral airspace enlargement and is thought to occur as a result of pneumothorax. One mouse knockout model, with allele deletion of the FBN1 gene, shows abnormalities in lung septation during development, although cellular distributions and matrix deposition appears normal.

There are four Notch receptor proteins, activated by ligands on neighbouring cells. During development the ratio of endocrine and non-endocrine airway cells seemed to be determined by Notch. Other functions of Notch can be categorised as lateral inhibition, the prevention of neighbouring cells developing the same characteristics, lineage specification, dictating the pathway of differentiation and boundary formation. Notch can determine the differentiation of cells into specific phenotypes. Notch has been identified on CD34 positive haematopoietic progenitor cells. Expression studies have shown that the four Notch proteins were present from the embryonic phase right through gestation. Different cell types expressed different Notch proteins, Notch4 is endothelial specific and Notch3 is present on epithelial cells. Another protein involved in the Notch pathway is Hes1, seen in cells which later become Clara cells (Collins, Kleeberger & Ball 2004).

Hox genes are capable of controlling expression of a number of genes that in the lung help control body segmentation and organogenesis. In adult lung Cillo *et al.* (2001) have shown the presence of Hox genes A2-A6, B2-B6 and D1. Golpon *et al.* (2001) have examined Hox genes in lung disease, the results showed presence of foetal Hox genes including; A4, A5, B2, B6, C6 and C8. Compared to normal adult the emphysema lung showed a loss of A1, A10, B6 and C6 and a gain of C8 which shows that emphysema is associated with both loss and gain of Hox genes. Hox genes are also associated with lung cancer, Lechner *et al.* (2002) found expression of A7, C5 and D13. Expression of these genes was also found in surrounding tissue showing changes affected local tissue as well as tumour.

Epithelial tubule development is additionally controlled by EGF/TGFα, hepatocyte growth factor and FGF7. FGF7 also mediates type II pneumocyte differentiation. TGFβ1 has the inverse effect and, anatomically, is thought to act by prevention of branching in the distal airways. VEGF is involved in blood vessel development. Alveolar septation is principally mediated by FGF and PDGF. Many lung conditions show a dysregulation of homeostatic mechanisms. Decreased VEGF and VEGF receptor 2 have been shown in emphysema patients. It has been proposed that these

decreased growth factors affect cell maintenance and cause capillary apoptosis (Kasahara *et al.* 2001). Other cells that may play a role are fibroblasts. Lung fibroblasts from patients with silicosis have an enhanced response to TNFα and TGFβ which increases collagen production (Desai & Cardoso 2002). Additionally, the fibrotic phase in severe ARDS is associated with higher levels of TGFα and procollagen peptide III (Madtes *et al.* 1998).

Matrix production can help determine structure during development. Tenascin C is abundant in foetal lung but virtually absent from basement membrane in adult lung. In adult or juvenile lung the presence of tenascin is usually indicative of damage, aberrant repair or tumour. Collagens I and III differ in distribution, being scarce in the developing lung but persisting in the adult to be the main contributors to extracellular matrix in vessels and present in airway basement membranes. Collagens I and III appear to be elevated in the infant lung in cases of BPD. TGFβ also influences the amount of collagen as well as tenascin and fibronectin (Thibeault *et al.* 2003, Kaarteenaho-Wiik *et al.* 2004, Kaarteenaho-Wiik *et al.* 2002). These disease state changes perhaps reflect a growth factor sensitivity more akin to those seen during development.

Puddicombe *et al.* (2000) have demonstrated in asthmatics that EGFR is more activated on airway epithelium than in normal individuals. This showed that EGFR expression in asthma is damage related. The group postulate that EGFR upregulation holds the epithelium in a repair phenotype with a continued response to inflammation. This could also contribute to COPD pathogenesis as there is damage, inflammation and repair. Another role of EGFR is mucus production upregulation in certain injury situations. The similarities in mucus profiles of chronic bronchitis and foetal lung may suggest that the disease state is reverting to a more foetal phenotype to try and repair the damaged COPD lung. During foetal development, there are increased numbers of mucus cells compared to adult lung and mucus produced is often sulphated (Gaillard *et al.* 1989).

Epithelial regeneration during repair has been seen to occur in similar ways to foetal development; epithelial clusters formed and branched out into granulation tissue until finally ductal lumen developed. Studies showed that mature epithelium put into 3D cultures could form gland-like structures along with dichotomous branching (Infeld, Brennan & Davis 1993). Assessment of the phenotype of the diffuse metaplastic population in COPD may give an indication of either a reversion to a foetal phenotype as part of a repair attempt or of a pre-neoplastic lesion.

1.7 Aims and Objectives

The aim of this thesis was to investigate the diffuse parenchymal epithelial metaplasia seen in COPD. Although rarely described in the literature, this metaplasia is usually considered to be Lambertosis, a reparative extension of airway epithelium. The possible origin, role and importance of these metaplastic cells will be examined.

1.7.1 Chapter 3: Characterisation of the pathology of COPD cases and the association with the metaplastic cells

The aim of chapter 3 was to investigate the histological characteristics and cellular morphology of the metaplastic cells and the lesion distribution.

What are the morphologies of the metaplastic cells?

Where are the metaplastic cells localized?

These were examined on histological sections from a cohort of 50 COPD patients.

Are the metaplastic cells associated with any histopathological changes or groups of individuals within the COPD cohort?

A set of pathological characteristics observed in COPD (section 2.7.1) were examined using a slide subset from COPD patients. These features were grouped using hierarchical clustering to create histopathological groups. Metaplastic cell distribution was then compared with the groups.

Is the loss of airways associated with the metaplastic cell lesion?

The number and diameter of the airways were assessed in COPD patients 1-10 and controls (tumour resection margins N1-N6).

Is the airway epithelial pathology predictive of surrounding metaplastic cells?

The pathology of the airways and surround was noted and airways with or without surrounding metaplastic cells were compared.

1.7.2 Chapter 4: Characterisation of the immune phenotype of the metaplastic cells in COPD

The aim of chapter 4 was to determine whether progenitor cells from within the lung or from multipotent progenitor populations were contributing to the metaplastic cell population.

> Do metaplastic cells demonstrate phenotypic markers suggestive of any resident lung progenitor populations?

Immunohistochemistry and electron microscopy were used to examine characteristics suggestive of basal cells, Clara cells, goblet cells, mesothelial cells, mucus gland progenitors and type II cells presence within the metaplastic cell population. Resident airway progenitor contribution would indicate Lambertosis through airway cell spread.

> Do metaplastic cells demonstrate surface markers suggestive of multipotent progenitor populations?

Multipotent progenitor markers were examined by immunohistochemistry on the metaplastic cell population.

1.7.3 Chapter 5: Does the metaplastic cell population resemble normal airways or reparative airways?

The aim of chapter 5 was to compare metaplastic cells with repair phenotypes of airway epithelium.

> Does the cytokeratin profile of the metaplastic cells resemble that of airway epithelial spread (Lambertosis) or other epithelial reparative phenotypes?

Simple, basal and more squamous cytokeratins were examined on the airways and metaplastic cells.

> Are the metaplastic cells producing mucins and are these similar to those seen in the airways?

Mucin presence and localization was investigated on the metaplastic cells to compare with the airways.

1.7.4 Chapter 6: Is there evidence for an abnormal microenvironment regulating the metaplastic cell population?

The aim of chapter 6 was to determine whether there were the necessary features within the local environment for the development of metaplastic cells. These included mesenchymal cells, matrix and local hypoxia.

Are metaplastic cells associated with underlying mesenchymal cells?

A range of mesenchymal cells were investigated using immunohistochemistry.

Is there a particular matrix composition associated with metaplastic cells?

A number of collagens and matrix constituents within the COPD tissue were examined for the possible association with the metaplastic cells.

Is there evidence for as association between local hypoxic conditions and metaplastic cell localization?

COPD damage including, airway obliteration, fibrosis and angiogenesis all indicate local hypoxia within the COPD lung. Hypoxic areas were investigated using immunohistochemistry for carbonic anhydrase IX (suggestive of hypoxia) and von Hippel Lindau (suggestive of normoxia).

1.7.5 Chapter 7: Is there evidence that the metaplastic cells represent a pre-neoplastic lesion?

Chapter 7 aimed to address whether the metaplastic cells were likely to constitute or develop into a pre-neoplastic lesion.

What is the distribution of proliferating metaplastic cells? Is it similar to a pre-neoplastic lesion?

This was examined using immunohistochemistry for Ki67 comparing both metaplastic cells with airways.

Are the epithelial alterations in metaplastic cells similar to those seen in tumour margins or neoplasia?

This was addressed using immunohistochemistry for involucrin, EGFR, ΔN-p63 and p63.

Is there evidence of molecular change within the metaplastic cell population consistent with changes observed in adenocarcinoma (L858R mutation in EGFR in the metaplastic cells)?

Laser microdissection, DNA extraction and PCR was carried out to assess the presence of an EGFR mutation L858R seen within some lung adenocarcinomas.

Do the metaplastic cells have a morphology or localization similar to the metaplasia observed surrounding tumours?

The position and morphology based on H&E staining were compared with those found in areas of bronchiolisation with alveolar bed associated with tumours.

Chapter 2. Materials and Methods

2.1 Sample acquisition

Lung tissue from patients 1-40 in the cohort for this study was obtained as a result of lung volume reduction surgery (LVRS) at the Glenfield Hospital, Leicester. The procedure is outlined below. Samples 41-50 were obtained from individuals undergoing lung transplantation for severe emphysema at the Freeman Hospital, Newcastle. 6 control samples (N1-N6) were obtained from tumour resection surgery where the tumour resection margins were used. These are considered to be normal areas of tissue. These samples were obtained from the Midlands Lung Tissue Consortium. All samples were obtained with full ethical consent; a copy of each consent form is attached in the appendix 8.1.1-8.1.3.

2.1.1 Surgical intervention for COPD

Patients with severe COPD may be offered the option of undergoing LVRS. This surgery is palliative not curative. The pathophysiology of COPD involves air trapping and therefore hyperinflation of the lung with a relative increase in dead space. One such measurement is the ratio of the residual volume to the total lung capacity (RV:TLC). The hyperinflation results in flattening of the diaphragm and hyper expansion of the chest wall, leading to an increase in the work of breathing. 'Target areas' for excision are identified using radio nucleotide perfusion scintigraphy and computed tomography (CT) scans. Upon excision of these functionally defective areas, there is improved chest wall and diaphragm mechanics, resulting in symptomatic improvement. LVRS was first carried out in the late 1950's by Brantigan, Mueller & Kress (1959). The surgery was heavily criticised, with high mortality rates and lack of objective clinical performance indicators. LVRS was not pursued further at the time, as lung transplantation seemed a better solution. This technique was revisited by Cooper & Patterson in 1995 because of the mortality and morbidity associated with lung transplantation. With a better understanding of the pathophysiology, improvements in surgical techniques and better intensive care facilities, LVRS became a viable option for COPD patients once again.

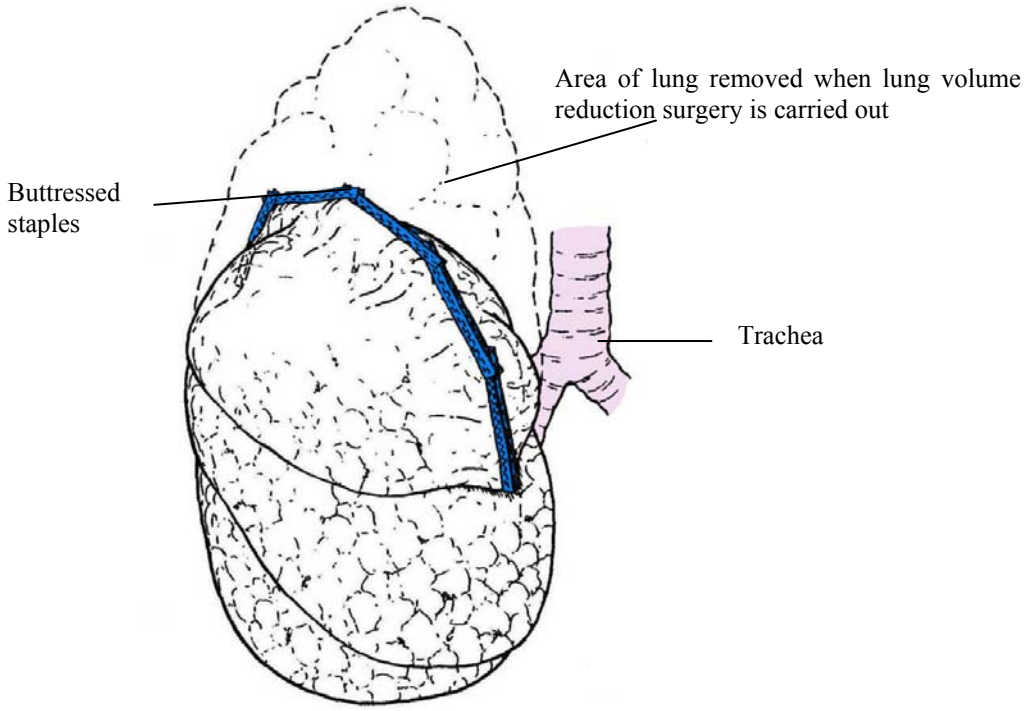


Figure 2.1 An example area removed during lung volume reduction surgery
A lung lobe diagram showing the typical area removed during LVRS surgery. Buttresses are applied to minimise gas trapping and aid recovery. This is a typical area of lung removed, the worst affected area is identified so may be in any part of any lobe. Diagram adapted from Cooper (1997).

2.1.2 Inclusion & exclusion criteria

Table 2.1 Inclusion & Exclusion Criteria for LVRS surgery

Indications	Contraindications
Pulmonary function tests FEV_1 20-40% Obstruction RV>120%, TLC>120% Distension DLCO>25%, pCO_2,7kPa Destruction *Symptoms* Dyspnoea, Medical Research Council scale 3-5 *'Target areas'* Anatomical Functional High-resolution CT Radionucleotide perfusion Scintography *General* Smoking Cessation Compliance with pulmonary rehabilitation programme Objective exercise test – 6 min walk or shuttle test Fitness for surgery Nutritional state	*Radiographic* Homogenous emphysema/no target areas on high resolution CT & perfusion scintography *Pulmonary* Giant bulla Pulmonary nodule of unknown histology Pulmonary hypertension Recurrent infections in lower resp tract Bronchiectasis Hypercapnia (pCO_2>7kPa) Ventilator dependant *Cardiovascular* Recent myocardial infarction (within 6 weeks) Impaired left or right ventricular function Significant arrhythmia *Others* Disease reducing life expectancy Previous chest surgery

Inclusion and exclusion criteria for LVRS patients (Vaughan & Waller 2005) can be seen in Table 2.1.

2.1.3 Patient cohort

Information about the patients compared can be found in Table 2.2 below.

Table 2.2 Patient Information, age, sex, smoking history & spirometry

Pat no.	Age	Sex	Smoking History	FEV_1 %pred	GOLD
1	53	M	Not available (NA)		
2	39	F	NA		
3	49	M	Ex 5 years	34	III
4	65	M	Ex for 2 years 22 pack years	22	IV
5	56	F	Ex 7 years 22.5 pack years	21	IV
6	67	M	Ex 15 years 150 pack years	22	IV
7	58	F	Ex 12 years 40 pack years	18	IV
8	61	F	Ex 3yrs 30 pack years	25	IV
9	65	F	Ex 20 pack years	35	III
10	62	M	Ex 25 pack years	24	IV
11	67	F	Ex 30 pack years	17	IV
12	55	M	NA		
13	55	M	Ex 44 pack years	31	III
14	57	F	Ex 30 pack years	34	III
15	54	M	NA	18	IV
16	69	M	Ex 50 pack year	33	III
17	60	M	Ex 30 pack year	27	IV
18	57	M	Ex 17 pack year	21	IV
19	63	F	Ex 40 pack year	20	IV
20	54	M	Ex 15 pack year	16	IV
21	51	M	NA	20	IV
22	60	F	Ex 40 pack year	36	III
23	66	F	Ex 30 pack year	33	III
24	65	M	Ex 40 pack year	24	IV
25	66	M	Ex 25 pack year	42	III
26	69	M	Ex 75 pack years	22	IV
27	62	M	Ex	24	IV
28	55	F	Ex 20 pack years	29	IV
29	63	M	Ex 40 pack years	26	IV
30	52	F	Non smoker	21	IV
31	65	M	Ex 50 pack years	25	IV
32	57	M	Ex 15 pack years	29	IV
33	43	M	Ex 45 pack years	26	IV
34	66	F	Ex 30 pack years	29	IV
35	58	M	NA		
36	58	M	NA		
37	60	M	NA		
38	63	M	NA		
39	65	M	NA		
40	63	F	NA		
41	46	M	Ex 14.5 pack years	18	IV
42	54	F	Ex 30 pack years	10	IV
43	53	F	Ex smoker	22	IV

44	51	F	Ex 30 pack years	25	IV
45	46	F	Ex 20 pack years	21	IV
46	58	M	Ex 20 pack years	15	IV
47	59	F	Ex 20 pack years	34	III
48	44	M	Ex smoker	14	IV
49	60	F	Ex smoker	26	IV
50	45	M	Ex smoker	26	IV
N1	74	M	Current smoker	104	
N2		F	Ex smoker 20 pack years	78	
N3	49	F	Non smoker	78	
N4	70	F	Current smoker 50 pack years	68	
N5		M	Non smoker		
N6	69	M	Ex smoker 50 pack years	58	

Pack Year – equivalent to 20 cigarettes a day for 1 year.; FEV_1 %pred – Forced expiratory volume in 1 second; percentage predicted The information in the table contains all the data available for the smoking history of each patient,

2.1.4 Panels used for analysis

The number of blocks available for each patient along with numbers used in each investigative panel are in appendix Table 8.1. Patients 1-10 were used in initial studies to interrogate a marker and examine the need to run on a larger cohort. Sub-panel was chosen to include at least 3 examples of lesion in all the locations identified. This set should gives an overview of the whole panel. Whole panel was selected to include the samples from all 50 patients. Control (N1-N6) samples had a representative sample taken from the limited resection tissue available, but, the sampling area was anatomically similar.

2.2 Sample preparation

The tissue samples were fixed for 48 hours at room temperature (RT) in 10% neutral buffered formalin (Pioneer Chemicals, Surrey). The tissue was then dissected into slices approximately 3mm thick. Tissues were processed overnight on a Leica Tissue Processor (LEICA TP 1050 fully enclosed vacuum tissue processor), which automatically takes tissue through graded alcohols and into paraffin. The tissue was then positioned and set into a paraffin wax block on a Leica Histoembedder. Blocks were stored at RT ready for use.

2.2.1 Sectioning

4 m sections were cut with a Leica Jung RM2155. Cut sections were floated on water (37 c) and transferred to Thermo Shandon Colourfrost Plus positively charged microscope slides. Positively charged slides are used to ensure that the tissue remained

adhered to the slide throughout the subsequent staining. The slides were dried overnight at 45 c and then stored at RT.

2.3 Tinctorial staining

All chemicals are from Fisher Scientific (Loughborough, Leicestershire) unless otherwise stated.

2.3.1 Haematoxylin and Eosin staining

The slides were dewaxed in xylene and taken through graded alcohols (3x xylene, 2x ethanol, 2x 100% IMS) and into distilled water. The slides were stained with Gills Haematoxylin (Pioneer Research Chemicals Ltd) for approximately 10-20 minutes. Slides were then washed in running water for approximately 10 minutes. The slides were stained in eosin Y (high purity, from Acros Organics, New Jersey, USA) for approximately 2 minutes, washed briefly in running water and cleared through graded alcohols to xylene. The slides were then cover slipped with Leica mounting media (Leica).

2.3.2 EVG (Elastic van Gieson)

Acid potassium permanganate 0.5% potassium permanganate (Fisher)
3% sulfuric acid
Van Gieson Saturated picric acid 50mls
1% aqueous acid fushin 9mls (Searle)
dH_2O 50mls
MillersVictoria Blue 10g (Sigma)
New Fuchsin 10g (Searle)
Crystal Violet10g (Polysciences)
Concentrated Hydrochloric acid 20cm^3 (Sigma)
Resorcin4g (Fisher)
Dextrin1g (Sigma)
30% Ferric chloride50cm^3 (Sigma)
95% Alcohol approx 3 litres

The slides were dewaxed in xylene and taken through graded alcohols (3x xylene, 2x ethanol, 2x 100% IMS) and into distilled water. The slides were incubated with acid potassium permanganate (Fisher) for 5 minutes (see above) and then rinsed in distilled

water. Next slides were immersed in 1% oxalic acid (Fisons, Loughborough, Leics) for 2 minutes to decolourise the potassium permanganate. After decolourisation the slides were rinsed in 95% alcohol and immersed in Miller's stain in an enclosed jar for 2 hours (see tinctorial reagents). Slides were then rinsed in 95% alcohol and submerged in Van Gieson (see above) stain for 1 minute. The slides were dehydrated and coverslipped with Leica mounting media. This stain is employed to examine elastic fibres. The elastic fibres and nuclei are highlighted in black staining, whilst collagen is seen as red and any other tissue is yellow.

2.3.3 Alcian Blue

Alcian Blue 1g in 100ml of 3% acetic acid (pH of 2.5)

The slides were dewaxed in xylene and taken through graded alcohols (3x xylene, 2x ethanol, 2x 100% IMS) and into distilled water. Slides were immersed in Alcian Blue for 10 minutes; the excess was washed off with distilled water. The slides were then incubated in Gill's haematoxylin (Pioneer Chemicals) for 2 minutes to give a nuclear counterstain, after which the haematoxylin was then altered to blue by brief submersion in saturated borax solution (di-Sodium tetraborate, Fisher) . Slides were then washed well in tap water, dehydrated and coverslipped with Leica mounting media. On lung tissue Alcian Blue highlights acidic and sulphated mucus with a turquoise blue colour, haematoxylin is used as a counterstain to show the nuclei of the tissue.

2.3.4 PAS (Periodic acid Schiff's)

The Schiff's reagent was warmed to room temperature for 1 hour before use. The slides were dewaxed in xylene and taken through graded alcohols (3x xylene, 2x ethanol, 2x 100% IMS) and into distilled water. Slides were immersed in 1% periodic acid solution for 5 minutes and then washed in distilled water. Schiff's reagent (Pioneer Chemicals) was applied to the slides for 8-10 minutes and washed in tap water for 10 minutes to develop the colour. The slides where then immersed in Gill's haematoxylin for 2 minutes to give a nuclear counterstain. Slides were then washed in tap water for 10 minutes, dehydrated and coverslipped with Leica mounting media (Leica). PAS staining is used to highlight glycogen, neutral mucus and acidic mucus, which are stained a purple or magenta colour. Haematoxylin is used as a counterstain to demonstrate tissue nuclei.

2.4 Immunohistochemistry

2.4.1 Standard Protocol

The diluent used for most of the solutions in immunohistochemistry was PBS 0.01M / BSA 1%, (Sigma) with 0.05% TWEEN 20 (National Diagnostics, Atlanta, Georgia). The exceptions were hydrogen peroxide where any dilutions were carried out in methanol and the tyramide step of TSA amplification step where reagents were provided in the kit. The wash buffer used was PBS 0.01M pH7.4 (Sigma) + 0.05% TWEEN 20 (National Diagnostics) unless the Envision kit was being used, which Envision x10 wash buffer was supplied with Dako Envision Flex kit (Dako, Glostrup, Denmark).

The slides were dewaxed in xylene and taken through graded alcohols (3x xylene, 2x ethanol, 2x 100% IMS) and into distilled water. Any necessary antigen retrieval was then performed. There are a range of antigen retrieval solutions that may be applied including: (i) Citrate (Sigma, Missouri, USA) which was a 10 mM solution, (2.1g citric acid monohydrate in 1L dH_2O), pH adjusted to 6.0 with NaOH, (ii) Dako low citrate based, pH6.1 solution (Dako) x10; 100mls in 900mls dH_2O, (iii) Dako high Tris/EDTA based, pH9.0 solution (Dako) x10; 100mls in 900mls dH_2O, (iv) EDTA (Fisher Scientific) produced using 1mM solution, (0.37g Diaminoethanetetra-acetic acid disodium salt in 1L dH_2O) pH to 9.0 with NaOH, (v) Vector Unmasking Fluid (VUF) 15mls antigen unmasking fluid in 1600mls dH_2O (Vector Laboratories, Burlingame, California, USA), (vi) 0.1% Trypsin made with dH_20 warmed to 37˚C. (0.1g Trypsin (Beckton Dickinson, Maryland, USA), 0.1g $CaCl_2$ (Fisher Scientific) in 100ml dH_2O) pH to 7.0. (vii) Proteinase K (ready to use) (Dako).

All protocol specific information may be found in Table 2.3.

Endogenous peroxidase activity was quenched using 0.5%-6% hydrogen peroxide (Fisher Chemicals) in methanol for 10 minutes or Dako peroxidase block (Dako EnVision) for 5 minutes. Slides were then washed in wash buffer.

In some protocols a 20% normal serum corresponding to host of secondary antibody was applied for 20 minutes. Slides were blotted prior to primary antibody application.

Slides were incubated in primary antibody at RT or at 4˚C, usually for 1 hour, times are shown in Table 2.3. Control slides were incubated with isotype Ig antibody at the same concentration as the primary antibody. Slides were then washed.

Bound antibody were detected using appropriate biotinylated secondary antibodies followed by Streptavidin ABComplex/HRP (horse radish peroxidase) or Streptavidin ABComplex/AP (alkaline phosphatase) (Dako) or TSA Biotin System (Tyramide Signal Amplification) kit, (PerkinElmer Life Sciences Inc, Boston, MA). Other antibodies were detected using Envision Flex (Dako), a ready made kit containing generic rabbit and mouse secondary, coupled to a polymer backbone, was applied for 20 minutes, with or without a 15 minutes amplification step prior to secondary application. HRP labelled slides were incubated with diaminobenzidine (DAB) chromagen (Dako). The amount of chromagen development was determined microscopically and counterstained for 10 seconds using haematoxylin. These slides were then dehydrated and coverslipped with Leica mounting media. Alkaline phosphatase slides were incubated with Fast Red (Sigma) for 10 minutes and aqueously mounted with AquaPerm (ThermoShandon, Pittsburgh, Pennsylvania, USA).

2.4.2 Immunohistochemistry optimisation

The Department of Cancer Studies and Molecular Medicine at Leicester Royal Infirmary kindly provided the protocols for antibodies CK 13 and CK 19. Mucin 2, Mucin 4 and Mucin 5AC were adapted from protocols provided by T Fehniger, (AstraZeneca) and the EGFR stain was a Ready To Use kit (Dako).

All other antibody protocols were optimised in Pathology Lab at AZ Charnwood. Optimisation was carried out on appropriate control tissue, most often lung tissue. A standard initial work up protocol used in the Charnwood Pathology department was employed and involved comparing a range of antigen retrievals run at one concentration of primary antibody Antigen retrieval. These included: -

High pH microwave, either EDTA pH 9.0, or Dako High pH retrieval solution

Low pH microwave, either Citrate pH 2.5, or Dako Low pH retrieval solution

Enzyme digestion, 0.1% trypsin, or proteinase K

No pre-treatment

The primary antibody was applied at a concentration recommended by the manufacturer or at a range of 2-10μg/ml. Secondary antibodies were used at 1/200 or 1/300 to give an excess and was appropriate to the host of the primary antibody. The Dako ABComplex HRP was used as the initial tertiary in most cases. Pre-treatments and concentrations, along with incubation time and temperature were altered according to the findings from the initial run. Primary antibody slides were always accompanied with an isotype control of the same immunoglobulin type at the same concentration to ensure there was no non-specific binding occurring.

Immunohistochemistry has been widely used throughout subsequent chapters. The initial step with all stains was to ensure the primary antibody used was producing reliable specific staining. An isotype control of the same immunoglobulin type and concentration are the primary antibody was applied. This should not bind to any specific protein or cell type, any pattern of staining seen with this control isotype would put the specific primary antibody into question. The highest concentration of each immunoglobulin type used in this study has been photographed and included below to demonstrate that none of the antibodies used showed any staining with a corresponding isotype. Each experiment had appropriate isotype slides run alongside primary antibody, the only slides with any staining on were CC10 antibody isotypes, these showed plasma and blood staining and minimal wash over some non-mucinous epithelial cells. In the interest of space only the highest concentrations are shown here.

Table 2.3 Immunohistochemistry protocols

Abbreviations VUF – Vector unmasking fluid; Strept HRP– Streptavidin Horse Radish Peroxidase; TSA – Tyramide Streptavidin Amplification; PC – Pressure Cook; MW – Microwave

Antibody	Retrieval	H_2O_2 (10min)	1˚ Ab	Dil^n/Conc (μg/ml)	Secondary	Tertiary	Cell types highlighted
Carbonic anhydrase IX	VUF MW	0.5%	AbCam Ab15086 60 min	1:1000 1	Goat α rabbit Dako E0432 1:200 20min	TSA Biotin System	Hypoxic cells
Clara cell prot (CC10)	Citrate MW	0.5%	RD181022220 BioVendor 60min	1:1000 10	Swine α rabbit Dako E0353 1:300 30 min	Strept AB/HRP 30 min	Clara cells
CD31	NONE	3%	AbCam Ab9498 30 min	1:40 3.75	Goat α mouse Dako E0433 1:300 30min	TSA Biotin System	Endothelial cells
CD34	VUF MW	6%	Dako M7165 2 hours	1:25 0.25	Rabbit α mouse Dako E0464 1:100 20 min	Strept AB/HRP 20 min	Haematopoietic stem/ endothelial cells
C-kit, CD117	VUF PC	3%	Dako A4502 60 min	1:800 12.5	Goat α rabbit Dako E0432 1:300 30 min	Strept AB/HRP 30 min	Haematopoietic stem cells
CD133	Low pH MW	Flex bk 5 min	AbCam Ab19898 20 min	1:400 0.425	EnVision Flex HRP 20 min		Bone marrow stem cells
Collagen 1	VUF MW	0.5%	AbCam Ab6308 60 min	1:1000 5.5	Goat α mouse Dako E0433 1:200 20 min	Strept AB/HRP 20 min	Extra cellular matrix; collagen
Collagen 3	VUF MW	Flex bk 5 min	Biogenex MU167-UC 20 min	1:100 0.13	Mouse Flex linker Dako DM804 15min	EnVision Flex HRP 20 min	Extra cellular matrix; collagen
Collagen 4	VUF PC	0.5%	Dako M0785 60 min	1:800 0.15	Horse α mouse Vector BA-2001 1:200 30min	TSA biotin system	Extra cellular matrix; collagen
CK (Pan)	EDTA MW	0.5%	Dako M0821 60 min	1:100 1.7	Goat α mouse Dako E0433 1:300 30min	Strept AB/HRP 20 min	Epithelial cells
CK5/6	EDTA MW	0.5%	Dako M7237 (5/16 B4) 60 min	1:25 2.2	Goat α mouse Dako E0433 1:100 20min	Strept AB/HRP 20 min	Basal epithelial cells
CK7	EDTA MW	0.5%	Dako M7018 60 min	1:100 2.6	Goat α mouse Dako E0433 1:300 30min	Strept AB/HRP 20 min	Simple epithelial
CK14	VUF MW	0.5%	NovoCastra NCL-LL002 60 min	1:144 0.25	Goat α mouse Dako E0433 1:300 20 min	TSA Biotin System	Squamous epithelial cells
CK18	Citrate MW 20min	None	Dako M7010 Overnight 4˚C	1:50 6.4	Rabbit α mouse Dako E0464 1:400 30 min	Strept AB/AP 30 min	Simple epithelial
CK19	Citrate MW	None	Dako M0888	1:100	Rabbit α mouse Dako	Strept AB/AP	Simple epithelial

	20min		Overnight 4˚C		E0464 1:400 30 min	30 min	
Desmin	NONE	0.5%	Dako M0760 60 min	1:100 2.3	Goat α mouse Dako E0433 1:200 20 min	Strept AB/HRP 20 min	Smooth muscle cells
E cadherin	High pH MW	0.5%	Santa Cruz SC-8426 60 min	1:1000 0.2	Horse α mouse Vector BA-2001 1:300 30min	TSA Biotin System	Epithelial cells
EGFR	Dako kit						Some epithelial cells
Involucrin	Low pH MW	Flex bk 5 min	AbCam ab68-100 20 min	1:4000 0.25	EnVision Flex HRP 20 min		Squamous epithelial cells
Ki67	VUF PC	0.5%	Vector VP-K452 60 min	1:100 0.56µg/ml	Goat α mouse Dako E0433 1:300 20 min	TSA Biotin System	Proliferation
Laminin 1&2	Trypsin 30min 37°c	Flex bk 5 min	AbCam Ab7463 20 min	1:250 Unknown	Rabbit Flex linker Dako DM805 15 min	EnVision Flex HRP 20 min	Extra cellular matrix
Mesothelin	VUF MW	0.5%	AbCam (SPM143) AB15230 60 min	1:400 2.5	Horse α mouse Vector BA-2001 1:300 30min	TSA Biotin System	Mesothelial cells
Mucin 2	Citrate MW	0.5%	Neomarkers 1037-P Clone AB2 60 min	1:25 8µg/ml	Horse α mouse Vector BA-2001 1:300 30min	Strept AB/HRP 20 min	Type 2 mucus some epi cells
Mucin 4	EDTA MW	None	Zymed 18-2322 60 min	1:50 1µg/ml	Horse α mouse Vector BA-2001 1:300 30min	Strept AB/HRP 20 min	Type 4 mucus some epi cells
Mucin 5AC	VUF MW	None	Neomarkers MS-551-P 60 min	1:200 1µg/ml	Horse α mouse Vector BA-2001 1:300 30min	Strept AB/HRP 20 min	Type 5AC mucus some epi cells
ΔN-p63	Citrate MW	0.5%	BioChem PC373 60 min	1:2000 7.5µg/ml	Swine α rabbit Dako E0353 1:200 30min	Strept AB/HRP 30 min	Truncated p63 progenitor cells
p63	NONE	6%	Pharmingen 559951 Overnight 4˚C	1:100 5µg/ml	Goat α mouse Dako E0433 1:100 20 min	Strept AB/HRP 20 min	Basal epithelial cells
S100a4	VUF PC	6%	Dako A5114 30 min	1:800 0.9	EnVision Flex HRP 20 min		Fibroblasts
Surfactant A	VUF MW	0.5%	Dako M4501 60 min	1:100 5	Goat α mouse Dako E0433 1:300 20 min	Strept AB/HRP 20 min	Type II/ Clara cells
(Pro)Surfactant C	Low pH MW	Flex bk 5 min	AbCam ab 28744 20 min	1:1000 10-16	EnVision Flex HRP 20 min		Type II cells
Vimentin	VUF MW	0.5%	Dako M0725 60 min	1:100 3.6	Goat α mouse Dako E0433 1:200 20 min	Strept AB/HRP 20 min	Mesenchymal cells
Von Hippel Lindau	VUF PC	0.5%	BD Biosciences 556347 60 min	1:500 1µg/ml	Goat α mouse Dako E0433 1:200 20min	TSA Biotin System	Normoxic cells

Table 2.4 Cell marker information & prior use

Cell types highlighted	Antibody	1˚ Ab	Clone	Reference
Hypoxic cells	Carbonic anhydrase IX	AbCam Ab15086		(Kim *et al.* 2005)
Clara cells	Clara cell prot (CC10)	BioVendor RD181022220	Polyclonal	(Coppens *et al.* 2007)
Endothelial	CD31	AbCam Ab9498	JC/70A	(Moriyama *et al.* 1997)
Endothelial/haematopoietic stem cells	CD34	Dako M7165		(Chauhan *et al.* 2003)
Haematopoietic Stem cells	C-kit CD117	Dako A4502	Polyclonal	(Went *et al.* 2004)
Stem cells	CD133	AbCam Ab19898	Polyclonal	(Monzani *et al.* 2007)
Epithelial cells	CK (Pan)	Dako M0821	MNF116	(Horkko *et al.* 2006)
Basal epithelial cells	CK5/6	Dako M7237	D5/16 B4	(Reis-Filho *et al.* 2003)
Simple epithelial	CK7	Dako M7018	OV-TL 12/30	(Tot 2001)
Squamous epithelial cells	CK14	NovoCastra NCL-LL002	LL002	(Hong *et al.* 2004)
Simple epithelial	CK18	Dako M7010	DC 10	(Sculean *et al.* 2001)
Simple epithelial	CK19	Dako M0888	RCK108	(La Rosa *et al.* 2007)
Extra cellular matrix; collagen	Collagen 1	AbCam Ab6308	COL-1	(Oswald *et al.* 2003)
Extra cellular matrix; collagen	Collagen 3	Biogenex MU167-UC	HWD1.1	(Kranenburg *et al.* 2006)
Extra cellular matrix; collagen	Collagen 4	Dako M0785	CIV 22	(Zeng, Cohen & Guillem 1999)
Smooth muscle cells	Desmin	Dako M0760	D33	(Kaarteenaho-Wiik *et al.* 2007)
Epithelial cells	E cadherin	Santa Cruz SC-8426	G-10	(Shen *et al.* 2006)
Some epithelial cells	EGFR	Dako	2-18C9	(Hirsch *et al.* 2008)
Squamous epithelial cells	Involucrin	AbCam ab68-100	SY-5	(Pendleton *et al.* 1996)
Proliferation	Ki67	Vector VP-K452	MM1	(Fernandez-Figueras *et al.* 2005)
Extra cellular matrix	Laminin 1&2	AbCam Ab7463	Polyclonal	(Catena *et al.* 2007)
Mesothelial cells	Mesothelin	AbCam AB15230	SPM143	(Anraku *et al.* 2008)
Mucin 2, some epithelial cells	Mucin 2	Neomarkers 1037-P	AB2	(Losi *et al.* 2004)
Mucin 4, some epithelial cells	Mucin 4	Zymed 18-2322	1G8	(Fischer *et al.* 2003)
Mucin 5AC, some epithel[ial] cells	Mucin 5AC	Neomarkers MS-551-P	1-13M1	(Kikuchi *et al.* 2004)
p63+ stem cells	ΔN-p63	BioChem PC373	Polyclonal	(Geddert *et al.* 2003)
Basal epithelial cells	p63	Pharmingen 559951	4A4	(Geddert *et al.* 2003)
Fibroblasts	S100a4	Dako A5114	Polyclonal	(Ward *et al.* 2005)
Type II/ Clara cells	Surfactant A	Dako M4501		(Kobayashi, Takeuchi & Ohtsuki 2008)
Type II cells	(Pro)Surfactant C	AbCam ab 28744	Polyclonal	(Gibbs *et al.* 2009)
Mesenchymal cells	Vimentin	Dako M0725	V9	(Kaarteenaho-Wiik *et al.* 2007)
Normoxic cells	Von Hippel Lindau	BD Biosciences 556347	Ig32	(Chen *et al.* 1995)

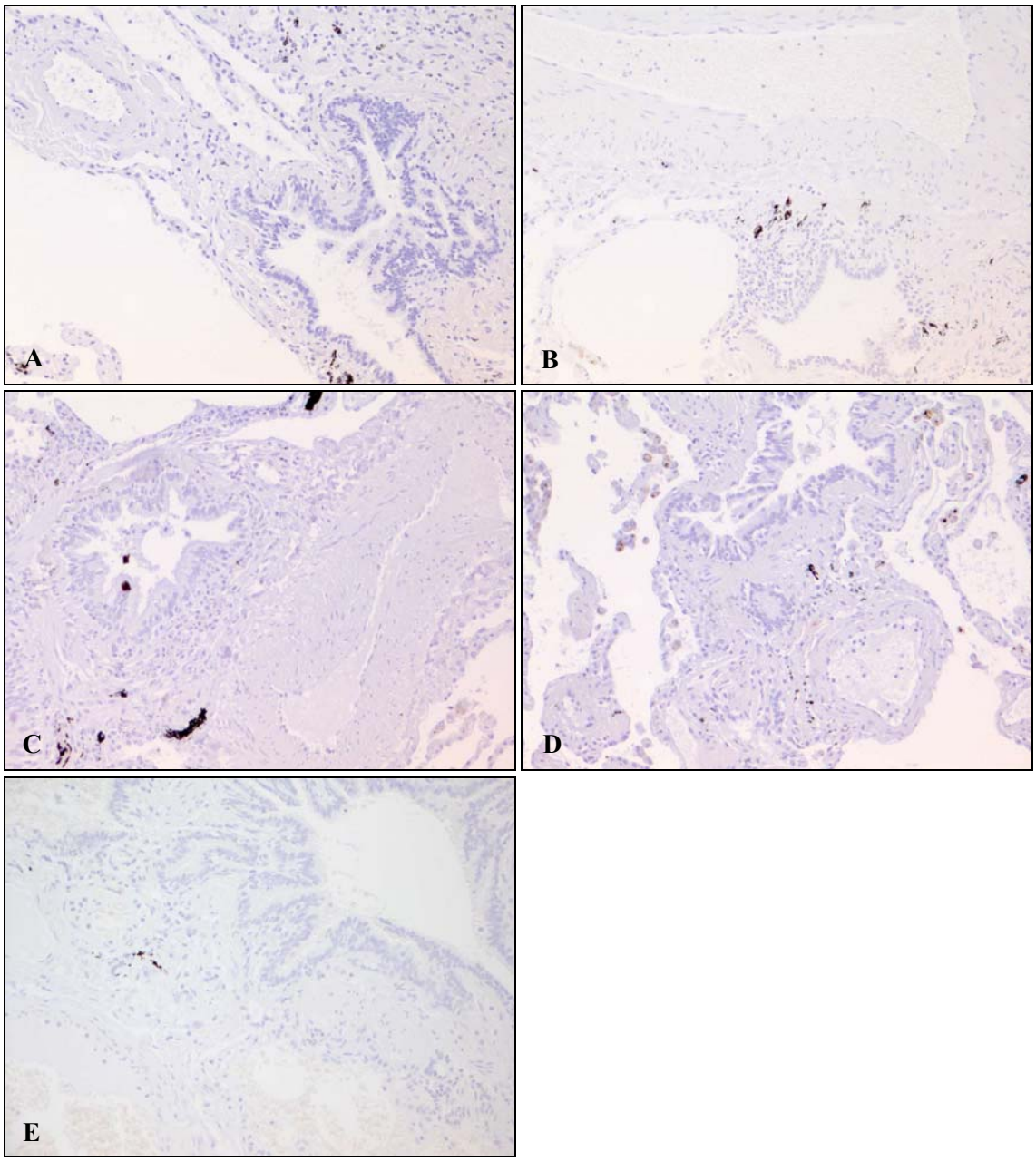


Figure 2.2 Examples of isotype antibody staining, to confirm specificity of primary antibody

Figure 2.2A) Isotype control slide for Surfactant A staining, mouse IgG2b used at 5µg/ml.
Figure 2.2B) Isotype control slide for Mucin 2 staining, mouse IgG2a used at 8µg/ml.
Figure 2.2C) Isotype control slide for Collagen I staining, mouse IgG1 used at 5.5µg/ml.
Figure 2.2D) Isotype control slide for c-kit staining, rabbit Ig fraction used at 12.5µg/ml.
Figure 2.2E) Isotype control slide for CC10 staining, rabbit Ig fraction used at 10µg/ml, this may have had different heat pre-treatment or other conditions that caused the non-specific staining at a lower concentration. This highlights the importance of using an isotype control with all experiments.

2.5 Electron Microscopy

Samples were chosen from archived formalin fixed, paraffin embedded tissue. These samples were taken from the same patients that were used in the other investigations here. Blocks similar to the immunohistochemistry blocks identified were chosen to contain metaplastic cells. This prevented the loss of sample necessary for immunohistochemical studies. 2mm core samples were taken out of the wax blocks using a Beechers tissue microarray machine. The areas to be taken were identified from an H&E section from the surface of the block. The samples were then dewaxed in xylene overnight on a rota-mix and post processed for resin blocks. The samples were incubated in: - xylene for 30 minutes, repeated twice, then 100% alcohol for 30 minutes, again repeated twice. Samples were then incubated in 95% alcohol for 30 minutes, 75% alcohol for 30 minutes, 75% alcohol for 30 minutes, 50% alcohol for 30 minutes and finally 30% alcohol for 30 minutes. Once these alcohol incubations were complete two incubations in sodium cacodylate were carried out, both of which were 30 minutes. The final step of 2.5% glutaraldehyde carried out overnight. The tissue was processed on a Leica TP processor using a 26 hour 19 step program, through 1% osmium tetroxide then through acetone. Tissue was embedded in araldite resin which was polymerised for 48 hours at 60ºC. Sections were cut at 1µm and stained with 1% toludine blue for 30 seconds on a hot plate at 50ºC. The toludine blue sections were then scanned using the Aperio, slide scanning microscope.

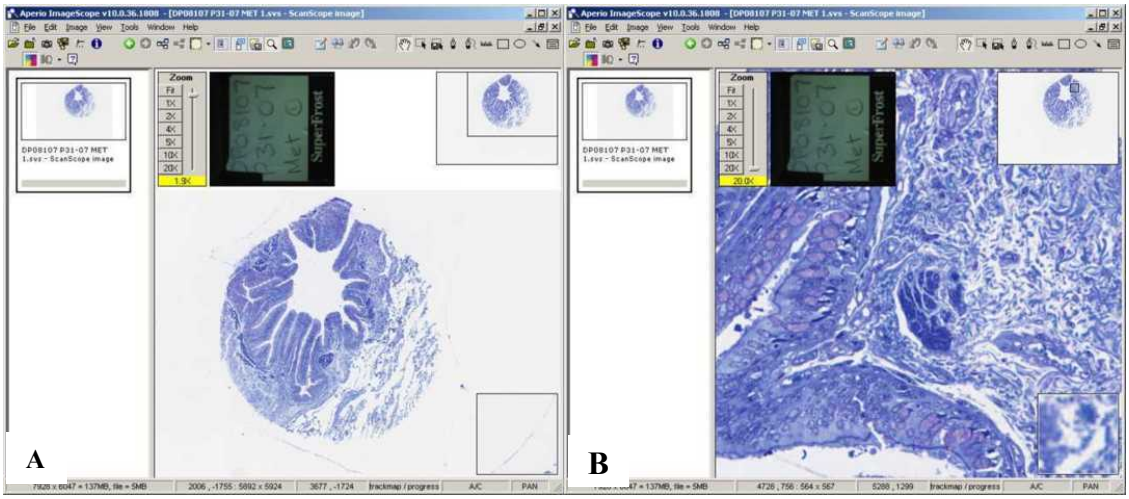


Figure 2.3 Images viewed on the Aperio system
Figure 2.3A is an overview of the core stained with toludine blue, Figure 2.3B shows the same scanned image zoomed in to x20. This system allows quite detailed review of the microscope slide without actually having the slide.

The appropriate areas were subsequently identified looking at adventital metaplastic cells and the blocks cut down to about 1.5mm^2. These small blocks were cut further using a diamond knife to a width of 100µm and picked up on nickel grids. The sections were dried overnight, stained with uranyl acetate for 10 minutes and washed in distilled water. Sections were then incubated in lead citrate for 10 minutes then washed and air dried. The samples were then ready to be examined on the Jeol JEM-1400 electron microscope. Photographs of the cells of interest were taken using a SIS-Morada camera.

Electron microscopy work was carried out by S Brocklehurst (AstraZeneca, Alderley Park).

2.6 EGFR Mutation detection

2.6.1 Laser Capture Microdissection

The wax sections were dewaxed in xylene for 10 minutes and allowed to air dry. Arcturus macro caps were used for capture. A minimum of 500-1000 captures were acquired for each sample.

Conditions set on the Arcturus PixCell IIe laser capture microdissection system were

Power 85mWTarget 0.2vSpot size 7.5µmRepeat 0.2ms Current approx 4.8Duration 5ms

The laser capture sticky disc with the captured cells on was carefully peeled from the cap and stored at 4ºC into tubes ready for DNA extraction.

2.6.2 DNA extraction using Qiagen kit

DNA extraction was performed using Qiagen kit (Qiagen, Crawley, West Sussex) as per manufacturers instructions. The following steps were carried out on the tubes containing the discs and cells. Incubated with 25µl of protease, 200µl of 0.9% NaCl and 200µl of buffer AL (with carrier RNA), each eppendorf containing this mixture was then closed and pulse vortexed for 15 seconds. These samples were then incubated at 56ºC overnight (minimum 16 hours). After overnight incubation samples were pulsed in a centrifuge before 250µl of ethanol was added. Samples were then pulse vortexed again and incubated at room temperature for 5 minutes. Samples were then pulsed in a centrifuge before adding the lysate into a Min Elute column. The remaining discs were discarded. Columns containing the lysate solution were centrifuged at 8000 rpm for 1 minute. Columns were placed in clean 2ml collection tube, 500µl of buffer AW2 was

added and columns were again centrifuged at 8000 rpm for 1 minute. Columns were placed in another clean collection tube, 500µl ethanol was added and columns were spun at 8000 rpm for 1 minute. Columns were placed in a clean collection tube and centrifuged at a higher speed of 13000 rpm for 3 minutes. Columns were placed in a clean 1.5ml eppendorf and 30µl of Buffer AVE elution buffer was added to the column this was incubated at room temperature for 5 minutes and the columns were then centrifuged at 13000 rpm for 1 minute to elute the DNA. The sample collected was stored at -20ºC.

2.6.3 Real-time PCR for detection of EGFR mutation

Real time PCR work was carried out to detect the L858R mutation of EGFR found on exon 21 using an Applied Biosystems StepOne PCR machine. The forward and reverse primers (Table 2.5) were designed using Primer 3 software to amplify an area surrounding the 858 amino acid position on the EGFR gene. Probes were then designed using Primer Express software. One probe was designed to label only wild type DNA sequence and the other probe was designed to label only the somatic single nucleotide mutation in the middle of amino acid 858.

Table 2.5 Primers & Probes used for mutation detection of EGFR

Type	Label	Sequence	Start	Stop	Tm
Forward Primer	None	AACACCGCAGCATGTCAAGA	550	569	59
Reverse Primer	None	CCGCACCCAGCAGTTTG	605	590	58
Probe 1 Mutant	FAM	CAGATTTTGGGCGGG	573	587	64
Probe 2 Wildtype	YY	ACAGATTTTGGGCTGG	572	587	64

YY=Yakima Yellow, Tm=predicted melt temperature

Figure 2.3 shows where the forward and reverse primers bind to the DNA sequence. The k in the sequence shows the position of the mutation that was investigated. This L858R point mutation changes the base from a T to G, of the 858 amino acid in the gene, counting from the N terminus. This changes the amino acid from a leucine (L) to an arginine (R).

2716 2726 2736 2746 2756 GGCATGAACT ACTTGGAGGA CCGTCGCTTG GTGCACCGCG ACCTGGCAGC
2766 2776 Forward primer 2806 CAGGAACGTA CTGGTGAAAA CACCGCAGCA TGTCAAGATC ACAGATTTTG
2816 Reverse primer 2846 2856 2781 GGCkGGCCAA ACTGCTGGGT GCGGAAGAGA AAGAATACCA GGCAAA TGCAGAAGGA

Figure 2.4 Primer locations within the EGFR DNA sequence
The base sequence of Exon 21, the numbers refer to the bases numbers starting at the beginning of the whole EGFR gene. The forward primer highlighted in red and the reverse primer highlighted in blue. This shows the area between the two primers that is amplified.

The probes were made up at a stock concentration of 100µM using milliQ water and diluted to a working concentration of 10µM. Primers were made up to a stock concentration of 200ρmol/µl and diluted to a working concentration of 10ρmol/µl. The PCR mastermix used was Ready To Use Taqman Genotyping Mastermix (Applied Biosystems). The PCR reactions were set up with the following components in each reaction, 5µl of DNA mastermix, 0.6µl forward primer, 0.6µl reverse primer, 0.2µl of wildtype probe, 0.2µl mutant probe and 3.4µl of the DNA sample of interest. Each sample was run in duplicate.

The wildtype probe was labelled with Yakima Yellow which possesses an absorbance maximum of 530.5 nm and an emission maximum of 549 nm. The mutant probe was labelled with FAM that has an absorbance maximum at 494 and an emission maximum at 522.

Pilot experiments determined that the best conditions were an annealing temperature of 57ºC and 50 cycles. All samples were run in duplicate. DNA from the cell line NCI-H1975 was used as a positive control for the EGFR mutation as this is heterogeneous for L858R EGFR mutation. DNA from normal tonsil was used as a wildtype control that did not show any mutation.

2.7 Slide analysis

2.7.1 H&E Examination

Initial assessment was carried out on all H&E slides available for each patient, the slides with the most obvious metaplastic lesions in a range of locations were identified for each patient, the number of slides selected ranged from 2-4 slides per patient. This full panel (see Table 2.6) of slides was then examined to gain an overview of the

background COPD pathology within the sample. The criteria of each lung compartment considered are outlined in Table 2.6 below.

Table 2.6 Criteria examined for each patient to gain an overview of pathology

Pleura	Parenchyma	Vasculature	Airways
Acute inflammation	Alveolitis	Adventitial hypertrophy	Basal cell hypertrophy
Fibrosis	Bronchiolisation	Fibrosis	Bronchiolitis
Lymphoid agg	Bullae	Intravascular leak	Damaged epithelium
Necrosis	Clubbing	Medial hypertrophy	
Odema	Fibrosis	Muscularisation	Fibrosis
Sclerosis	Epithelial lined cysts	Myolysis	Goblet cell hypertrophy
	Honeycombing	Odema	Hypertrophy
	Hyaline	Vasculitis	Inflammation
	Hyperinflation		Metaplastic cells
	Inflammation		Mucus in lumen
	Leak		Muscularisation
	Loss		Normal epithelium
	Lymphoid agg		Obliterative bronchiolitis
	Macrophage agg		
	Muscularisation		Pyknosis
	Pneumonitis		Squamous epithelium
	Type II hyperplasia		

Hierarchical clustering was then carried in Spotfire Decision site 10.0 to determine the presence of any similar pathology patterns between patients and assess the presence of general pathological phenotypes.

2.7.2 H&E analysis of the metaplastic lesion

The full panel of slides containing the most extensive examples of pre-neoplastic lesion were examined and the location frequency and morphology of the metaplastic cells were noted within each slide.

2.7.3 Airway analysis – epithelial characteristics

All available H&E slides for patients 1-10 were examined and the following was examined for each airway.

Table 2.7 Criteria examined for each airway in patients 1-10

Criteria Examined for each airway	Assessment
Airway Diameter	mm
Presence of metaplastic cells around external adventitia	Yes / No
Villus forms in airway	Yes / No
Predominant epithelial phenotype	Normal damage Hypertrophy Hyperplasia Goblet cell hypertrophy Goblet cell hyperplasia Squamous Reparative

These results were then analysed for significance using an unpaired Student's T test with unequal variances, see statistics section for further information.

Inflammation was graded as 0-3. Examples of each grade are shown in Figure 2.4 below.

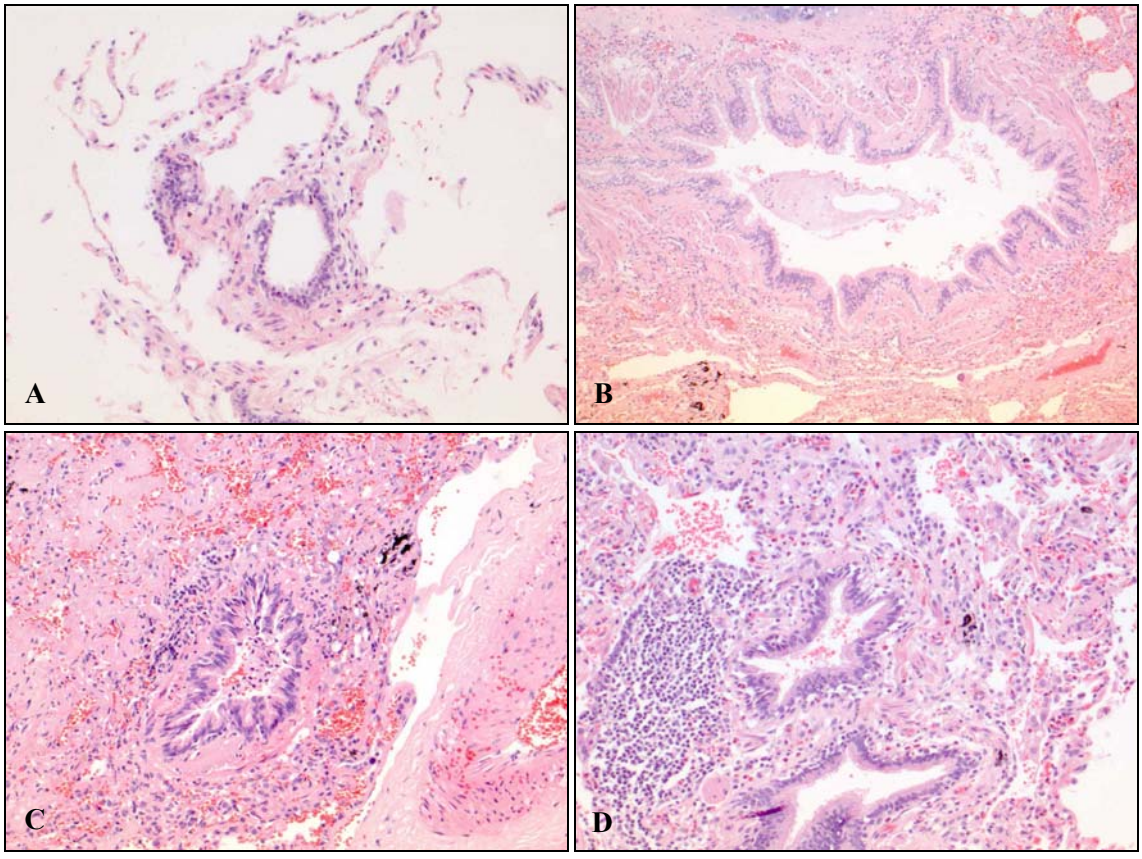


Figure 2.5 Airway inflammation example grades
Figure 2.5A) 0 = No inflammation x 5 objective
Figure 2.5B) 1 = Mild inflammation x 5 objective
Figure 2.5C) 2 = Moderate inflammation x 10 objective
Figure 2.5D) 3 = Severe inflammation x 10 objective

The level of intact attachments to the alveolar bed were graded as 0-4 as severity increased. Examples of these grades are shown below.

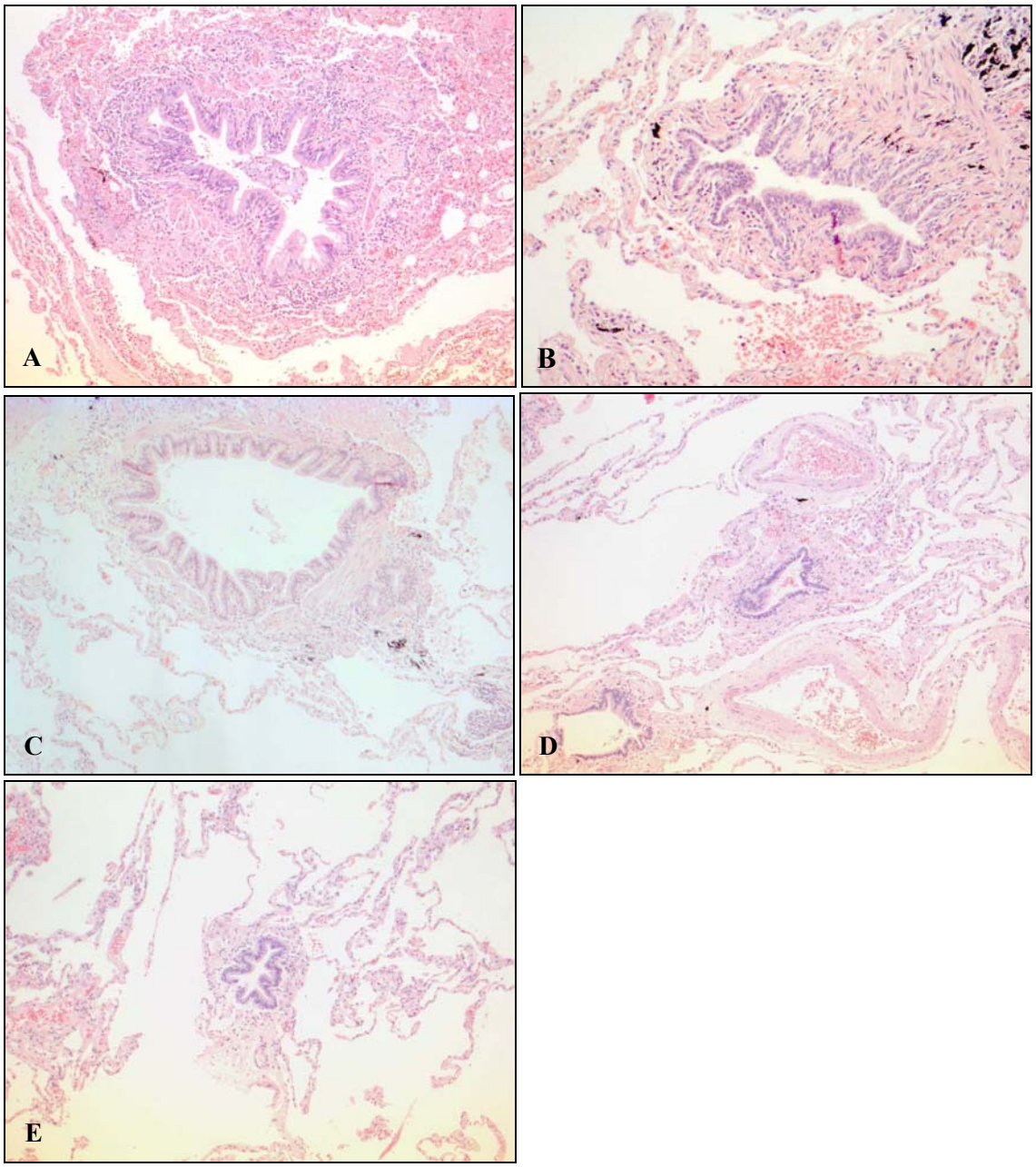


Figure 2.6 Loss of alveolar attachments example grades

Figure 2.6A) 0 = intact x 5 objective
Figure 2.6B) 1 = mild loss x 5 objective
Figure 2.6C) 2 = moderate loss x 5 objective
Figure 2.6D) 3 = severe loss x 5 objective
Figure 2.6E) 4 = none remaining x 5 objective

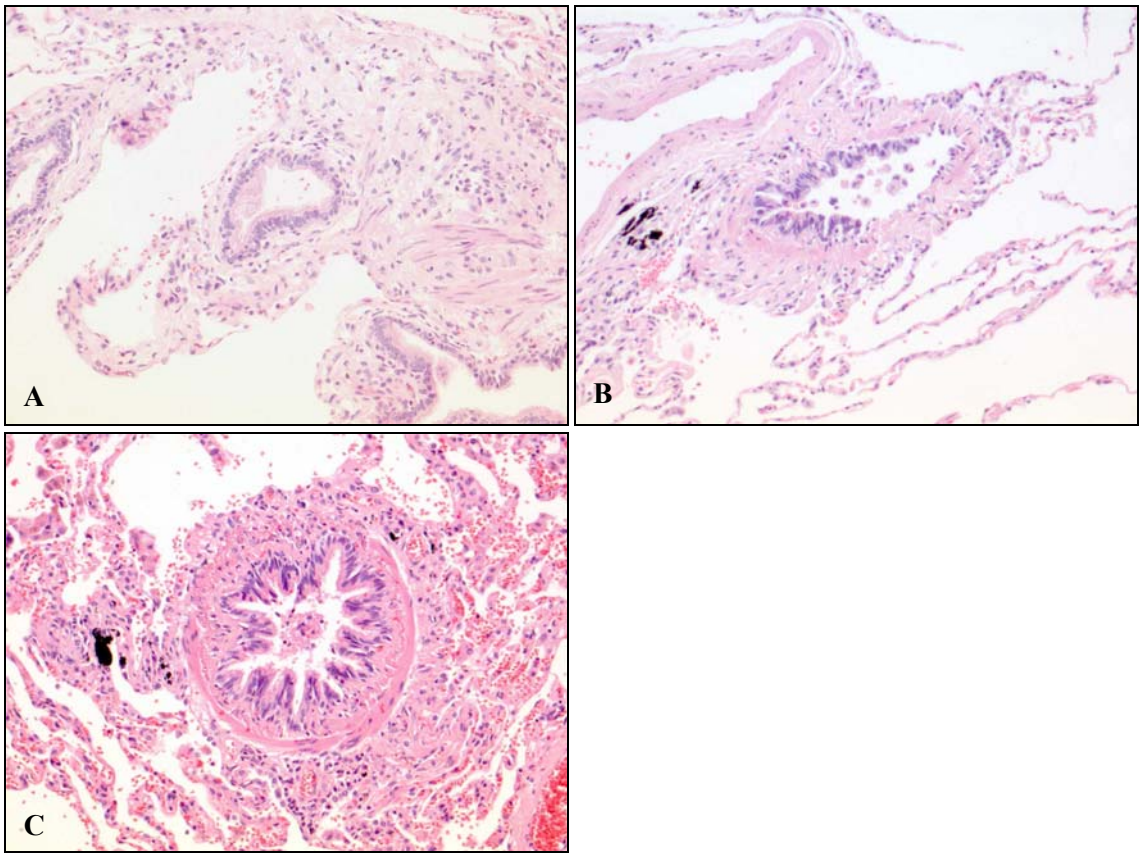


Figure 2.7 Airway fibrosis example grades
Figure 2.7A) 1 = mild fibrosis x 10 objective
Figure 2.7B) 2 = moderate fibrosis x 10 objective
Figure 2.7C) 3 = severe fibrosis x10 objective

2.7.4 Airway analysis – diameter and frequency

The area of tissue on the slide was traced with the use of a light box. The outlines were scanned into the computer with care to keep the document the same size. The area of the tissue was then calculated using GIMP 2.4.2, a free image software package. A Gaussion blur filter was applied to each scanned image, followed by a Laplacian edge-detection algorithm. The area inside airways could then be selected and accurately measured in pixels^2. The measurement in pixels^2 was converted into mm^2 using a known 1 cm marker on the scanned image. 5 measurements of the marker were taken which were 94.5, 94.5, 94.4, 94.5 and 94.5 pixels. The mean of these measurements was used (94.48 pixels per cm) to calculate the area that 1 cm^2 equated to (8926.47pixels per cm^2). The standard deviation of these measurements was 0.04472, which can be used to assess the error of measurement. The airways present on each slide were photographed on Leica DC300 camera and the basement membrane calculated using the line drawing tool on the Leica QWin system. The traced circumference measurement was converted into an average diameter measurement by division by π, using the rule: circumference =

2πr or πd where r is the radius and d is the diameter. This method assumes that the overall circumference of the basement membrane remains unaltered, although it may be distorted through disease, sampling and processing. This assessment was carried out for all the slides available for patients 1-10.

2.7.5 Image and tissue analysis – laminin immunohistochemistry staining

For the assessment of intensity of the laminin antibody which detected both laminins 1 and 2, Leica DC300 camera was used to take the photographs and Leica QWin image analysis was used to determine the colour intensity and hue of the staining to give an arbitrary value for the amount of laminin present. Staining was assessed in a range of locations underneath the airways, metaplastic cells in several locations including adjacent to inflammation.

2.7.6 Image acquisition

Photographs of all H&E and immunohistochemical stains of interest were taken using a Leica DC300 camera. Photographs were taken using an objective of x10, x20 or x40. The images were then adjusted to 7.42cm in width giving a scale of 0.7mm or 700µm across the picture width x10 pictures, 350µm picture width for x20 pictures and 175µm picture width for x40 pictures or alternatively 100µm is 1.14cm on x10 picture, 2.28cm on x20 or 4.56cm on x40. Figure 2.7 below shows a 1mm graticule at x10 objective.

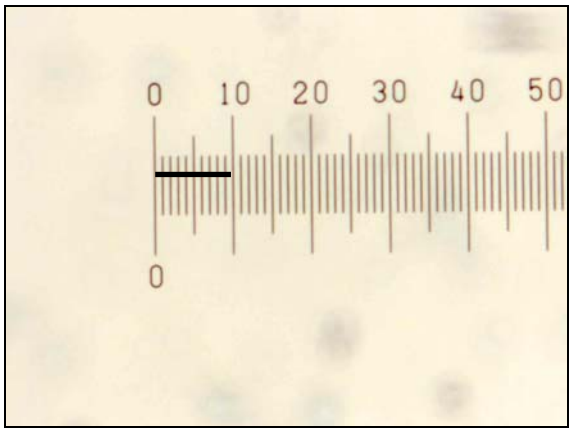


Figure 2.8 Sample Graticule (1mm)
A photomicrograph of a sample 1mm graticule at x10 objective, the width of all pictures was adjusted to 7.42cm. The black bar represents 100µm.

2.8 Statistical analysis

There was a range of statistical techniques applied to the data obtained in this study.

2.8.1 Parametric tests

The students unpaired T test was applied to the analysis of the epithelial observations for all the airways within all slides of patients 1-10. It was used to examine the difference between those airways with and without surrounding metaplastic cells. As there were several hundred values the data can be assumed to follow a normal distribution. Statistical significance is reached if the p value is lower than 0.05, 0.01 or 0.001. Z statistics were applied to examine whether proportions are statistically different. It were applied to the proportions produced for grading of fibrosis, inflammation and loss of alveolar attachments around each of the airways in all slides for patients 1-10.

2.8.2 Non parametric tests

The Mann-Whitney test was used to compare quite small data sets where there is not a normal distribution of results. This test was carried out to compare the diameter of the airways with and without metaplastic cells for each of the patients 1-10 separately. The smaller of the two U values is then taken to examine whether the value is significant or not. The calculations carried out to determine the z and two-tailed p values were calculated using an add-on package in excel. Chi squared (χ^2) statistics were used to examine a population to determine if it varies from normal distribution. This test was carried out on the values gained for mucin 5AC staining (goblet cells) and CC10 staining (Clara cells).

2.8.3 Hierarchical clustering

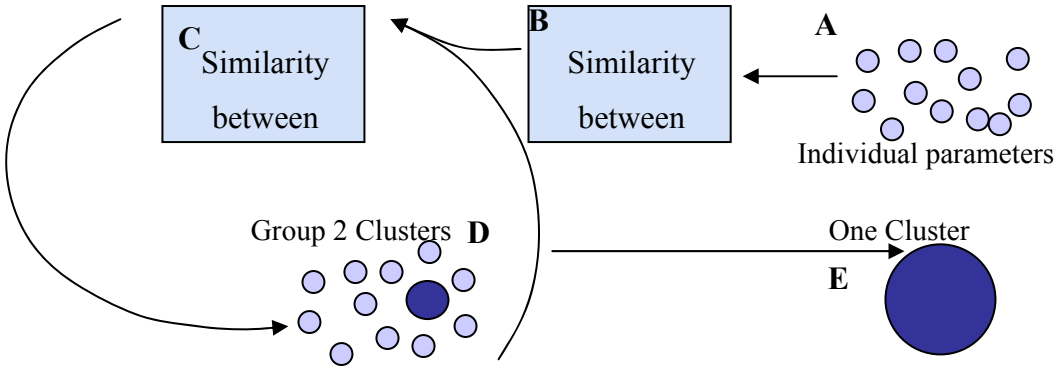


Figure 2.9 Diagram of Hierarchical Clustering
The individual parameters are entered at A and the similarity is assessed using a complex mathematical algorithm (B). Each parameter is placed into its own cluster, with the two most similar classified as one (D). The process continues until there is only one cluster (E).

The clustering carried out as was achieved using Spotfire Decision Site 10.0.

The values are pasted in as a 0 and 1 format for presence of absence of each feature for each patient. The criteria to examine as clusters are then chosen manually and the clustering is carried out with regard to feature and patient.

Hierarchical clustering works by calculating the similarity between all possible combinations of independent observations, in this case the pathological features and the distribution data for the metaplastic cell population. Each separate criterion is initially placed in a separate cluster. The two most similar clusters are then grouped together into a new cluster and the similarities recalculated. The next most similar cluster is then taken and so forth, until all probesets eventually form one large cluster. This is known as agglomerative clustering. The alternative is divisive clustering that begins as one cluster that is split as alterations occur. A diagram representing agglomerative clustering is shown in Figure 2.9.

Chapter 3. Characterisation of the epithelial metaplastic lesion in COPD

An overview of the baseline, well-documented range of pathologies seen in COPD is outlined in section 1.5.5. Despite the growing literature on the pathological features of COPD inflammatory influence has been the focus of most research. There is little published describing the diffuse epithelial response within the parenchyma of the COPD lung. The hypothesis of disease pathogenesis considered here is the lack of integration between lung compartments. The lung regions have lost communication with one another. This concept makes the diffuse epithelial lesion more important to consider.

3.1 Aims and Objectives

The aim of chapter 3 was to investigate the histological characteristics and cellular morphology of the metaplastic cells and the lesion distribution.

> What are the morphologies of the metaplastic cells? Where are the metaplastic cells localized?

The morphology and distribution of metaplastic lesions were assessed on H&E from a cohort of 50 COPD patients.

> Are the metaplastic cells associated with any histopathological changes or groups of individuals within the COPD cohort?

A set of pathological characteristics (section 2.7.1) observed in COPD were examined using a slide subset from 50 COPD patients. These characteristics were grouped using hierarchical clustering to create histopathological groups. Metaplastic cell distribution was then compared with the groups.

> Is the loss of airways associated with the metaplastic cell lesion?

Airway numbers and diameters were assessed in COPD patients 1-10 and control samples (tumour resection margins N1-N6). Numbers per cm^2 tissue were calculated and compared between COPD and control samples.

> Is the airway epithelial pathology predictive of surrounding metaplastic cells?

The pathology of the airways (defined in 2.7.2) and surroundings was noted for airways with or without surrounding metaplastic cells.

These were compared using unpaired students t-tests and z-statistics to determine any statistical differences between the population with surrounding metaplastic cells and the population without surrounding metaplastic cells.

3.2 Results of COPD Tissue and Lesion Characterisation

Histologically COPD is very variable. The samples looked at in this study were all peripheral lung, with airway diameters of 3mm or less. All aspects of the lung architecture were altered in the COPD lung.

Vasculature: The vasculature showed a remodelling reaction, mainly in the media segment of the vessel wall, however there was considerable variability within any one sample.

Parenchyma: The lung parenchyma had frequent loss of alveolar wall connectivity with some reactive fibrosis, hyalinisation and type II pneumocyte hyperplasia. There is also evidence of chronic and acute inflammation in the parenchyma. The capillary network often showed extensive with congestion, sclerosis, microthrombosis and vessel casts were all relatively common features.

Pleura: The pleura was sometimes thickened and fibrosed. This possibly related to pleural effusion or hyperinflation where physical interaction with the thoracic cage may stimulate fibrosis.

Airways: The epithelium particularly of medium and small bronchioles may exhibit epithelial loss, goblet cell hyperplasia, epithelial cell hyperplasia or metaplasia. There may be mucus plugging within the airway lumen and the submucosal may have a thickened basement membrane, muscle block invagination (bronchoconstriction), submucosal muscle hyperplasia and myolysis, peribronchiolar fibrosis or inflammation. In severe examples there may be a complete breach of the epithelial wall, airway wall and lumen become replaced with connective tissue in the more obliterative phenotypes.

Figure 3.1 shows the range of epithelium from within the airways of single COPD patient. There are very inflamed areas of lung with mucus plugs packed with inflammatory cells visible within the airway lumen. Figure 3.1A and Figure 3.1B show a reparative basal cell hyperplasia. Basal cell proliferation is seen in Figure 3.1C and Figure 3.1D with a goblet cell metaplasia. Goblet cell metaplasia may be stimulated by inflammation or damage. Figure 3.1E and Figure 3.1F show a basal cell metaplasia

whereas the upper epithelial layers retain the CK5/6 basal marker staining. This demonstrates the heterogeneity of response within COPD. Inflammation is present in all three epithelial areas perhaps suggesting that the presence of inflammation is not the only disease driver.

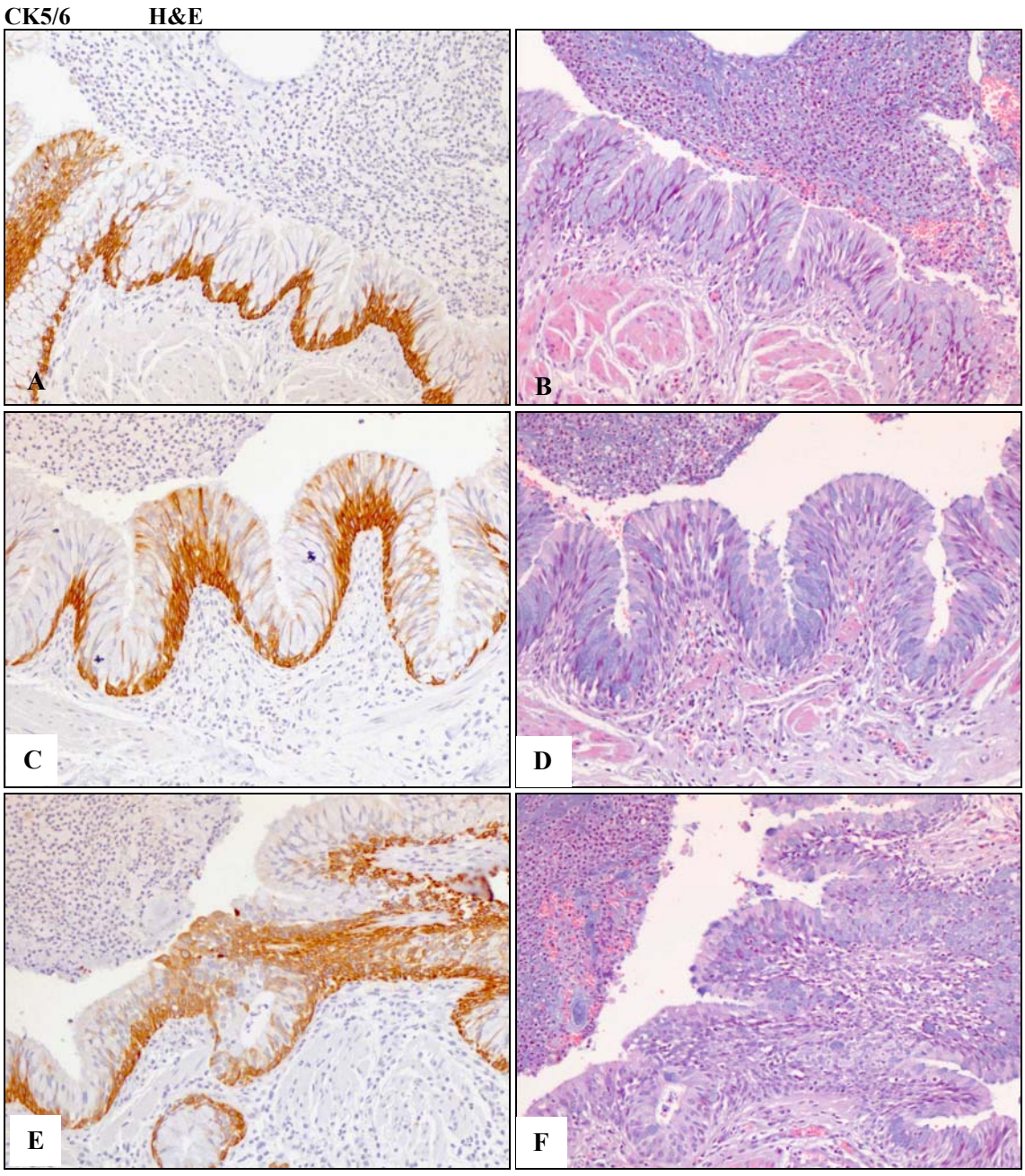


Figure 3.1 Epithelial pathology seen within the airways of COPD patients
Figure 3.1A) CK5/6 basal cell hyperplasia
Figure 3.1B) H&E basal cell hyperplasia
Figure 3.1C) CK5/6 basal cell hyperplasia with goblet cell metaplasia
Figure 3.1D) H&E basal cell hyperplasia with goblet cell metaplasia
Figure 3.1E) CK5/6 squamous metaplasia
Figure 3.1F) H&E squamous metaplasia

3.2.1 Hierarchical clustering of Pathological criteria

The pathology of each patient was overviewed by examining the slides used to investigate the metaplastic lesion (full panel). The pathologies noted are outlined in section 2.5.1. The summary of pathological changes is tabulated in the appendix section 8.2.1. This pathological information was then used for hierarchical clustering to examine which patients were similar and if groups demonstrated had consistent pathological features present. Specialist software, Spotfire, was employed to the data to compare all the patients to each other and based on the pathological criteria and location of metaplastic cells. The concept of this is described in section 2.6.4. The output of this analysis is a dendogram and a clustering heatmap where red is criteria present and green is absent, these shows the links in similarity between samples. The two patients that are most similar are grouped together. This process is repeated until all of the patients are linked in a single group. The clustering carried out here is based on pathological features identified and the frequency and location distribution of the metaplastic cell population. The dendogram (Figure 3.2) was used to divide the cohort into similar groups, a threshold line was applied to split the cohort, moving the line to the left would have created fewer groups and moving to the right would have divided the groups further. The groups produced were then further examined using the clustering heatmaps (Figure 3.3 and 3.4) to determine the features of each group.

Figure 3.3 above shows 8 patient groups. The first group contains all the control resection samples and two of the COPD patients. Pathology assessment was only carried out on selected slides chosen to represent the metaplastic lesions within the whole patient. This may mean that slides chosen have few pathological changes but are more extensive changes elsewhere in the sample. The groups were examined according to the pathological criteria present in the hierarchical clustering diagrams.

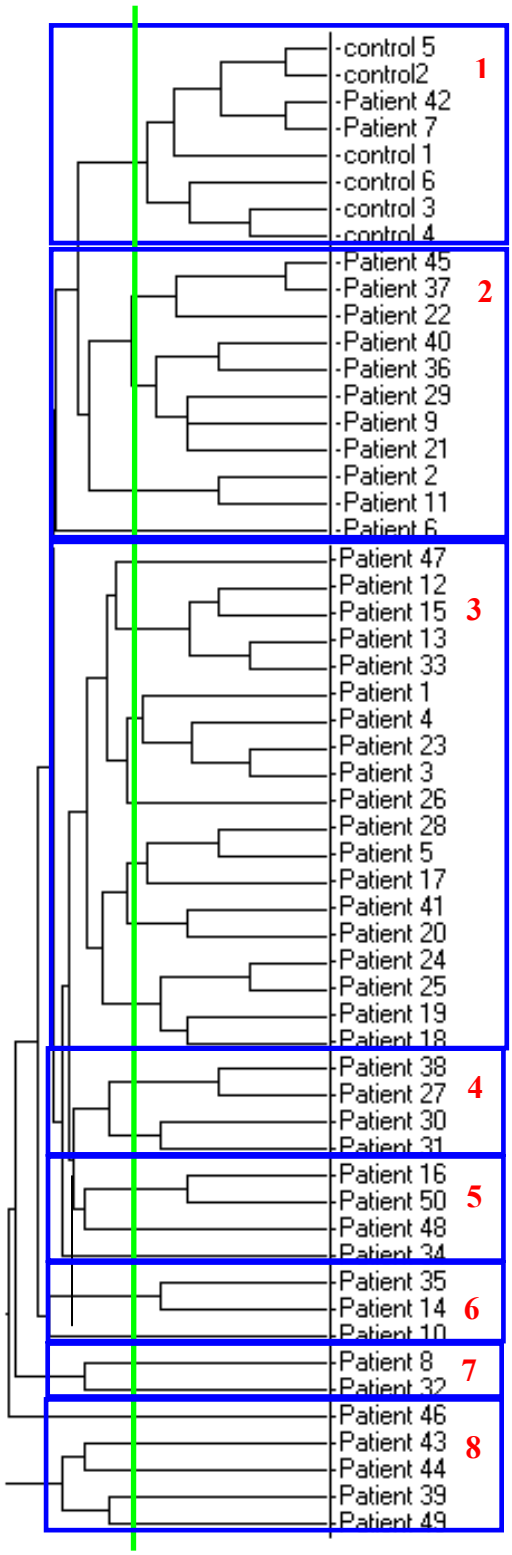


Figure 3.2 Dendogram clustering produced by pathological criteria
This dendogram was produced using a clustering of all the pathological criteria assessed. The blue boxes demonstrate the groupings achieved when the green line is considered to be a threshold point, this is an arbitrary line that can be moved left to give fewer groups that are less similar within the group or right to further sub-divide groups. The dendogram here correlates with the clustering diagram in Figures 3.3 and 3.4.

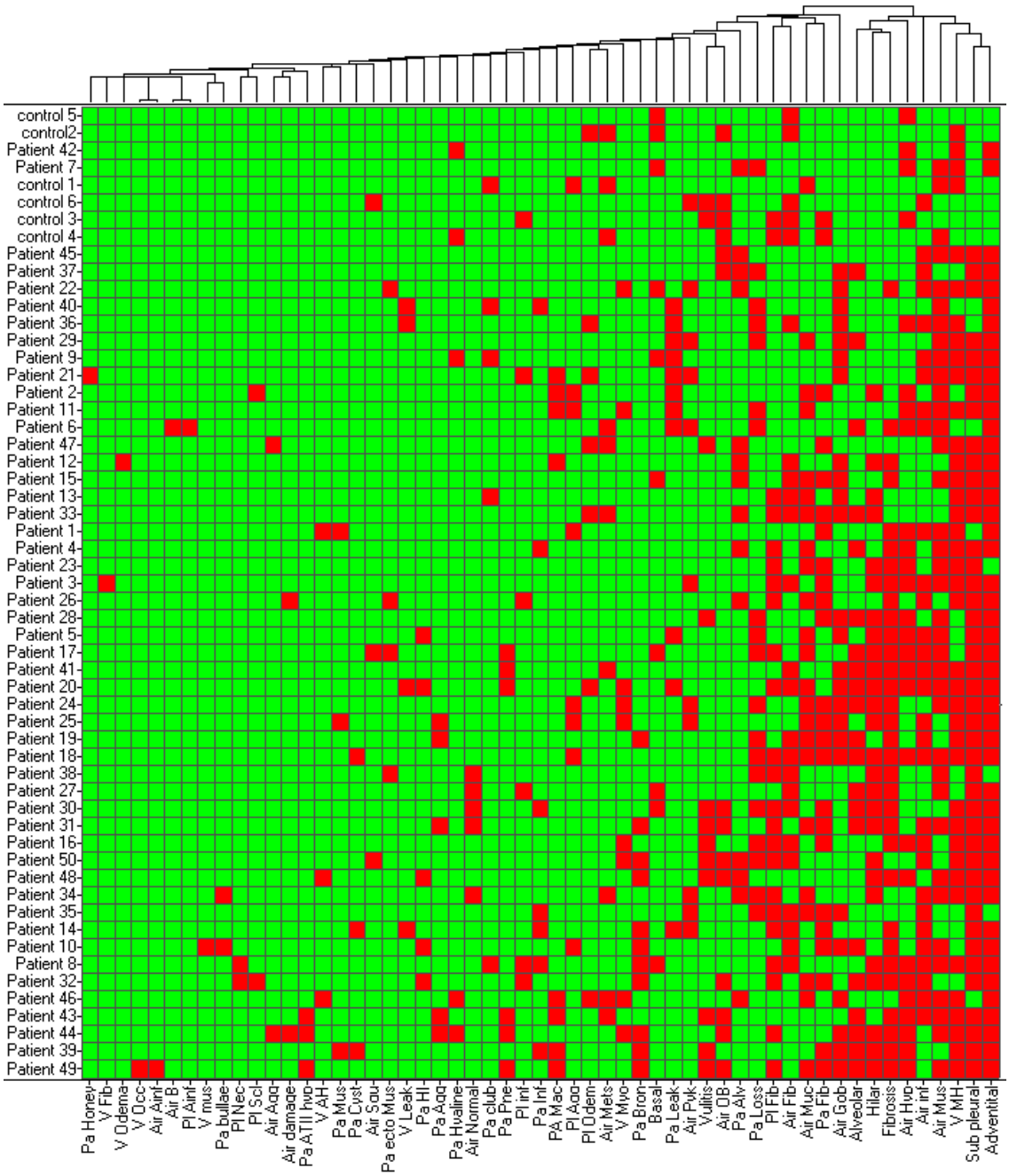


Figure 3.3 Hierarchical clustering of pathological criteria in relation to patient

The hierarchical clustering of all pathological criteria. This clustering correlates with the dendogram shown in Figure 3.2. The patients are clustered according to similarity, red boxes show a criteria present. The pathological criteria can be seen across the x-axis with the patient numbers up the y-axis.

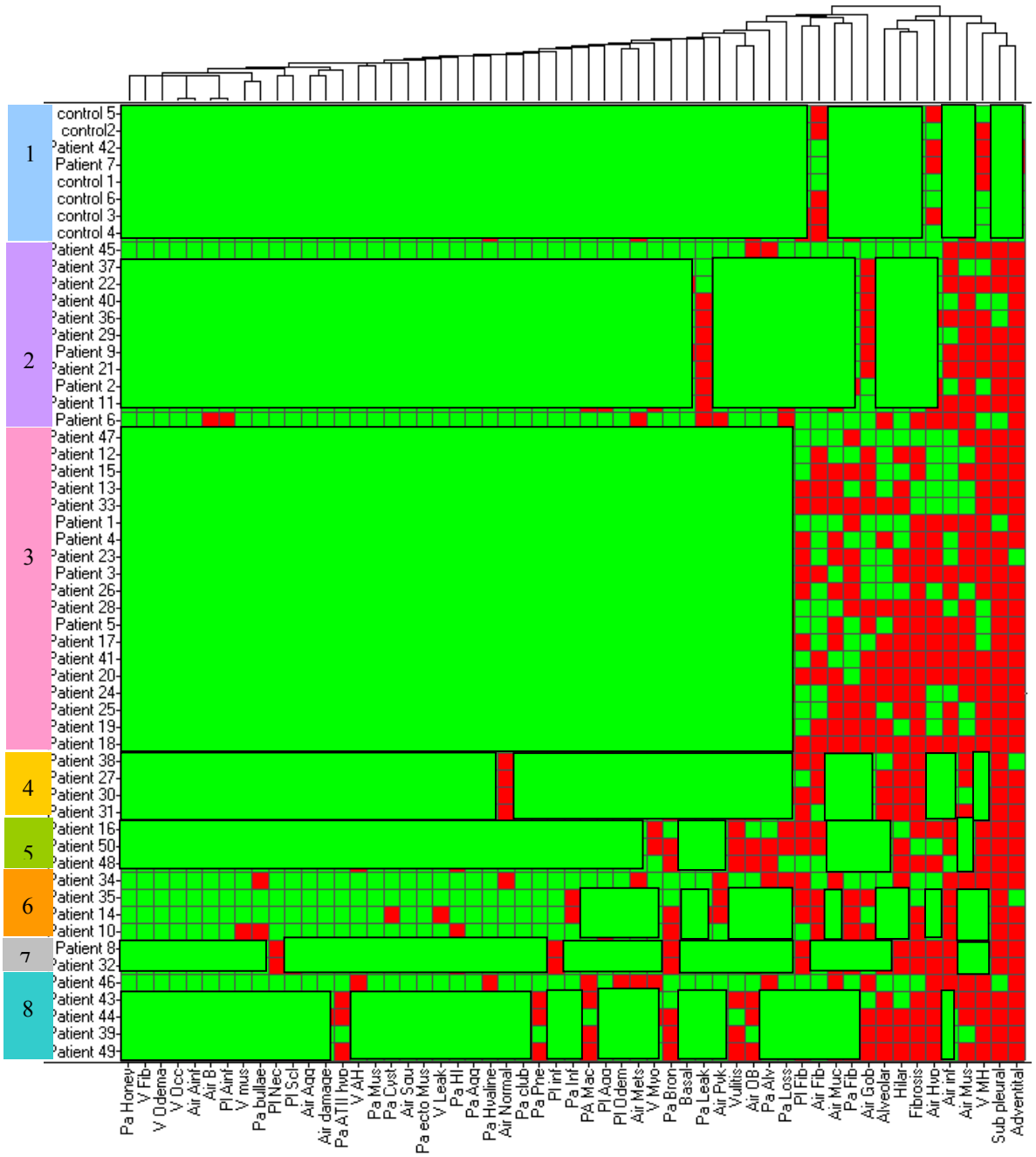


Figure 3.4 Hierarchical Cluster with groupings overlaid

The hierarchical clustering of all pathological criteria; this clustering correlates with the dendogram shown in Figure 3.2. The patients are clustered according to similarity; there are few clear groups with clustering on all criteria. Further investigation looking at separate compartments may show other associations. Patient 6 is to be included into group 2 as it is closer to this group than group 3, however there is a slightly different profile to this patient that does not cluster well with any other. Patient 34 will be included into group 6 as it clusters nearest to this group. Patient 46 will be included into group 8 as it is most similar to this group.

Figure 3.4 has blocked out in green any features present in less than half of the individuals in the group. This leaves the key criteria for each group. These are shown in Table 3.1. These groupings have had a name assigned to reflect key criteria.

Table 3.1 Key features for each group identified by hierarchical clustering

Group	Pat	Criteria	Location	Overall feature
1	C5, C2, 42, 07, C1, C6, C3, C4	Airway fibrosis Airway hyperplasia Vascular medial hypertrophy		Normal
2	45, 37, 22, 40, 36, 29, 09, 21, 02, 11, 06	Parenchymal leak Airway goblet cell hyperplasia Airway inflammation Airway mucus Vascular medial hypertrophy	Sub pleural Adventitial	Goblet cell airway changes
3	47, 12, 15, 13, 33, 01, 04, 23, 03, 26, 28, 05, 17, 41, 20, 24, 25, 19, 18	Pleural fibrosis Airway fibrosis Airway mucus Parenchymal fibrosis Airway goblet cell hyperplasia Airway hyperplasia Airway muscularisation Vascular medial hypertrophy	Alveolar Hilar Fibrosis Sub pleural Adventitial	Airway changes and fibrosis
4	38, 27, 30, 31	Normal airways Pleural fibrosis Airway fibrosis Airway muscularisation	Alveolar Hilar Fibrosis Sub pleural Adventitial	Fibrosis
5	16, 50, 48, 34	Vascular myolysis Basal cell hyperplasia Vasculitis Obliterative bronchiolitis Parenchymal alveolitis Parenchymal loss Pleural fibrosis Airway fibrosis	Hilar Fibrosis Sub pleural Adventitia	Widespread ongoing damage
6	35, 14, 10	Parenchymal inflammation Parenchymal bronchiolisation Airway pyknosis Pleural fibrosis Airway fibrosis Parenchymal fibrosis Airway goblet cell hyperplasia Airway inflammation	Fibrosis Sub pleural Adventitia	Fibrosis and inflammation
7	8, 32	Pleural necrosis Pleural inflammation Parenchymal bronchiolisation Pleural fibrosis Airway hyperplasia Airway inflammation	Hilar Fibrosis Sub pleural Adventitia	Airway and pleural change
8	43, 44, 39, 49, 46	Type II cell hyperplasia Parenchymal pneumonitis Parenchymal macrophages Parenchymal bronchiolisation Vasculitis Obliterative bronchiolitis Airway goblet cell hyperplasia Airway hyperplasia Airway muscularisation Vascular medial hypertrophy	Alveolar Hilar Fibrosis	Airway and parenchymal change

Using the pathological criteria further co-localisation of features was examined. A heatmap has been produced by looking firstly only at the criteria that occur 5 or more times in the 50 patient cohort. Figure 3.5 and 3.6 shows for all the instances of the criteria running along the top of the page the frequency of co-incidence with the criteria running down the side of the page. The results for this are presented as a percentage of the top criteria also have the criteria on the left. The results are then coloured according to frequency. Pathological changes that are only rarely observed may suggest some co-incident patterns that are not accurate due to the low incidence levels.

The criteria that co-localise in 100% of cases are shown in the Table 3.2 below.

Table 3.2 Pathological criteria that are 100% overlapping with other features

Criteria present	Incidence in cohort n=50	Comparison criteria
Vascular myolysis	9	Medial hypertrophy Adventitial metaplastic cells
Pleural odema	7	Medial hypertrophy
Parenchymal leak	11	Adventitial metaplastic cells
Parenchymal alveolitis	14	Adventitial metaplastic cells
Parenchymal pneumonitis	6	Airway muscularisation Airway hyperplasia Adventitial metaplastic cells Sub pleural metaplastic cells Fibrotic metaplastic cells Alveolar metaplastic cells
Parenchymal bronchiolisation	11	Adventitial metaplastic cells Sub pleural metaplastic cells
Parenchymal macrophages	8	Adventitial metaplastic cells
Alveolar metaplastic cells	21	Adventitial metaplastic cells

		1	2	3	4	5	6	7	8	9	10	11	12	15	14	15	16	17
Medial hypertrophy	1	0	100	75	62.5	73.0	70	64.3	78.3	76	63.6	70.4	80	70	69.6	100	75	50
Myolysis	2	23.08	0	16.7	12.5	16.2	23.3	14.3	21.7	24	27.3	18.5	10	10	17.4	28.6	37.5	0
vasculitis	3	23.08	22.22	0	20.8	16.2	16.7	57.1	8.7	12	9.1	25.9	20	10	26.1	14.3	0	16.7
Air Fibrosis	4	38.5	33.3	41.7	0	29.7	43.3	42.9	30.4	44	36.4	33.3	40	60	60.9	57.1	25	50
Air muscularisation	5	69.2	66.7	50	45.8	0	66.7	50	69.6	68	63.6	77.8	80	70	56.5	71.4	87.5	50
Air inflammation	6	53.9	77.8	41.7	54.2	54.1	0	50	56.5	56	81.8	55.6	50	40	56.5	57.1	62.5	66.7
Air Oblit bron	7	23.1	22.2	66.7	25	18.9	23.3	0	13	12	9. 1	22.2	30	20	34. 8	14.3	0	33.3
Air Mucus	8	46.2	55.6	16.7	29.2	43.2	43.3	21.4	0	52	45.5	40.7	40	20	56.5	42.9	75	33.3
Goblet hyperplasia	9	48.7	66.7	25	45.8	46	46.7	21.4	56.5	0	54.6	37	30	30	30.4	71.4	50	16.7
Air Pyknosis	10	18	33.3	8.3	16.7	18.9	30	7.1	21.7	24	0	7.4	20	10	17.4	14.3	25	16.7
Air Hyperplasia	11	48.7	55.6	58.3	37.5	56.7	50	42.9	47.8	40	18.2	0	40	40	52.2	42.9	50	50
Air Metaplasia	12	20.5	11.1	16.7	16.7	21.6	16.7	21.4	17.4	12	18.2	14.8	0	10	13	57.1	12.5	0
Basal cell hypertrophy	13	18	11.1	8.3	25	18.9	13.3	14.3	8.7	12	9.1	14.8	10	0	13	14.3	0	33.3
Pl Fibrosis	14	43	44. 4	50	58.3	35.1	43.3	57.1	56.5	28	36.4	44.4	30	30	0	28.6	12.5	66.7
Pl Odema	15	18	22.2	8.3	16. 7	13.5	13.3	7.1	13	20	9.1	11.1	40	10	8.7	0	0	16.7
Pl Aggregates	16	15.4	33.3	0	8.3	18.9	16.7	0	26.1	16	18.2	14.8	10	0	4.3	0	0	0
Pl Inflammation	17	7.7	0	8. 3	12.5	8.1	13.3	14.3	8.7	4	9. 1	11.1	0	20	17.4	14.3	0	0
Pa Fibrosis	18	41	33.3	66.7	50	35.1	40	42.9	56.5	44	45.5	40.7	30	20	60.9	28.6	75	50
Pa Alveolar Loss	19	28.2	44.4	33.3	33.3	35.1	40	21.4	39.1	40	45.5	33.3	20	30	34.8	14.3	37.5	0
Pa Alveolar Leak	20	15.4	22.2	0	12.5	27	26.7	0	21.7	28	36.4	22.2	10	10	8.7	42.9	25	16.7
Pa Alveolitis	21	33.3	33.3	25	16.7	24.3	23.3	28.6	26.1	24	18.2	14.8	40	30	21.7	42.9	0	16.7
Pa Pneumonitis	22	12.8	22.2	16.7	8.3	16.2	13.3	21.4	13	16	0	22.2	20	10	17.4	14.3	0	0
Pa Bronchiolisation	23	20.5	22.2	41.7	20.8	16.2	23.3	42.9	17.4	20	9. 1	22.2	0	10	30.4	0	12.5	33.3
PA Macrophage	24	18	22.2	25	4.2	16.2	13.3	14.3	17.4	20	9. 1	22.2	20	0	4.3	28.6	25	16.7
Pa Inflammation	25	10.3	0	16. 7	16.7	8.1	10	7.1	8.7	12	18.2	11.1	0	20	21.7	0	0	16.7
Adventitial mets	26	92.3	100	83.3	70.8	89.2	93.3	71.4	87	96	81.8	88.9	70	80	78.3	85.7	87.5	83.3
Sub pleural mets	27	82.1	88.9	83.3	75	78.4	83.3	71.4	91.3	88	81.8	70.4	50	70	91.3	57.1	75	83.3
Hilar mets	28	48.7	55.6	58.3	50	48.7	40	50	60.9	52	36.4	55.7	30	40	69.6	28.6	50	50
Fibrosis mets	29	59	66.7	66.7	58.3	62.2	63.3	50	60.9	60	54.6	70.4	30	60	69.6	3	62.5	66.7
Alveolar mets	30	38.5	44.4	50	37.5	43.2	40	50	52.2	56	27.3	48.2	40	50	47.8	42.9	37.5	33.3

Figure 3.5 Heatmap of pathological criteria co-incidence (n>5)

The values 1-30 in the second column represent the pathological criteria listed in column one. These values are also used across the top of the heatmap to show the same pathological criteria. Colour darkens as the frequency increases, 0-20%, 21-40%, 41-60%, 61-80%, 81-99% and 100%.

		18	19	20	21	22	23	24	25	26	27	28	29	30
Medial hypertrophy	1	66.7	57.9	54.6	92.9	83.3	72.7	87.5	57.1	76.6	74.4	73.1	71.9	66.7
Myolysis	2	12.5	21.1	18.2	21.4	33.3	18.2	25	0	19.2	18.6	19.2	18.8	14.3
Vasculitis	3	33.3	21.1	0	21.4	33.3	45.5	37.5	28.6	21.3	23.3	26.9	25	28.6
Air Fibrosis	4	50	42.1	27.3	28.6	33.3	45.5	12.5	57.1	36.2	41.9	46.2	43.8	38.1
Air muscularisation	5	54.2	68.4	90.9	64.3	100	54.6	75	42.9	70.2	67.4	69.2	71.9	71.4
Air inflammation	6	50	63.2	72.73	50	66.7	63.6	50	42.9	59.6	58.1	46.2	59.4	52.4
Air Oblit bron	7	25	15.8	0	28.6	50	54.6	25	14.3	21.3	23.3	26.9	21.9	33.3
Air Mucus	8	54.2	47.4	45.5	42.9	50	36.4	50	28.6	42.6	48.8	53.9	43.8	52.4
Goblet hyperplasia	9	45.8	52.6	63.6	42.9	66.7	45.5	62.5	42.9	51.1	51.2	50	46.9	61.9
Air Pyknosis	10	20.8	26.3	36.4	14.3	0	9.1	12.5	28.6	19.2	20.9	15.4	18.8	14.3
Air Hyperplasia	11	45.8	47.4	54.6	28.6	100	54.6	75	42.9	51.1	44.2	57.7	59.4	57.1
Air Metaplasia	12	12.5	10.5	9.1	28.6	33.3	0	25	0	14.9	11.6	11.5	9.4	19
Basal cell hypertrophy	13	8.3	15.8	9.1	21.4	16.7	9.1	0	28.6	17	16.3	15.4	18.8	14.3
Pl Fibrosis	14	58.3	42.1	18.2	35.7	66.7	63.6	12.5	71.4	38.3	48.8	61.5	50	47.6
Pl Odema	15	8.3	5.3	27.3	21.4	16.7	0	25	0	12.8	9.3	7.7	3.1	9.5
Pl Aggregates	16	25	15.8	18.2	0	0	9.1	25	0	14.9	14	15.4	15.6	14.3
Pl Inflammation	17	12.5	0	9.1	7.1	0	18.2	12.5	14.3	10.6	11.6	11.5	12.5	9.5
Pa Fibrosis	18	0	36.8	18.2	28.6	16.7	63.6	37.5	57.1	42.6	48.8	50	56.3	52.4
Pa Alveolar Loss	19	29.2	0	54.6	28.6	16.7	18.2	12.5	42.9	36.2	34.9	34.6	31.3	42.9
Pa Alveolar Leak	20	8.3	31.6	0	0	16.7	9.1	37.5	28.6	23.4	18.6	11.5	12.5	14.3
Pa Alveolitis	21	16.7	21.1	0	0	0	18.2	25	14.3	29.8	27.9	19.2	18.8	14.3
Pa Pneumonitis	22	4.2	5.3	9.1	0	0	18.2	25	0	12.8	14	19.2	18.8	28.6
Pa Bronchiolisation	23	29.2	10.5	9.1	14.3	33.3	0	25	42.9	23.4	25.6	30.8	31.3	33.3
PA Macrophage	24	12.5	5.3	27.3	14.3	33.3	18.2	0	14.3	17	16.3	15.4	12.5	14.3
Pa Inflammation	25	16.7	15.8	18.2	7.1	0	27.3	12.5	0	12.8	14	11.5	15.6	14.3
Adventitial mets	26	83.3	89.5	100	100	100	100	100	85.7	0	93	92.3	93.8	100
Sub pleural mets	27	87.5	79	72.7	85.7	100	100	87.5	85.7	85.1	0	92.3	93.8	95.2
Hilar mets	28	54.2	47.4	27.3	35.7	83.3	72.7	50	42.9	51.1	60.5	0	65.6	66.7
Fibrosis mets	29	75	52.6	36.4	42.9	100	90.9	50	71.4	63.8	69.8	80.8	0	85.7
Alveolar mets	30	45.8	47.4	36.4	21.4	100	63.6	37.5	42.9	66	48.9	57.7	59.4	0

Figure 3.6 Heatmap of pathological criteria co-incidence (n>5) continued

The values 1-30 in the second column represent the pathological criteria listed in column one. These values are also used across the top of the heatmap to show the same pathological criteria. Colour darkens as the frequency increases, 0-20%, 21-40%, 41-60%, 61-80%, 81-99% and 100%.

3.2.2 Analysis of COPD metaplastic lesion

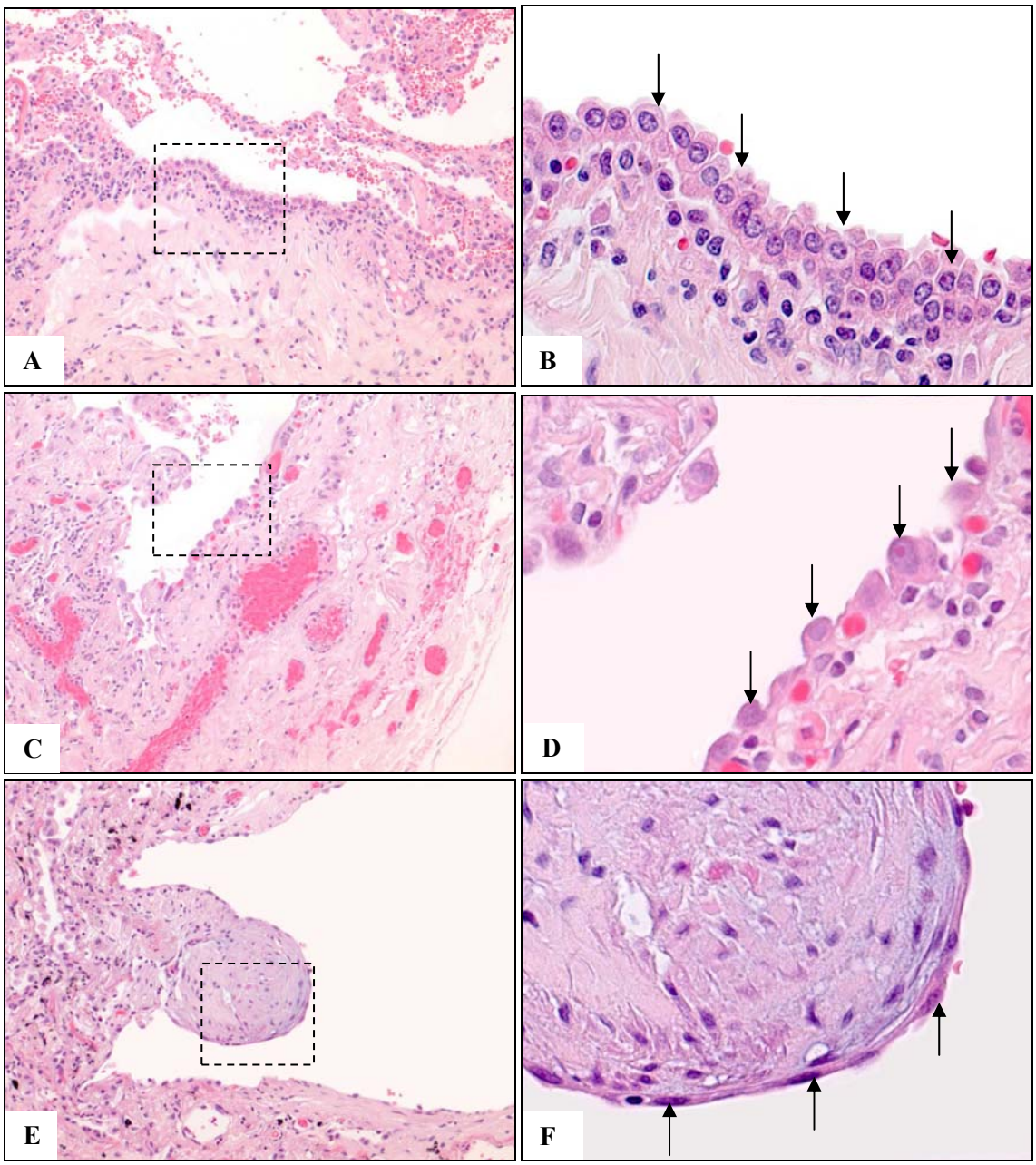


Figure 3.7 Metaplastic cell phenotypes

Figure 3.7A) x10 objective. Uniform metaplastic cells lining the external adventitia of a vessel
Figure 3.7B) x40 objective. Area in box in Figure 3.7A. Uniform metaplastic cells lining the vascular external adventitia
Figure 3.7C) x10 objective. Macrophage-like metaplastic cells on sub pleural fibrosis
Figure 3.7D) x40 objective. Area in box in Figure 3.7C. Macrophage-like metaplastic cells on sub pleural fibrosis
Figure 3.7E) x10 objective. Squamous metaplastic cells surrounding an area of parenchymal fibrosis
Figure 3.7F) x40 objective. Area in box in Figure 3.7E. Squamous metaplastic cells surrounding an area of parenchymal fibrosis

Diffuse parenchymal metaplastic cells were identified in all 50 COPD samples and in 3/6 resection control samples. These cells have a simple epithelial morphology when observed on H&E. These cells are seen in strings running along the surface of the tissue,

usually in a single layer, but occasionally forming multiple layers. The cells have three distinct morphological types; Figure 3.7 shows these morphologies. These phenotypes are,

Uniform: the cells are all round a similar size and have a prominent nucleolus

Macrophage-like: cells very large with variable shape and large nucleus

Squamous: the cells are very flattened and quite elongated

Adventitial – Cells may be found on the external adventitia of vessels, airways or the adventitia that surrounds the broncho-vascular unit. The cells are lining the external surface, adhered to the solid matrix of the airway or vessel structure. The tissue used in this study is mainly peripheral as a result of the nature of LVRS surgery, but these cells have been observed on airways of up to 3mm in diameter which have some cartilage surrounding them and out to the periphery as far as the terminal transitional airways (Figure 3.8A).

Sub pleural – The metaplastic cells sit on the internal surface of the pleura. Pleura may be a very thin layer or a thick area of tissue if fibrosed (Figure 3.8B).

Hilar – There are often fibrotic ingrowths of pleura in COPD patients. This is a response to the multi-directional stretching as gas trapping and tissue loss occurs. These are called hilar regions. These areas have a similar composition to the pleura, largely acellular and sometimes ultimately associating with the adventitia of bronchovascular units. Metaplastic cells are found on the outside of this tissue (Figure 3.8C).

Fibrosis – Cells may be found on the surface of blocks of fibrotic tissue. These areas of fibrosis are largely acellular and may or may not be associated with structures such as vessels or airways within the lung (Figure 3.8D).

Alveolar – Very occasionally there may be colonising cells within the alveolar bed with no apparent link to any other structure. In these cases the alveolar septae are usually thickened and fibrosed. These are usually isolated cases with some alveolar loss and some thickening (Figure 3.8E, F).

All the above mentioned lesions usually appear in obvious strings of cells, where occasionally a more diffuse lesion has the cells arranged in clusters as opposed to strings. These are usually found at the vascular pediment where the vessel attaches to the alveolar bed.

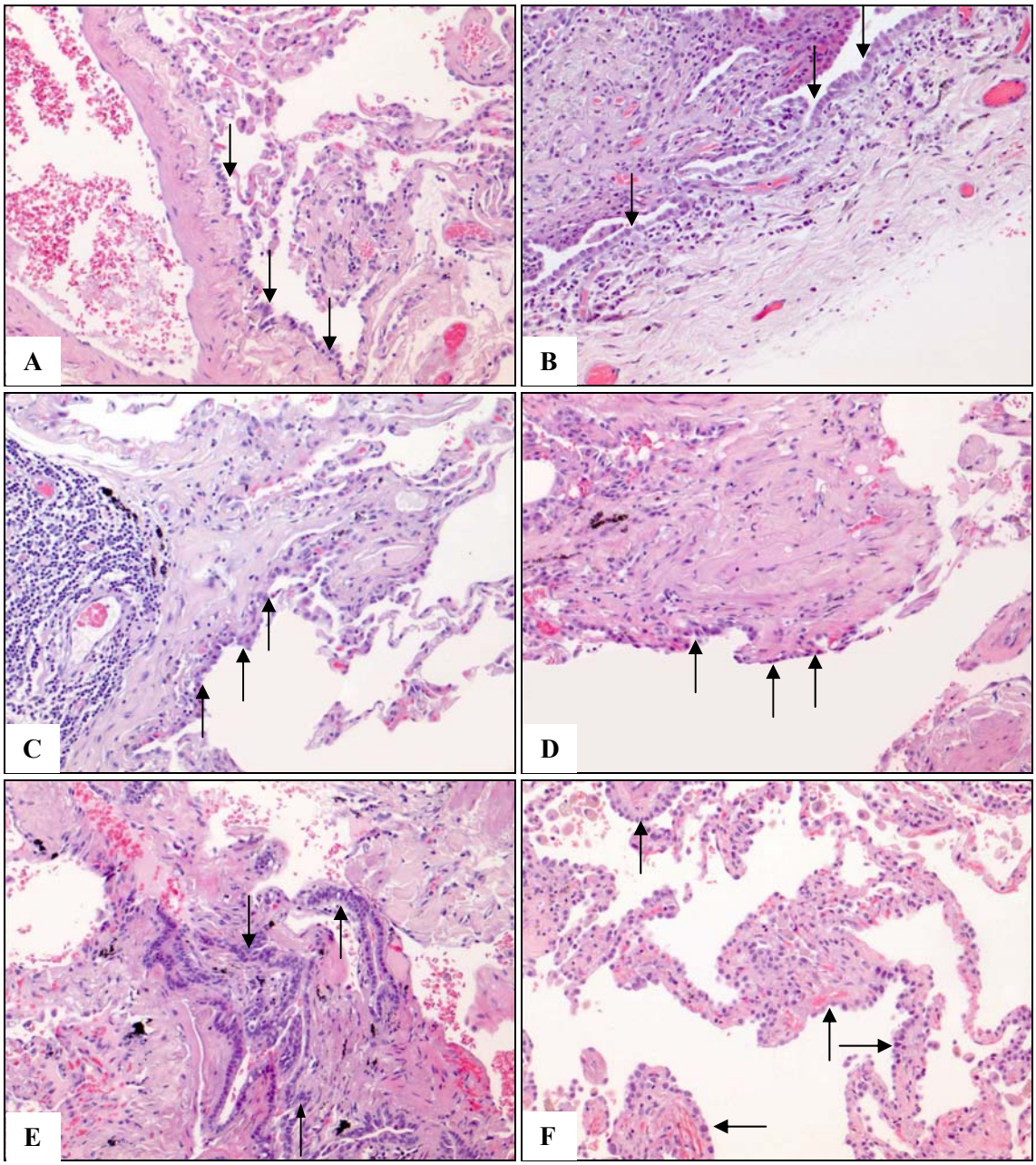


Figure 3.8 Location of metaplastic cells

Figure 3.8A) Adventitial lining cells sitting outside an airway.
Figure 3.8B) Sub pleural lining cells adhering to the under lying surface of the pleura.
Figure 3.8C) Hilar lining cells, these are pleural invaginations projecting into the parenchyma of the lung.
Figure 3.8D) Fibrosis seems to have metaplastic cells coating the external surface of the lesion.
Figure 3.8E) Alveolar lining cells, these cells are infrequent and here may represent the escaped remnants of an airway.
Figure 3.8F) Alveolar lining cells, more classic example.
Figures 3.8 A-E are examples all chosen from one slide to demonstrate that these localisations may all be found within a small area of tissue.

Other lesions occurring in the samples that must be considered are the damaged remains of obliterated airways. This damage is associated with a vessel and shows areas of intact basement membrane but often proliferate onto the surrounding surfaces. Bronchiolisation has also been observed. This is inappropriate deposition of ciliated

epithelium within the parenchyma of the lung. It occurs in normal human lung but is more prevalent in COPD.

The location of the metaplastic cells was noted for each patient. The percentage of patients that had metaplastic cells in each location is shown in Figure 3.9 below.

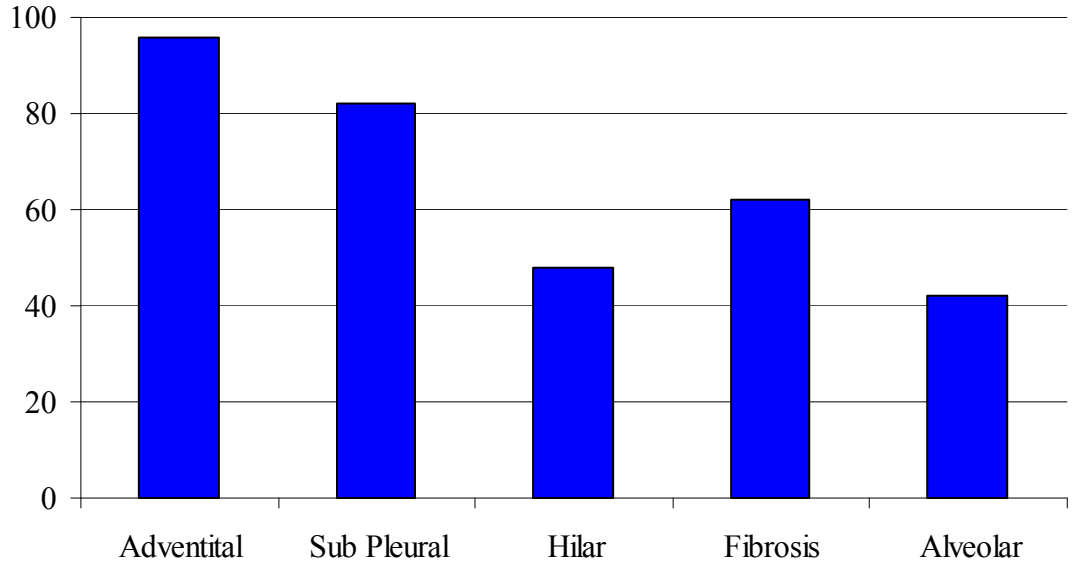


Figure 3.9 Graph showing location distribution of colonising metaplastic cells
The overall proportion of patients that have metaplastic cells in each of the five locations. Adventitial (48/50), sub pleural (41/50), hilar (24/50), fibrosis (31/50) and alveolar (21/50).

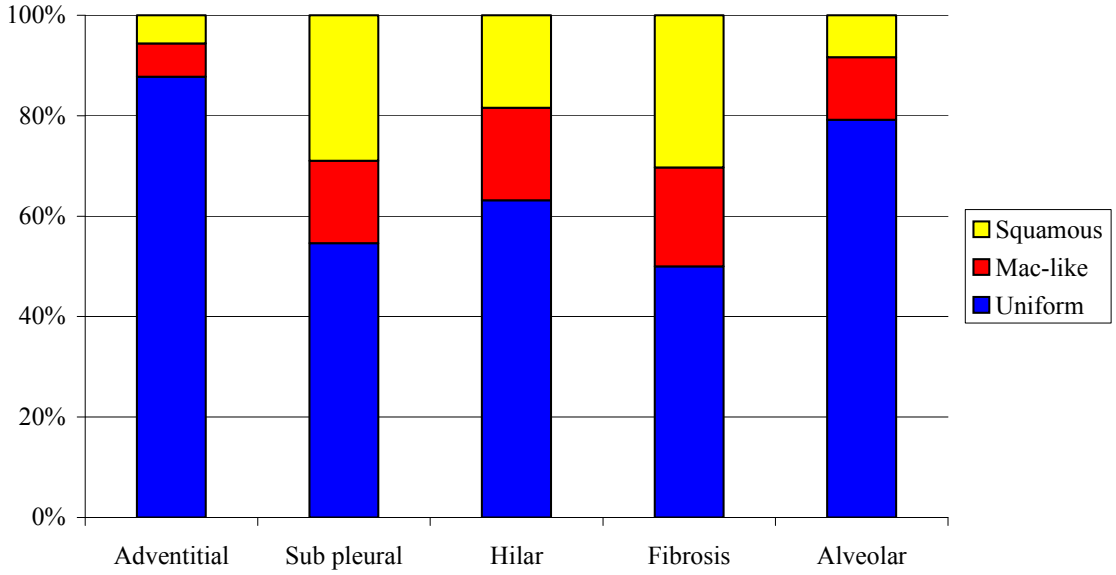


Figure 3.10 Graph showing morphology and location distribution of colonising metaplastic cells
The proportion of metaplastic cells with each of the three cell phenotypes, this suggests that there are more squamous cells seen sitting on fibrosis and sub pleural surfaces than in other locations.

Six of the seven samples that only had metaplastic cells in one location type were samples with adventitial cells. This location had examples of cells present in 48/50

patients and adventitia was the most frequently occurring location. This may suggest that this location is more prone to developing metaplastic cells. It is difficult to consider the pattern of spread from one location to another as the incidence of some locations is altered by the anatomical location for pleura and disease severity for hilar and fibrosis areas.

Figure 3.10 shows the distribution of the three morphological phenotypes of metaplastic cell. The proportions are shown for each anatomical location. 50% or more cells are of a uniform morphology in all locations. The sub pleural and fibrosis locations showed the greatest proportion of squamous morphology metaplastic cells, around 30%. Macrophage-like morphology was the most infrequent, ranging from 6.6%-19.7%.

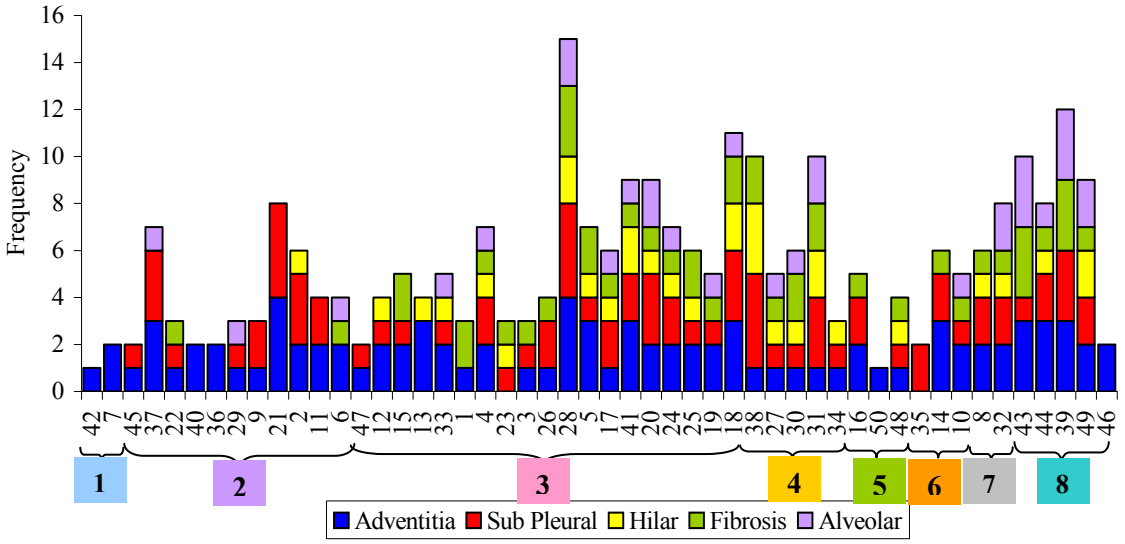


Figure 3.11 Frequency & distribution of metaplastic cells within representative blocks

The location of metaplastic cells by colour and the frequency at which these locations are colonised for each patient when representative blocks were examined. These blocks were chosen to demonstrate the metaplastic cells within each patient, the best and most widespread examples were picked. The numbers and brackets underneath show the pathological groups identified by hierarchical clustering

Figure 3.11 shows that there are no examples of alveolar colonisation until at least two other areas are showing metaplastic cells. Alveolar spread is a much less frequent lesion that may occur later in colonisation progression. There is only one example patient 13 that has hilar colonisation without the sub pleura also having cells. The hilar projections are ingrowths from the pleura so it may be expected that if there are projections present then these will not be colonised unless the sub pleura is also colonised. Fibrosis and hilar may be expected to be less frequent as these locations may not exist within a sample. Both phenomena arise as part of COPD disease changes and damage. The two patients that are grouped with the control patients in group 1 (normal) are patients 7 and

42, both these patients show only adventitial metaplastic cells. Generally as the number of locations with metaplastic cells increases the overall number of lesions increases.

3.2.3 Airway analysis of patients 1 to 10 – epithelial characteristics

All slides for COPD patients 1-10 were examined for the presence or absence of surrounding metaplastic cells. Epithelial and airway characteristics were also examined. The criteria and example grading are outline in section 2.5.3.

The presence of epithelial hypertrophy, squamous cells and basal cell hyperplasia indicative of repair were all noted. These did not show significant differences in frequency between airways with or without the presence of metaplastic cells around the external adventitia of the airways. Although normal, epithelial cell hyperplasia, goblet cell hyperplasia and goblet cell hypertrophy criteria were all statistically significant between the airways with and without surrounding metaplastic cells, the overall frequency of occurrence was nowhere near 100%. The frequency for airways with and without metaplastic cells is shown in Figure 3.12. If there was a specific airway change necessary for the presence of metaplastic cells around the outside, the difference between airways with and without cells may be expected to be much greater. Table 3.3 shows all the epithelial criteria examined with the frequency of presentation for the airways with and without. These frequencies were then compared using students unpaired T test.

Table 3.3 Epithelial characteristic proportions for airways with & without metaplastic cells

* = p value of <0.05, ** = p value of <0.01 and *** = p value of <0.001

Epithelial characteristic	Frequency with metaplasia	Frequency without metaplasia
Hyperplasia	21% *	14%
Hypertrophy	37%	42%
Squamous	2%	1%
Reparative	6%	7%
Normal	4% ***	11%
Damage	9%	10%
Goblet Hyperplasia	9% **	2%
Goblet Hypertrophy	14% ***	5%

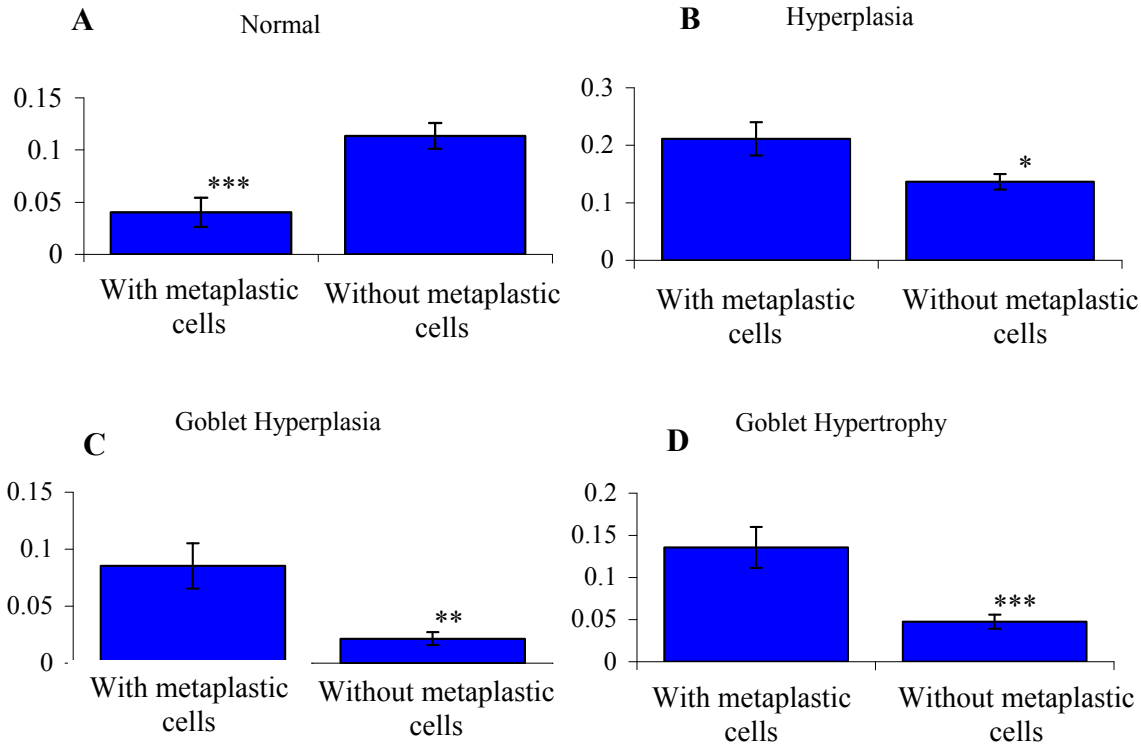


Figure 3.12 Airway criteria showing significant differences between airways with & without metaplastic cells

The percentage of airways with and without metaplastic cells surrounding the adventitia Figure 3.12A represent where normal epithelium was present, Figure 3.12B with hyperplastic epithelium present, Figure 3.12C with goblet cell hyperplasia present and Figure 3.12D with goblet cell hypertrophy present. * p=0.05, **p=0.01, ***p=0.001.

Details about inflammation, the amount of fibrosis and the loss of attachments surrounding the airways were also noted. Inflammation and fibrosis were assigned a grade of 0-3 with 3 having the most inflammation or fibrosis present. The airway attachments were given a grading of 0-4 where 0 had all the alveolar attachments attached and 4 was a virtually isolated airway with no intact alveolar attachments.

Z-statistics were then carried out to determine whether any of these proportions were significantly different between the with and without populations. If the z value is greater than 1.96 or less than –1.96 the value is significant to 95%, if values are greater than 3.27 or less than –3.27 the value is significant to 99.9%. See section 2.6.3 for further explanation. The proportions for each grading are shown in Figures 3.13, 3.14 and 3.15 below. The z statistic values are listed in the legend of each figure.

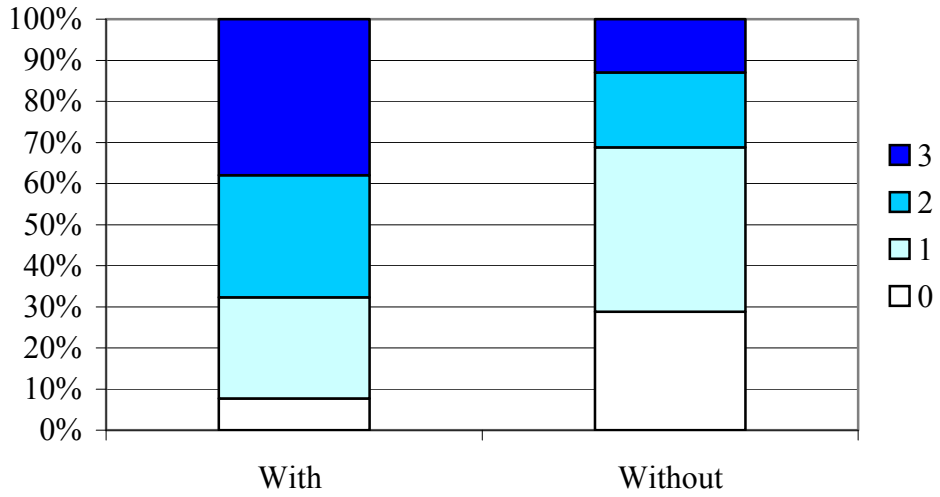


Figure 3.13 Inflammation grade of airways with & without metaplastic cells

The proportion of airways achieving a grade of 0-3 inflammation within and around the airways. (0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation and 3 = severe inflammation.) z-statistic values were 0 = -6.13,. 1 = -4.41, 2 = 4.04, 3 = 8.00 (3.27 represents 99.9% confidence)

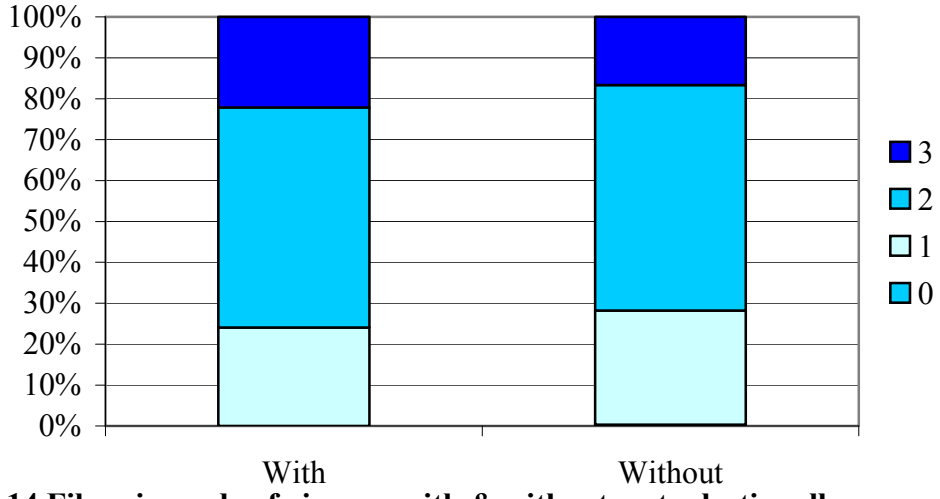


Figure 3.14 Fibrosis grade of airways with & without metaplastic cells

The proportion of airways with and without metaplastic cells surrounding the adventitia with regard to amount of fibrosis graded 0-3. (0 = no fibrosis, 1 = mild fibrosis, 2 = moderate fibrosis, 3 = severe fibrosis.) z statistic values for these grades were 0 = -0.55, 1 = -0.19, 2 = -0.94. 3 = 1.47.

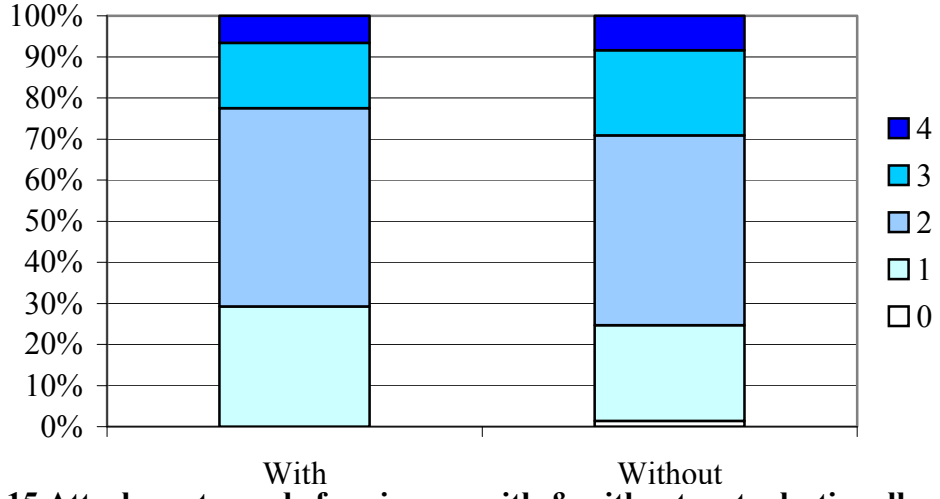


Figure 3.15 Attachments grade for airways with & without metaplastic cells

The proportion of airways with and without metaplastic cells with regard to the amount of alveolar attachments graded 0-4. (0 = intact, 1 = mild loss, 2 = moderate loss, 3 = severe loss, 4 = none remaining.) z statistic values for these grades were 0 = -1.57, 1 = 1.86, 2 = 0.39, 3 = -1.83, 4 = -0.47.

The proportion of grade 1 fibrosis in airways with metaplastic cells was compared to the proportion of grade 1 fibrosis in airways without metaplastic cells. If the z values are <± 1.96 then the proportions of the same grade of those with and without are statistically significantly different. When examining the inflammation grades, all levels were significantly different between those with surrounding metaplastic cells and those without to 99.9% confidence. Those with metaplastic cells had fewer grade 0 and grade 1 and more grade 2 and grade 3. This suggests that the presence of inflammation in and around the airways is predictive of metaplastic cells surrounding the airways.

3.2.4 Airway analysis diameter and frequency of airways within COPD

The airways of all the slides available for patients 1-10 were examined. The diameter was calculated and it was noted whether metaplastic cells surrounded the airway or not. The number of airways per centimetre squared was calculated for COPD patients 1-10 and the control samples. The airway density in the COPD patients were significantly different to the numbers seen in the airways

Figure 3.16 below shows the overall average airway density for COPD patients 1-10 vs the 6 control samples. The number of airways was assessed in anatomically matched areas. This shows the marked difference indicative of airway loss in the COPD cohort. This demonstration makes the previous findings more important as there are airway associations with the presence of metaplastic cells even though there is significant airway loss within the tissue.

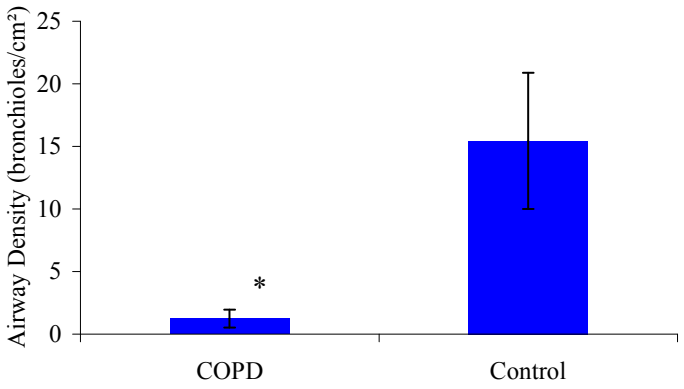


Figure 3.16 Overview of airway density in COPD & control tissue

The overall airway density for COPD patients vs. control samples, an unpaired students T test showed that this was a statistically significant difference. * Denotes that the p value was <0.01.

The proportion of airways with and without surrounding metaplastic cells is shown below in Figure 3.17 for each of patients 1-10. The range of airways with metaplastic cells was as low as 7.14% for patient 10 and as high as 41.44% for patient 6. It shows that there are different proportions of airways with metaplastic cells surrounding them in different patients. There may be several reasons for the differences in proportions between patients. The diameter of the airways within the sample may determine the number with a metaplastic cell surround. The level of inflammation or COPD change may influence the development of metaplastic cells.

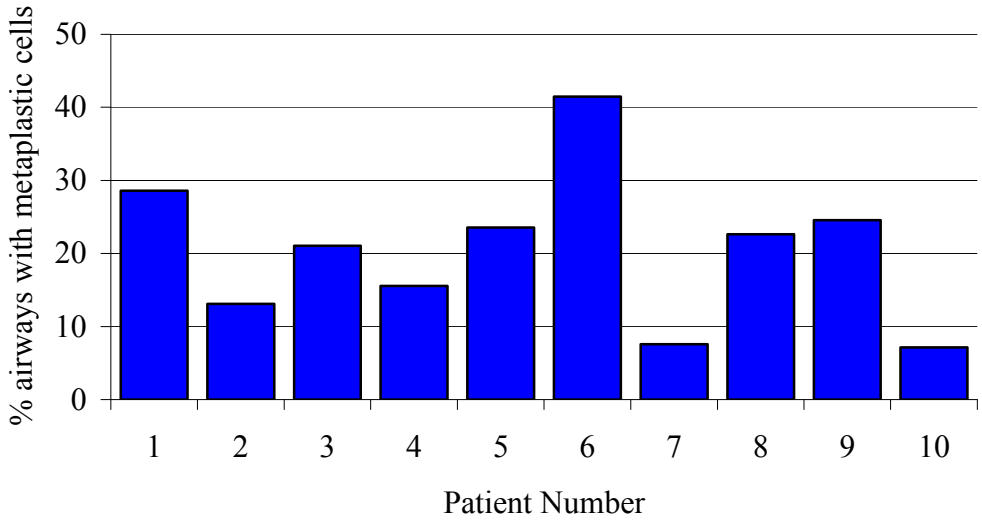


Figure 3.17 The proportion of airways with surrounding metaplastic cells percentage to total per patient
The proportion of airways within the whole sample for each patient that has metaplastic cells around the adventitia of the airways. This ranges from over 40% in patient 6 to around 15% in patient 10.

The diameter of the airways was plotted, dividing out those with and without metaplastic cells to give an indication about whether the airway size predicted the presence of metaplastic cells. The median values were calculated for those with and without for each patient, Mann-Whitney statistics were applied to calculate whether the diameters were statistically significant between those airways with and those without surrounding metaplastic cells.

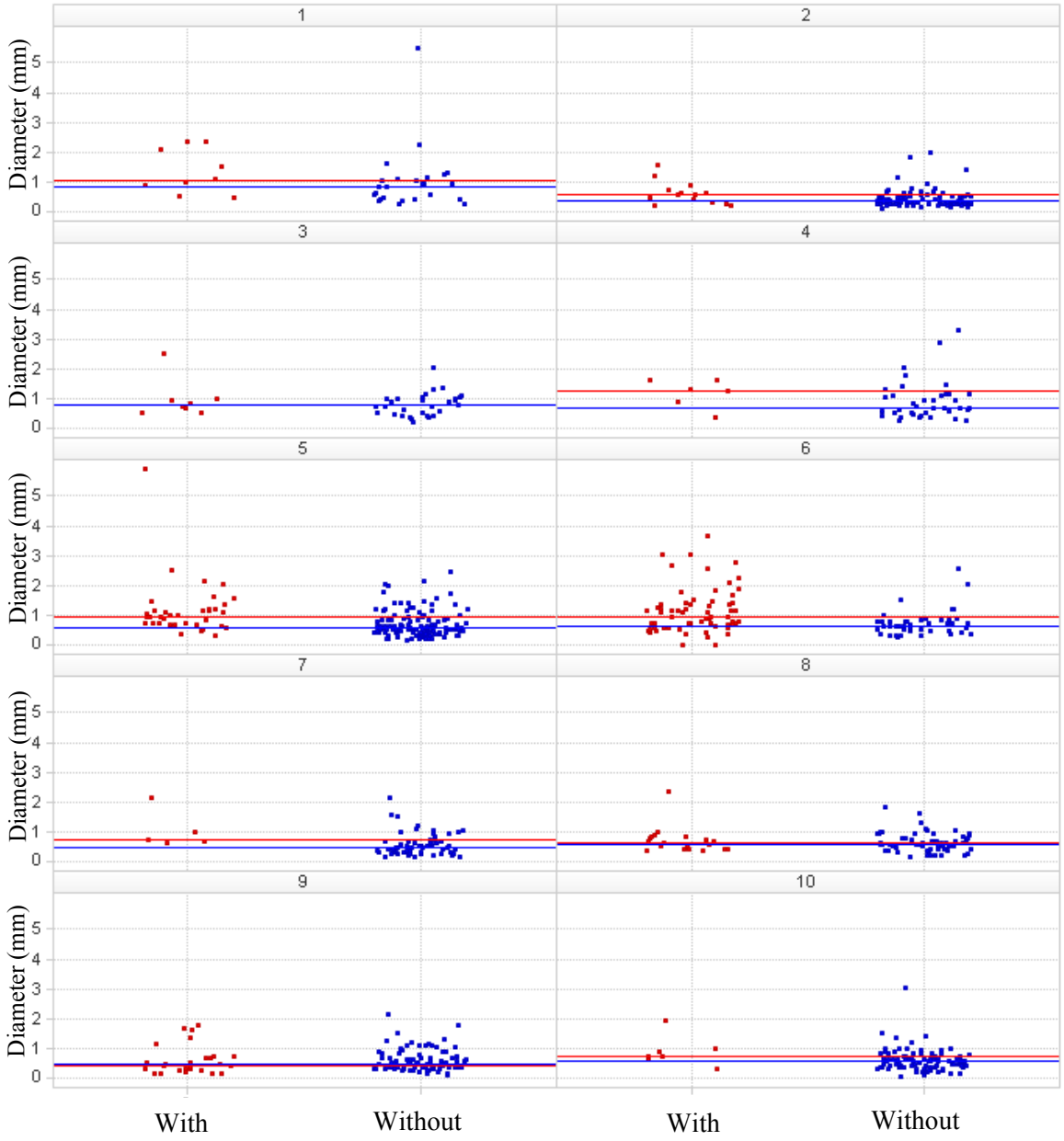


Figure 3.18 Airway diameter with and without surrounding metaplastic cells all data

The red markers show diameter (mm) of airways with surrounding metaplastic cells and the blue markers show diameter (mm) of airways without metaplastic cells. The number above each table shows the patient number. The red line shows the median airway diameter with metaplastic cells for each patient and the blue line shows the median diameter for the airways without surrounding metaplastic cells. These median values are tabulated below in Table 3.4. Patient 6 seems to show that the larger airways have surrounding metaplastic cells, whereas patient 4 does not. There does not seem to be an obvious upper or lower limit to the size of airway and the presence of metaplastic cells.

The results are summarised in Table 3.4. Patient 7 and patient 8 show similar median values but there are far fewer airways with metaplastic cells. In patient 7 this may indicate less severe disease. Patient 7 clusters with the control samples in the normal group showing that there are few pathological changes.

Table 3.4 Statistical analysis of airway diameters with & without metaplastic cells

Pat No	Median Without	Median With	Mann-Whitney $U_{without}$	Mann-Whitney U_{with}	Z statistic	2-tail p
1	0.83	1.06	165	85	-1.46	0.441
2	0.35	0.57	851	451	-1.85	0.0647
3	0.76	0.80	445	283	-1.04	0.2984
4	0.70	1.24	406	231	-1.21	0.2274
5	0.56	0.95	3254	1186	-4.26	<0.0001***
6	0.64	0.97	2071	827	-3.82	0.0001***
7	0.49	0.72	372	55	-3.20	0.0014*
8	0.60	0.63	656	579	-0.41	0.6806
9	0.48	0.43	1028	1213	0.64	0.5206
10	0.56	0.74	466	171	-2.03	0.0419

2-tailed p values of < 0.05 the diameter of the groups with and without are statistically significant. This means patients 5, 6 and 7 show significantly different airway diameters between the airways with and without surrounding metaplastic cells. *** denotes a p value of <0.001, ** denotes a p value of <0.005 and * denotes a p value of <0.001

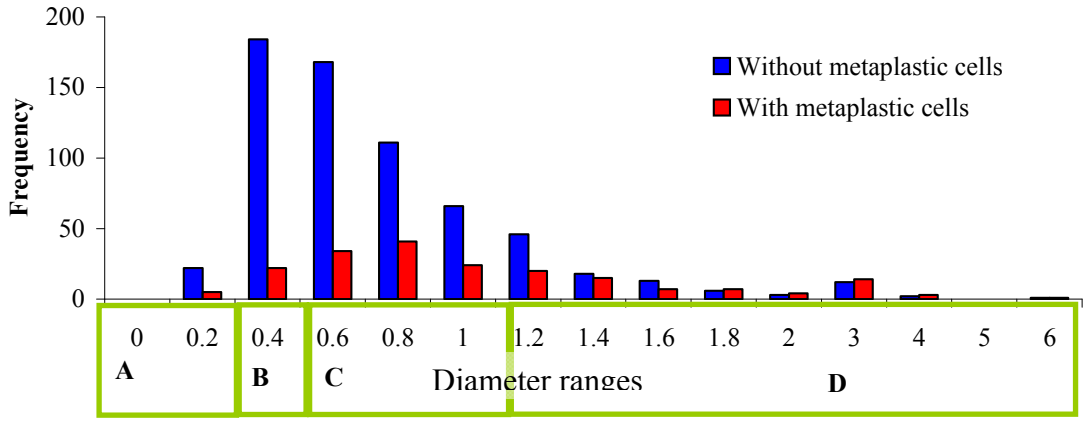


Figure 3.19 Combined airway diameters of airways with & without metaplastic cells

The distribution of airway diameter with the results amalgamated across patients 1-10. The lines represent a line of fit suggested by the graphing software. Box A shows the respiratory bronchioles at 0.3mm or less, box B 0.3-0.5mm shows the terminal bronchioles, box C 0.5-1mm shows the lobular bronchioles and box D 1-5mm shows the bronchioles. The distribution of airway diameter is reflective of the airway loss that may have occurred and the anatomy and location of the LVRS sample taken. It is therefore of interest to compare those with metaplastic cells and those without rather than the actual distribution of airway diameters.

Figure 3.19 shows the numbers of airways with and without metaplastic cells present based on airway diameter. The data of COPD patients 1-10 has been pooled to provide greater n numbers. This allows interrogation as to whether airway diameter and therefore anatomical location influences the presence of metaplastic cells. The airways with metaplastic cells peaks at 0.6mm, these are lobular bronchioles. Anatomically there are a greater number of respiratory bronchioles. This suggests that the incidence of metaplastic cells is less in the very small respiratory bronchioles.

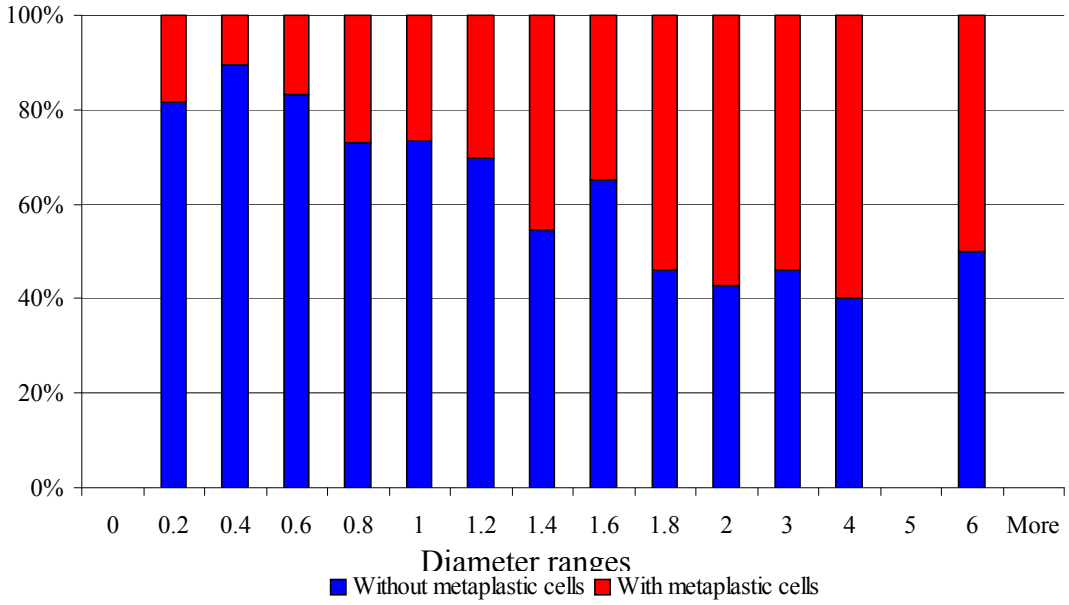


Figure 3.20 Percentage of airways with and without metaplastic cells according to diameter

The same data as Figure 3.19. This demonstrates the proportion of those with metaplastic cell according to size rather than showing actual numbers counted. This shows that the proportion of airways with metaplastic cells increases to almost 60% at approximately 1.8mm diameter and remains at this level until at least 4mm in diameter possibly higher.

3.3 Discussion of Characterisation of the metaplastic lesion in COPD

3.3.1 COPD pathology and general COPD phenotypes

The hierarchical clustering based on pathological feature and metaplastic cell location divided the COPD cohort and controls into 8 groups. The control samples were all in the same group alongside 2 COPD patients. Interestingly the two COPD patients 7 and 42 show few areas of metaplastic cells and only in the adventitial location. This frequency and distribution is shown in Figure 3.11.

There was also a separate group of COPD patients that perhaps represented the worst affected individuals. Groups 3 and 4 were associated with metaplastic cells in all locations. Group 3 showed fibrosis in the pleura, airways and parenchyma along with airway changes. Group 4 had pleural and airway fibrosis and showed few other changes suggesting fibrosis was the dominant lesion in this group. This perhaps reflects the general fibrosis throughout the tissue leading to surface changes throughout the peripheral lung. These samples may show similar characteristics to IPF lung where there is extensive fibrosis and the production of surface lining cells is well documented

(Haddad & Massaro 1968). The influence of fibrosis and fibroblasts will be an important factor to consider in later chapters.

Medial hypertrophy seemed to be a feature through several of the groups including the more normal tissue. This suggests that there is a background of vascular change that is greatly increased in those patients with widespread damage. This does not give an indication of whether the vascular changes are primary or secondary, merely that there were very few patients where vasculature was unaffected.

The heatmap work showed several associations between pathological features. Vascular myolysis was always present with medical hypertrophy and adventitial metaplastic cells. This shows that the more frequent perhaps minor change of medial hypertrophy preceded the more severe myolysis. Also this damage may trigger the development of adventitial metaplastic cells. Many of the other associations were also with adventitial metaplastic cells and medial hypertrophy. This may be expected because the incidence of adventitial metaplastic cells and medial hypertrophy were so high within the cohort. There are no obvious pathological progression indications from this work.

3.3.2 The metaplastic lesion in COPD

The distribution of metaplastic cells between the five identified locations within the lung may indicate of the origin and spread of these cells. If metaplastic cells were 'escaped' airway cells, movement outwards would suggest that airway and vessel adventitia would be colonised first. Then moving further into the lung it may be expected that hilar then sub pleural would follow. The presence of fibrosis or fibrosed alveoli can occur anywhere in the parenchyma so is difficult to link to airway proximity. Whatever the association with airways, fibrosis and alveolar colonisation should be more frequent than sub pleural proliferations as these are at the extreme borders of the tissue. The presence of alveolar metaplastic cells must be determined by something other than progressive spread from the airways because the incidence would be much higher if proximity to airways was the only factor. The presence of hilar areas and fibrosis is dependent on the disease state and changes occurring in the lung. These features are a result of damage not a normal anatomical aspect of the lung making the frequency with which these features occur less regular so the subsequent colonisation of these areas is much more difficult to predict. Also due to the random anatomical sampling, not all slides investigated had pleura. This shows that the only location really comparable across samples is adventitial as pleura may be absent through sampling,

hilar and fibrosis are disease associated and alveolar spread has a factor other than presence of parenchyma determining colonisation.

Due to the paucity of literature regarding metaplastic epithelial cells in the COPD parenchyma it is difficult to conclude whether the three morphologies, uniform, macrophage-like and squamous were expected. Within IPF patients there are cells that have a similar appearance and range of morphology to the metaplastic cells seen in the peripheral lung of COPD patients. The alveolar bed of the lung is fibrosed in IPF so the normal anatomical locations are indistinct. The lining cells of IPF all seem to adhere to fibrosis making it difficult to draw parallels with the locations of colonising cells observed in COPD (Iyonaga *et al.* 1997).

The three morphologies of metaplastic cell seen are observed across all five locations. The uniform phenotype appears similar to several tumour growths, for example lepidic BAC (Awaya *et al.* 2004). The uniform and squamous cell types are more similar to those observed in IPF lining the alveolar bed. The uniform cells also look rounded in the same way as hyperplastic type II cells do (Osanai, Igarashi & Yoshida 2001). These morphologies are all suggestive of an epithelial phenotype.

It is difficult to compare colonising locations to other lung diseases such as BPD and pulmonary sequestration. In these conditions the lung has failed to form completely and so airways are much harder to distinguish. In IPF there is so much fibrosis that again the general tissue structure is lost and cystic with large areas of fibrosis. It will have to be determined immunohistochemically and tinctorially whether there are any similarities between the colonised surfaces in these different conditions.

The observation of metaplastic epithelial cells within the parenchyma of COPD is perhaps unsurprising based on the previous observations by Berkheiser (1959). Berkheiser noted the presence of epithelial metaplasia in the lung periphery in bronchiectasis, pulmonary infarcts and anthracosis. The observation of epithelial metaplasia tended to be associated with smoking and epithelial damage. The main risk factor for the development of COPD and the contributory conditions all cause epithelial damage at different levels of the respiratory tree.

3.3.3 Airway analysis, epithelial characteristics in association with metaplastic cells

Airways on all slides for COPD patients 1-10 were assessed for the presence of a number of epithelial criteria and the presence or absence of metaplastic cells. Airway

epithelial hypertrophy, airway goblet cell hypertrophy and airway goblet cell hyperplasia were all significantly associated with the presence of surrounding metaplastic cells. It should be noted however that this relationship although significantly different to the airways without metaplastic cells surrounding was only observed on around 15% of airways. This means that these characteristics are suggestive; it is not a 100% predictor. The normal epithelium was associated with an absence of surrounding metaplastic cells. These factors all suggest that metaplastic cells are more likely when the airways are under repair or damage responses. The presence of goblet cells within the smaller airways has been associated with inflammation and initiation of a type II cytokine response (Kondo *et al.* 2008, Park *et al.* 2007). This presence of goblet cells coincides with of inflammation and irritation and may explain why goblet cells do not have to be present to illicit the generation on metaplastic cells. Some individuals are more prone to Th2 responses that include goblet cell production whereas others are predominantly Th1, without goblet cell hyperplasia.

The airway surround was also examined. Fibrosis and loss of alveolar attachments did not differ significantly between the airways with and without colonisation. The inflammation grades were significantly different between those with and without metaplastic cells. There were more grade 0 and grade 1 airways without metaplastic cells and more grade 2 and grade 3 airways with metaplastic cells. This showed that as the level of inflammation increased so did the likelihood of metaplastic cells.

3.3.4 Airway analysis, the frequency and diameter of airways in COPD

Airway loss was considered by measuring the area of the tissue sample and looking at the diameter, based on the basement membrane, of the airways present in the sample. It has been shown previously that although there may be tissue distortion or shrinkage due to pathology or processing the basement membrane length remains fairly consistent (James 1987). The distribution of the airways should highlight any loss of airways within the tissue. The number of airways per cm^2 has been used for determination of airway loss in emphysema patients previously (Depierre *et al.* 1972). The values observed in this previous study were, for normal lung 0.73 bronchioles/cm^2 whilst the emphysema group was separated into minimal (0.87 br/cm^2), panlobular (0.55 br/cm^2), centrilobular (0.46 br/cm^2) and miscellaneous emphysema (0.65 br/cm^2). These values were calculated from examining the whole lobe whereas the cohort here uses tissue from the outer rind of the lung in most cases. This may lead to higher overall values as

there are many more small airways in the periphery, the range of values in the emphysema patients which are all COPD and not classified further was 0.65-2.90 br/cm^2. The range for the control samples was between 9.02-20.66 br/cm^2, although these values differ greatly from the Depierre study. This is probably a consequence of the tissue portion examined. The number of airways present is significantly lower in the COPD samples showing extensive damage and airway loss in the COPD cohort. To try and negate the anatomical differences it would be helpful to examine the whole lobe within the COPD cohort or to examine more than 10 patients.

Three metaplastic cell morphologies were identified rounded and uniform, large and irregular resembling macrophages or squamous and flattened. The lesions were found to be localised to the external adventitia of the airways and vessels, sub pleural zone, hilar regions, ectopic fibrotic deposits and the external facet of alveolar epithelium.

Hierarchical clustering produced eight histopathological groups. Group 1 with fewer pathological changes was not strongly associated with the presence of any metaplastic cells. This group included the control resection samples. Groups 3 and 4 demonstrated more pathological change particularly near the airways and these were associated with metaplastic cells in all five localizations identified. The remaining five groups 2, 5, 6, 7 and 8 all showed an association with some metaplastic cell localizations but not all locations. This would suggest that as COPD pathology progresses, the number and distribution of metaplastic lesions also increases.

The number of airways was significantly decreased in the COPD cohort compared to the control samples (N1-N6).

The airway pathologies of hyperplasia, goblet cell hypertrophy and goblet cell hyperplasia were found to be significantly higher on airways with surrounding metaplastic cells. Conversely the airways with normal epithelium were found to be statistically higher in the group of airways without surrounding metaplastic cells. Z-statistics also confirmed increased airway inflammation was associated with the presence of metaplastic cells surrounding the airways. Although these findings were all statistically significant, none were totally predictive.

Chapter 4. Phenotype of the metaplastic lesion in COPD

A diffuse metaplastic response was observed within the lung periphery of all COPD samples examined. The metaplastic response has the appearance of epithelial cells when observed by H&E. To further investigate this phenotype, it was necessary to be aware of the lung epithelial phenotypes and appropriate cell markers.

4.1 Lung epithelial population and progenitors

The aim of this chapter was to ascertain the population contributing to the epitheloid cells within the COPD lung parenchyma. This was carried out by examining lung resident epithelial phenotypes and possible progenitor cells from outside of the lung, for example the bone marrow. The lung is more complex than many organs with numerous resident progenitor cells types outlined below.

4.1.1 Basal cells

Traditionally considered the main airway progenitor, basal cells are located adjacent to the airway basement membrane throughout the respiratory tree. Basal cells are more abundant in the upper airways. Basal cells can produce basal cells or mature into ciliated cells, goblet cells and secretory cells (Inayama *et al.* 1988). Basal cells can become hyperplastic when there is targeted Clara cell destruction. This is the other main progenitor of the airways. This hyperplasia is a part of normal repair, where the basal cells mature and differentiate (Hong *et al.* 2004).

One basal cell marker is p63. This is part of the p53 family; p63 has two isoforms, transactivating TA-p63 and truncated ΔN-p63 (p40). TA-p63 and ΔN-p63 have different opposing functions. TA-p63 functions like p53 and dominant negative ΔN-p63 has a negative effect on p53. TA-p63 is involved with the cell homeostasis triggering apoptosis if the cell is damaged. ΔN-p63 is involved in cell turnover and is the isoform of p63 that highlights basal cells. Chilosi (2002) suggests that p63 may play a role in dysregulated epithelial growth and repair. Cytokeratins are discussed in more detail in section 5.1.1.

Another marker often used to highlight progenitor cells is the intermediate filament marker cytokeratin 5 or 6. This cytoskeletal protein is found within basal cells and squamous cells (Reis-Filho *et al.* 2003).

4.1.2 Clara cells

Clara cells are also found throughout the respiratory tree, but are more dominant in the peripheral airways of humans. Clara cells are thought to act as a progenitor cell within the peripheral airways. Snyder, Zemke & Stripp (2009) recently reviewed the data surrounding lung progenitor cells and highlighted that 3 types of Clara cells have been identified in mouse.

1) CC10 positive, found near airway bifurcations, associated with neuroendocrine bodies and destroyed by naphthalene treatment.
2) CC10 positive, found near airway bifurcations, not associated with neuroendocrine cells and resistant to naphthalene acting as a progenitor cell.
3) CC10 positive, surfactant C positive, cells have been shown to express *sca-1* and CD34. These are capable of behaving as a pluripotent progenitor cell.

Identification of these populations has yet to be carried out in humans but suggest a rare pluripotent cell possibly originating from haematopoietic progenitor cells. If stimulated by damage or local cell death resulting in growth factors release, these cells can give rise to ciliated and non-ciliated cells. This stimulation involves Clara cells de-differentiation to facilitate proliferation and alternative differentiation. This is not the usual maturation of one cell type to another (Evans, Cabral-Anderson & Freeman 1978, Randell 2006). Fehrenbach *et al.* (2002) have shown in rat lungs that stimulation with keratinocyte growth factor (KGF) leads to upregulation and proliferation of Clara cells in the terminal bronchioles. As well as acting as a local progenitor cell, these cells contribute to airway secretions producing surfactants and surfactant-like proteins such as Clara cell 10 protein (CC10) (Soerensen *et al.* 2005). CC10, also called Clara cell specific protein, is produced exclusively by Clara cells and is often employed as a cell marker. Clara cells also secrete antiproteases, which act to counteract proteases seen arising from smoke damage and inflammation. Clara cells are epithelial cells and are highlighted by pan cytokeratin, a cocktail antibody that marks all cytokeratins within a sample. The specific cytokeratins seen in these cells are simple cytokeratins including CK17, but these cells do not show squamous marker CK14 (Nakajima *et al.* 1998).

4.1.3 Goblet cells

Goblet cells are prolific in the central airways becoming sporadic in distal lung. Goblet cells can act as transit amplifiers maintaining the goblet cell population, but may also be

replaced by Clara cells (Evans *et al.* 1986). The primary function of goblet cells is mucus production, mainly mucin 5AC. Goblet cells may be identified by morphology; unciliated, nucleus near the cell base and the cell body filled with mucus of open blue appearance on H&E. Mucin 5AC immunohistochemistry may also be used. Alternatively tinctorial stains such as PAS that highlights most mucus magenta may be used. When observed using electron microscopy, secretory cells have short microvilli and contain fibrillar granules.

4.1.4 Pulmonary neuroendocrine cells

Airway contain cells called neuroendocrine cells and also clusters of cells referred to as neuroepithelial bodies. Neuroepithelial bodies can regenerate epithelium, giving rise to neuroendocrine cells and Clara cells when the principal progenitor populations are damaged. Neuroendocrine cells are found in epithelial basal layer predominantly near bronchiolar bifurcations. These decrease in frequency, moving down the respiratory tree and are rarely found in terminal bronchioles. Neuroendocrine cells are found in higher numbers in fetal rather than adult lung, however cell numbers may increase in hypoxic conditions and in several disease states such as pulmonary fibrosis. These cells may have a sensory function or produce several messengers and hormones. Pulmonary neuroendocrine cells can be specifically highlighted with calcitonin gene-related peptide IHC in rat (bombesin in human), or identified using electron microscopy (Reynolds *et al.* 2000).

Other resident lung cells that are not thought to act as progenitors are ciliated cells and brush cells. Ciliated cells are integral to muco-ciliary transport; the cilia beat pushing mucus and debris up the respiratory tract. The brush cell is a controversial cell type. It has only been observed in animal samples and diseased human tissue, for example desquamative interstitial pneumonitis (DiMaio *et al.* 1988). There remains debate over the existence of brush cells in the lung. Other structures within the lung that are outside the airways also contain epithelial cells.

4.1.5 Submucosal glands

Submucosal glands sit adjacent to the larger airways connecting through small epithelial lined ducts. Glands are a mixture of serous cells and mucus cells supplying mucin 5B to the airways. The serous glands produce other secretions including surfactant A, that mix with the mucus (Saitoh *et al.* 1998). The submucosal glands have a progenitor cell

population within the gland (Hajj *et al.* 2007). This population is highlighted with p63 (Wang *et al.* 2002).

4.1.6 Type II pneumocytes

Type II pneumocytes form the main alveolar progenitor cell and can replicate, to either replace type II pneumocytes or differentiate into type I pneumocytes. Type I pneumocytes are very elongated flattened cells that interact closely with capillaries to facilitate gaseous exchange. Type I pneumocytes occupy the greatest area of the alveolar bed. There is some evidence that type I cells can be triggered to revert to type II cells in culture if seeded on the correct gel matrix, which suggested that the correct matrix must be maintained in order to maintain a type I phenotype (Danto *et al.* 1995). Type II pneumocytes are found at alveolar junctions, although around double in number when compared to type I pneumocytes, these cells occupy only around 7% of the alveolar surface. Type II pneumocytes produce several forms of surfactant, A, B, C and D (Buckingham *et al.* 1966). These are released onto alveolar surfaces increasing compliance and reducing friction between surfaces making inspiration easier. Surfactant A and D are also involved with innate immune responses and immunomodulation. Whilst precursor uncleaved surfactant C is only produced by type II pneumocytes in the lung, surfactants A and B are also produced by Clara cells. Broers *et al.* (1989) also examined adult lung and showed simple epithelial markers, CK7 and CK19 expression on type II cells. Paine showed that CK19 is usually seen on type II cells. When cultured and seeded sparsely so cell-cell contact is lost, cytokeratin expression switched to CK18. Cells have a distinctive cytokeratin profile, however other cytokeratins can be rapidly upregulated to adapt to environment change (Paine *et al.* 1995). Confluent kidney cells show mainly perinuclear cytokeratin filaments at cell-cell junctions, whereas subconfluent kidney cells without cell-cell contact demonstrate cytoplasmic cytokeratin filaments in wavy lines (Benitez-King, Cazares & Meza 1989). This demonstrates that repair and cell-cell contact influence cytokeratin expression.

4.1.7 Mesothelial cells

The external surface of the pleura is lined with mesothelial cells. These are transit amplifier epithelial cells. Unless activated these cells have a very flattened morphology. Mesothelial cells do not have a distinct cytokeratin profile therefore it is necessary to use an alternative marker to highlight this specific population. Mesothelin is a protein

specific to mesothelial cells that can be used as a marker (Mayall, Goddard & Gibbs 1992).

Numerous cell types have been outlined above, the key EM characteristics are summarised in Table 4.1 below. EM will help confirm normal phenotype and may also provide an insight into similarities of metaplastic cells, dividing cells, cancer cells and foetal epithelial cells.

Table 4.1 Electron microscopy features for key lung cells

Cell type	Electron microscopy features
Columnar	Clear cilia in apical surface
Goblet	Domed apical surface, filled with pale secretory packages
Neuroendocrine	Small solitary granules with a halo look around the granule, generally darker than other epithelium, surface has microvilli
Clara	Few short microvilli, secretory granules both translucent and opaque, filamentous granules, may contain lamellar body
Basal	Linked to basement membrane, anchoring fibrils
Type II	Multilamellar bodies, microvilli on surface, resting on membrane,
Type I	Flat shape, pinocytotic vesicles
Brush	Larger villi, flattened tips, often multi-lobed nuclei, vesicles at apical end of cell. (Info for rat cells)
Macrophages	Large vesicles & electron dense debris areas, filipodia, may contain lamellar body, may stick to fibronectin on type I cells, tubular myelin
Endothelium	Flat similar to type I cell.

Features taken from following reviews and papers (DiAugustine, Sonstegard 1984, Penney 1988, Stockstill *et al.* 1995, Scheuermann 1997)

Previous investigators have examined atypical adenomatous hyperplasia, where cells have variable nuclei and some were quite rounded whereas others had irregular grooves on the membrane surface. Nuclear intensity varied from grey to black. Some cells appeared to be horizontally connected to one another but had intercellular spaces. The cell surface had a few short microvilli. The cells contained lamellar bodies suggestive of type II cells and inclusions usually seen within the Clara cell (Osanai, Igarashi & Yoshida 2001).

Bruder *et al.* (2007) examined two infants with fatal interstitial pneumonia and the lungs showed type II hyperplasia. These had occasional inclusions that contained tightly packed membrane whirls, referred to as ‘fried egg’ inclusions. The two infants had surfactant gene mutations which probably accounted for the production of cytoplasmic inclusions. There were no other ultrastructural alterations. This suggests that hyperplasia is simply increased cell numbers without structural changes.

4.2 Adult Progenitor cells

Haematopoietic stem cells (HSC) or bone marrow stem cells both originate in the bone marrow. HSC's can give rise to blood cells including lymphocytes, macrophages and eosinophils. HSCs also produce endothelial, muscle and some dendritic cells. There are stem cells specific to embryonic development, by definition are only found in embryonic tissue. These cells are identified with markers such as Oct-4 and stage specific embryonic antigens (SSEA) found on totipotent stem cells, which can become any cell type.

There are several markers that are employed to determine the presence of progenitor cells, some fibroblasts and mesenchymal cells. CD34 is a marker of HSCs but it may also be found on endothelial cells and mast cells (Katz *et al.* 1985, Chauhan *et al.* 2003). It is present on endothelial cells except those which are fully mature with intact tight junctions (Fina *et al.* 1990). The endothelial population can be excluded with the use of endothelial specific marker CD31 (PECAM-1). CD34, the receptor for L-selectin (CD62), is thought to mediate leukocyte adhesion during inflammation. CD34 positive progenitor cells have been examined using electron microscopy once stimulated cell characteristics altered within a few hours. This suggests that CD34 positive progenitor cells present will not be identifiable with electron microscopy as the cell will have altered rapidly to resemble the subsequent mature phenotype (Krauter *et al.* 2001).

A sub-population of CD34 positive haematopoietic progenitor cells also express CXCR4. Mouse knockout studies suggest that stromal derived factor-1 (SDF-1) may act as a chemoattractant to CXCR4 positive haematopoietic progenitor cells. The capability and function of this sub-population of CXCR4 positive cells appears to be similar to the CXCR4 negative population. Although negative cells are more likely to become erythroid cells whereas CXCR4 positive cells become granulocytes (Rosu-Myles *et al.* 2000). A review by Forbes *et al.* (2002) highlights mixed findings in mouse studies regarding cell number and type that may be replenished by HSCs or bone marrow cells. This may be in part a result of strain and model variation between studies.

C-kit, also known as CD117 is a progenitor cell marker. It may be found on haematopoietic progenitor cells, multipotent progenitors and inflammatory cell progenitors. CD117 is a tyrosine kinase receptor that acts as receptor for stem cell factor, which is capable of activating the bone marrow.

CD133 or AC133 is another HSCs marker. CD133 is found on the apical surface of immature epithelial cells associated with apical surface microvilli. Corbeil *et al.* (2000) demonstrated apical epithelial staining in several embryonic tissues and demonstrated using an intestinal epithelial cell line that CD133 is rapidly down regulated upon cellular differentiation. This may lead one to conclude that apical staining is an indication of recent cell proliferation. CD133 appears to be found on cells which are CD34 ^{bright} i.e. strongly stained with CD34 (Yin *et al.* 1997).

Previous lung progenitor cell work has shown progenitor contribution to a range of cell types. Albera *et al.* (2005) examined both autopsy and resection samples from females who received a male donor bone marrow transplant or from men who had received female lung transplants. Female samples were examined for male bone marrow cells presence: sporadic macrophages, epithelial and endothelial cells were identified. Donor lungs were examined for host Y chromosome containing cells. Transplanted lungs displayed a Y chromosome in type II cells, macrophages, endothelial and smooth muscle cells. One quarter of patients examined also showed occasional epithelial cells of the airway. Suratt *et al.* (2003) carried out a similar study and showed one patient with donor bone marrow cells contributing to bronchiolar epithelium. Finally Mattsson *et al.* (2004) examined four female patients receiving male bone marrow transplants. These showed lung cells that were cytokeratin and surfactant positive and could be seen using FISH to have a Y chromosome indicating cells from male donor bone marrow.

Recent work has identified a very small sub-population of progenitor cells that are positive for embryonic marker, Oct-4 and are present in adult tissue. A murine study identified very small cells that were positive for Oct-4, Sca-1 and negative for Lin and CD45. These cells were found in many organs including the lung. Researchers concluded these cells may be mobilised by stress or injury and that hypoxia damaged tissues produce several chemoattractants (Ratajczak *et al.* 2009). This suggests that perhaps damaged and possibly hypoxic COPD lung would contain some mobilised progenitor cells. Other work looking at human patients shows that Oct4 positive cells are present in adults in very small numbers but may be mobilised after injury such as myocardial infarction (Wojakowski *et al.* 2009).

There are several technical difficulties with detection of progenitor cells which arise from protein differences between mouse and human homologues, such as mouse prominin and human CD133, cell fusion and cell maturation. If cell fusion occurs, cells

acquire characteristics and markers of both cell types, making confirmation of progenitor cell maturation more difficult (Camargo, Chambers & Goodell 2004). The other issue with identifying progenitor cells is that as the cell matures into an alternative phenotype, the markers of progenitor cell origin are lost.

4.3 Aims and objectives

The aim of this chapter was to determine whether lung or multipotent progenitor cells were contributing to the metaplastic cell population. Possible cell populations and markers are shown in Figure 4.1.

The following markers were all applied using immunohistochemistry techniques.

Are the metaplastic cells epithelial?

A pan cytokeratin antibody was used as a marker to determine whether the metaplastic cells were of epithelial phenotype.

Do the metaplastic cells contain basal cells?

CK5/6 and p40 antibodies were used to examine whether basal cells were present in the metaplastic population

Do the metaplastic cells contain Clara cells?

A CC10 antibody was used to examine whether Clara cells were present in the metaplastic population

Is there evidence for goblet cell presence in the metaplastic cell population?

Mucin 5AC immunohistochemistry and tinctorial stains PAS and Alcian blue were used to examine the presence of goblet cells

Is there evidence of a mucus gland progenitor cells within the metaplastic cells?

A p40 antibody combined with a mucin 5AC antibody, PAS and Alcian blue staining were used to examine whether mucus gland progenitor cells were present in metaplastic cell population

Are type II pneumocytes present in the metaplastic cell population?

Surfactant A and C were used to examine whether type II pneumocytes were present in metaplastic population

Do the metaplastic cells contain mesothelial cells from the pleura?

Mesothelin was used to examine whether mesothelial cells were present in the metaplastic population

Do the metaplastic cells demonstrate pluripotent progenitor cell markers?

CD34, c-kit and CD133 are all multipotent progenitor cell markers and the presence of these would suggest multipotent progenitor contribution.

Do the electron microscopy characteristics of metaplastic cells suggest a specific phenotype?

Features found on electron microscopy examination were compared to other cells in the COPD tissue and published cell phenotypes.

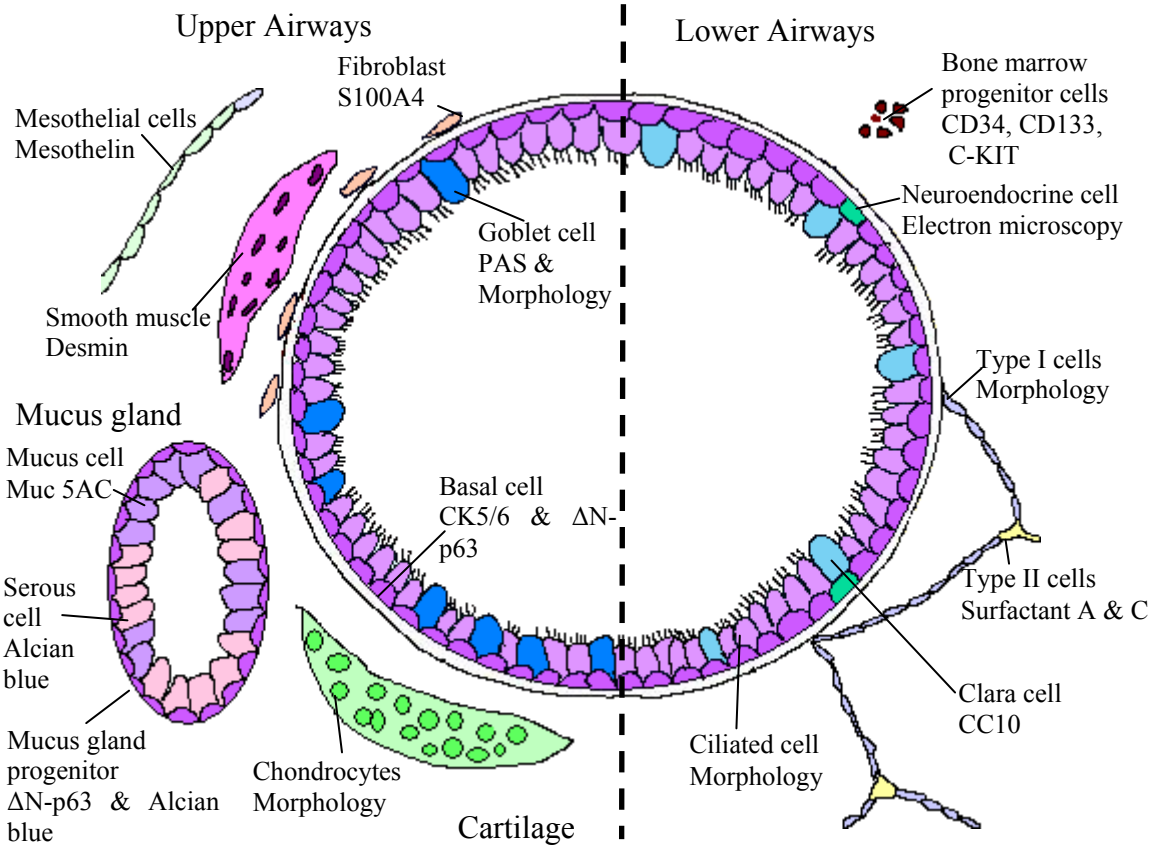


Figure 4.1 Cell populations that may contribute to the metaplastic cell lesion
Shows cells of the airways, alveolar bed and pleura along with associated markers. Multipotent progenitor cells will also be considered as possible metaplastic cell progenitors.

4.4 Results for Epithelial Phenotype

It was important to first confirm that all metaplastic cells were epithelial.

4.4.1 Results for Epithelial phenotype: Pan cytokeratin

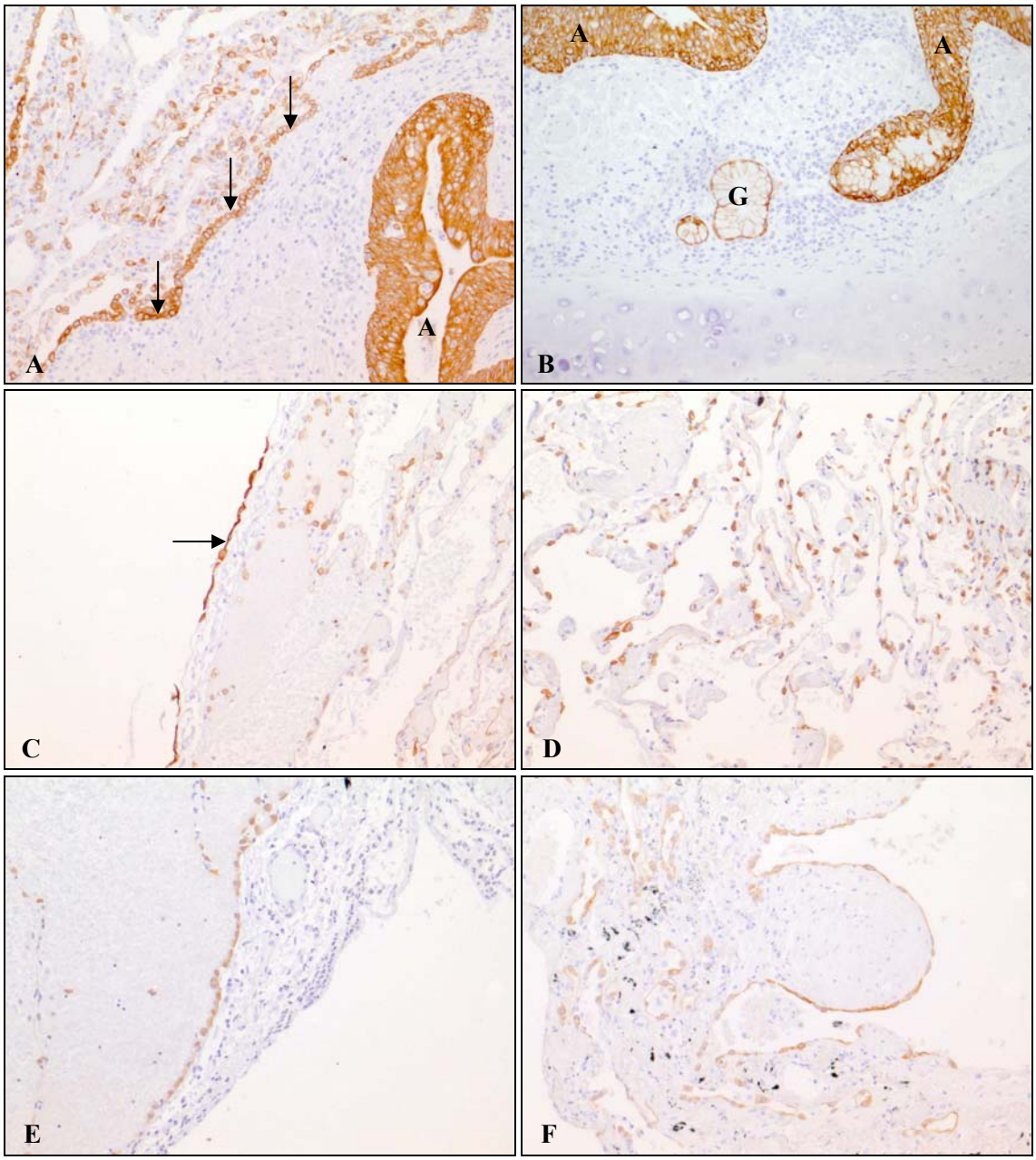


Figure 4.2 Pan cytokeratin IHC

Figure 4.2A) Pan CK staining (Patient 6: Goblet cell airway changes), on large airways (A), metaplastic cells (arrows) and sporadic type II cells
Figure 4.2B) Pan CK staining (Patient 6: Goblet cell airway changes), on large airway (A) and gland (G).
Figure 4.2C) Pan CK staining (Patient 6: Goblet cell airway changes) on an area of mesothelium (arrow).
Figure 4.2D) Pan CK staining on an area of parenchyma showing positive type II cell staining.
Figure 4.2E) Pan CK staining (Patient 4: Airway changes & fibrosis), positive sub pleural metaplasia
Figure 4.2F) Pan CK staining (Patient 17: Airway changes & fibrosis), fibrosis metaplastic cells.

A pan cytokeratin antibody was used to confirm the epithelial phenotype of the metaplastic cells. The whole panel COPD samples 1-50 and 6 control samples was

stained, examples are shown in Figure 4.2. This consisted of 124 slides in total. This antibody highlights all airway epithelium (Figure 4.2A, B), type II cells in the parenchyma (Figure 4.2D), mesothelium (Figure 4.2C) and the diffuse metaplastic response (Figure 4.2E, F). This confirms the parenchymal diffuse metaplastic response has an epithelial phenotype.

4.5 Progenitor cells within the lung

This section discuss the markers for lung progenitor cells. Basal cells, Clara cells, goblet cells, sub mucosal gland cells and mesothelial cells are examined here.

4.5.1 Results for Basal cells CK5/6 and ΔN-p63

CK5/6 was run on the subset panel that included 28 COPD patients and 6 controls, staining is represented in Figure 4.3. All patients showed perinuclear airway basal and suprabasal staining of CK5/6 as expected (Figure 4.3A, B). Small airways tended have a few positive cells in the basal layer (data not shown). Large airways often had the whole basal layer and many suprabasal cells positively stained. Positive cells varied between and within patients, and were influenced by submucosal inflammation, airway damage and repair. There was no pattern associated with metaplastic cell presence: airways with few positive basal cells and those with numerous positive basal cells both had examples with and without metaplastic cells.

There was perinuclear staining of cells within the parenchyma of five patients, locations are summarised in Table 4.2. CK5/6 positive cells were not seen on hilar regions or within the alveolar bed. The morphology of positive parenchymal cells was usually a tessellate squamous appearance. These areas were usually disconnected from surrounding tissue, fibrosed and in some cases very inflamed with lymphocytes. On initial inspection cells appeared to be metaplastic in all cases, however cells were actually areas of bronchiolisation with transitional cells present a range of stains are shown co-localising in Figure 4.4. Bronchiolisation was determined by the multilayer nature of the cells, the presence of transitional epithelial cells and positive staining with other markers such as ΔN-p63 and involucrin.

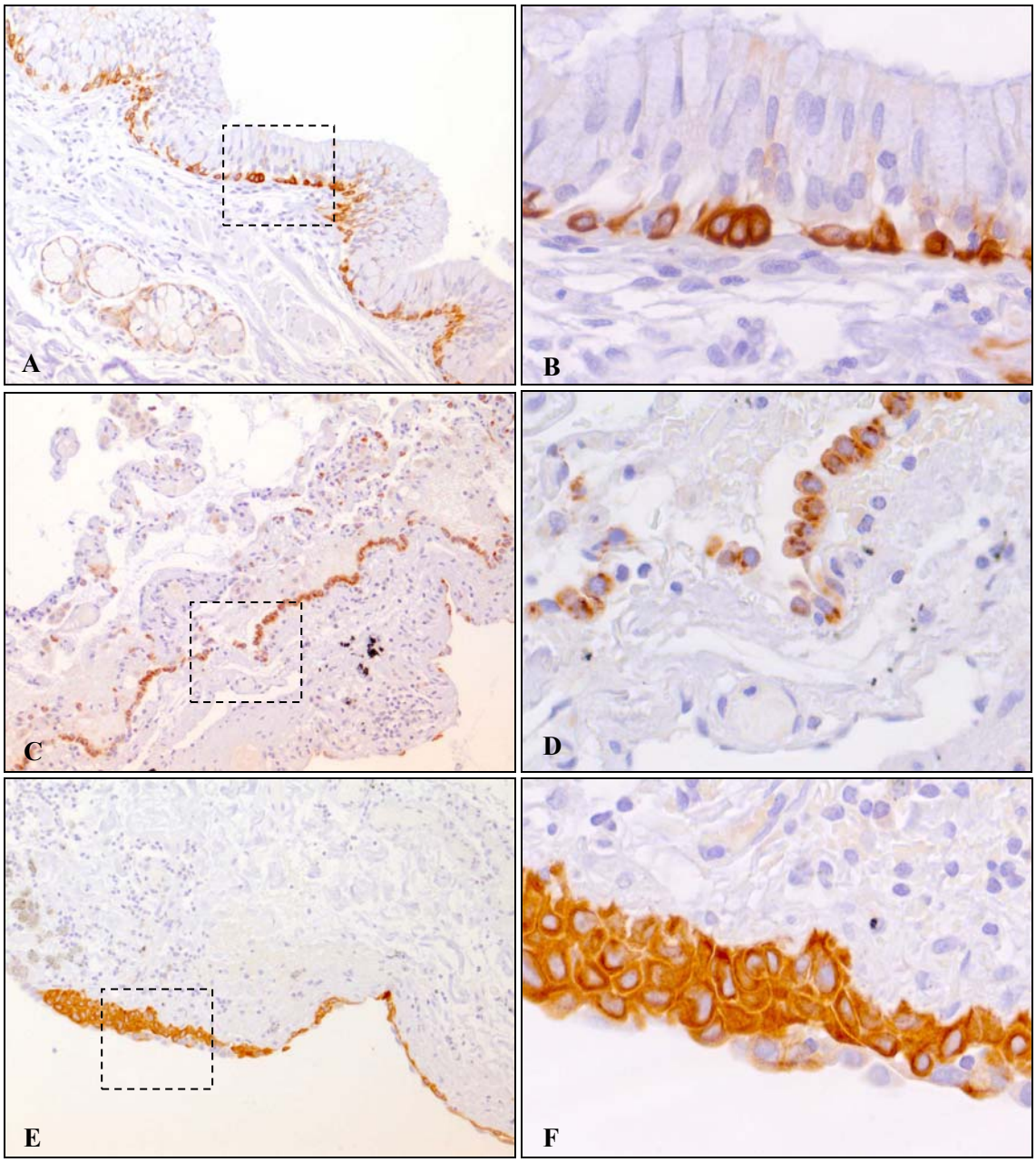


Figure 4.3 Basal cell CK5/6 IHC staining

Figure 4.3A) CK5/6 staining (Patient 13: Airway changes & fibrosis) on a typical COPD airway, the basal cell population is stained positive, with some suprabasal cells also still showing some stain. In the bottom left part of the photograph there is positive staining within the basal cells of the mucus glands.

Figure 4.3B) x40 objective of CK5/6 this shows the boxed area from Figure 4.3A. The basal cells of the large airway show a normal cellular distribution of cytokeratin.

Figure 4.3C) CK5/6 granular positive staining (Patient 27: Fibrosis). This photograph shows metaplastic cells across the centre of the picture, there are numerous positive type II cells within the tissue.

Figure 4.3D) x40 objective CK5/6 granular staining. This photograph shows the boxed area from Figure 4.3C. The positive staining seen here is small perinuclear vesicles within metaplastic cells. This vesicular distribution is not normally seen with any cytokeratin.

Figure 4.3E) CK5/6 staining (Patient 13: Airway changes & fibrosis) on parenchymal squamous cells: an example of bronchiolisation.

Figure 4.3F) x40 objective CK5/6 staining from the hatched area. Cells show intense staining in the cytoplasm and perinuclear of the squamous cells.

Table 4.2 Summary of Parenchymal CK5/6 staining

Pat	Pathology Code	Location of positive cells
13	Airway changes & fibrosis	Sub Pleura & Adventitia
19	Airway changes & fibrosis	Sub Pleura
21	Goblet cell airway changes	Adventitia
25	Airway changes & fibrosis	Fibrosis
49	Airway & parenchymal change	Sub pleura

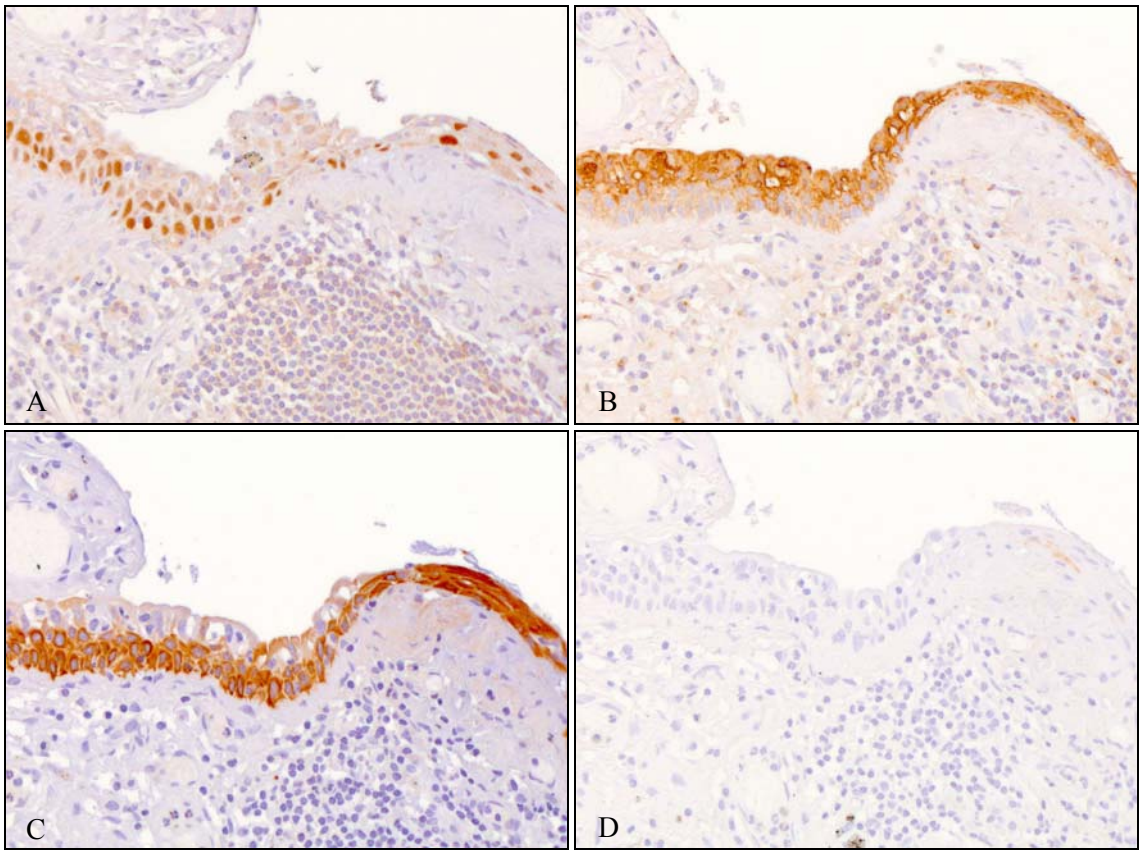


Figure 4.4 Bronchiolisation demonstration using a range of IHC markers

Figure 4.4A) ΔN-p63 positive (Patient 19: Airway changes & fibrosis) cells can be seen across the centre of the picture. These cells are on an area of sub pleura. The unstained cluster of cells underlying the positive signal is a lymphocyte aggregate. This is not metaplastic cells, this is a cluster of stem cells in an area of bronchiolisation.

Figure 4.4B) Involucrin positive staining on upper layers of the bronchiolisation, this is a serial section of tissue with the corresponding area of ΔN-p63 staining in Figure 4.4A.

Figure 4.4C) CK5/6 positive staining on a corresponding serial section. This shows positive cells in the same area as the ΔN-p63 positive cells of 4.8a the staining may appear more extensive as the cytoplasm is positive as opposed to the nuclei which cover a much smaller area.

Figure 4.4D) CK14 is negative on the cells corresponding to the CK5/6 cells seen in Figure 4.4C

There was an unusual granular stain observed in 4 COPD samples and 2 control samples. This vesicular stain appeared to highlight metaplastic cells but also, airway goblet cells, ciliated cells and type II cells (Figure 4.3 C, D). Granular staining occurred in both blocks of those patients with multiple samples of the sub panel used, suggesting the reaction was patient specific. Samples with granular staining all showed parenchymal fibrosis, in one sample fibrosis was more prominent around the airways

and vessels. When present, the vesicular staining was seen in type IIs, airway and metaplastic cells across the whole slide. The reason for this vesicular pattern of staining is unclear. Vesicular staining was present on the following patients, 27 (Fibrosis), 28 (Airway changes & fibrosis), 32 (Airway & pleural change), 46 (Airway & parenchymal change) and control samples N2 and N6.

A ΔN-p63 antibody was used to confirm basal cell presence within the airways, mucus glands and to investigate the metaplastic cells. The full panel of samples including 50 COPD patients and 6 controls was stained. This consisted of 124 slides. As CK5/6 highlights basal cells but also squamous cells, ΔN-p63 was employed to confirm the basal nature of any positive cells found outside airway basal layer, staining with this antibody is shown in Figure 4.5. All patients demonstrated ΔN-p63 staining in airway basal layer when present (Figure 4.4A, 4.7A, B). There were also a few patients with ΔN-p63 positive cells within submucosal gland basal cells (Figure 4.10E, F). There were areas of airways remnants with discernable basement membrane that contained positive cells. There were a number of patients that showed ΔN-p63 staining outside the airways aside from the obvious airway remains. Details of the parenchymal staining is outlined in Table 4.3. These areas are all cystic colonisation within very fibrosed areas of tissue or areas of bronchiolisation that shows a multilayer airway epithelial structure rather than the single layer of epithelial cells seen in the metaplastic population. It can therefore be concluded that all samples demonstrated basal progenitor cells.

Table 4.3 Parenchymal ΔN-p63 staining in COPD samples

Pat	Pathology Code	ΔN-p63 positive cells
8	Airway & pleural change	2 areas of bronchiolisation/ obliterated small airway with positive cells
10	Fibrosis & inflammation	1 area of bronchiolisation on fibrosis
14	Fibrosis & inflammation	Cystic and bronchiolisation areas all show some positive cells
18	Airway changes & fibrosis	Cystic area in solid fibrosis, numerous positive cells
19	Airway changes & fibrosis	Several areas of bronchiolisation with positive cells
32	Airway & pleural change	Cystic area in solid fibrosis, numerous positive cells
39	Airway & parenchymal	Several areas of cystic colonisation and bronchiolisation, numerous positive cells

4.5.2 Results for Clara cells: CC10 IHC

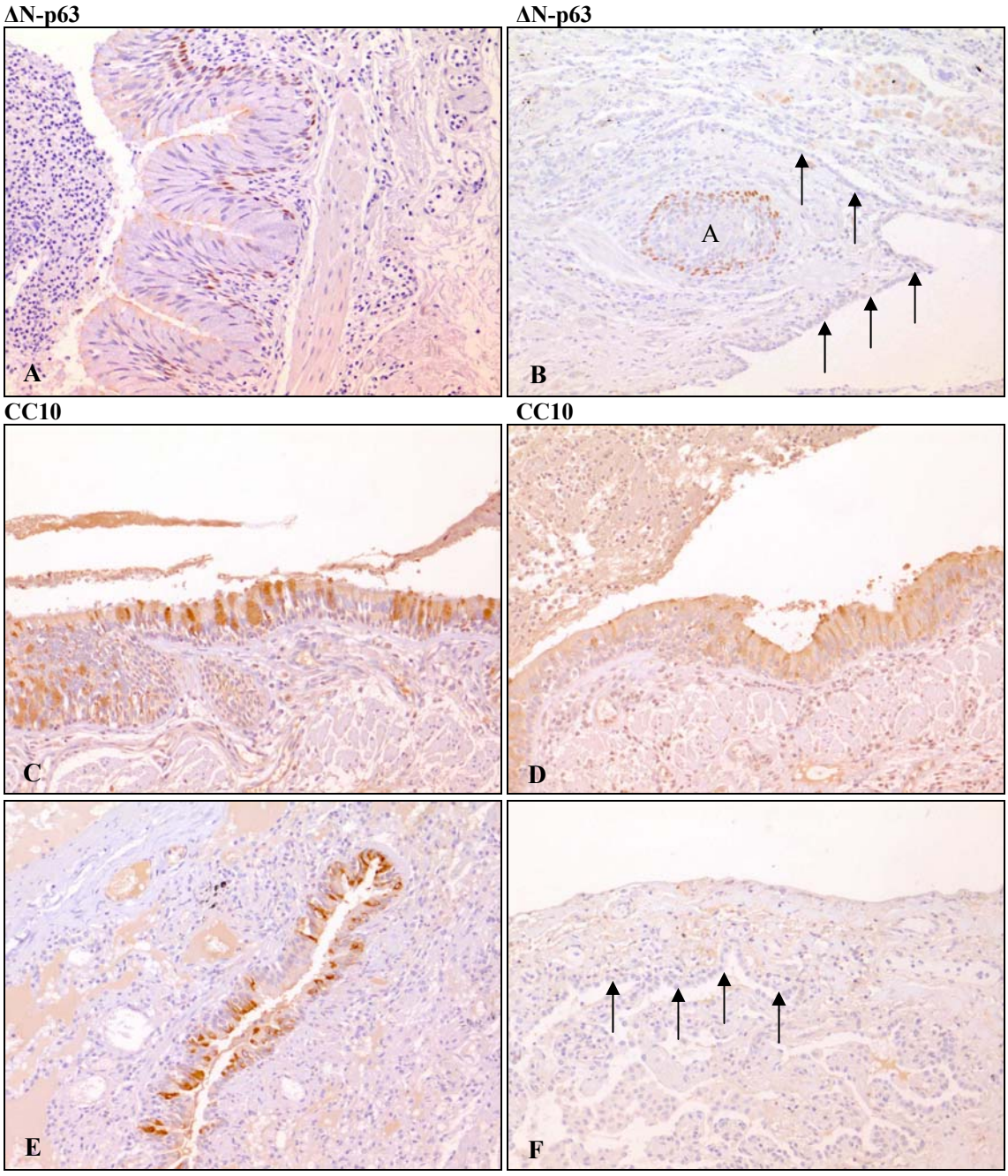


Figure 4.5 ΔN-p63 basal cells and CC10 Clara cells

Figure 4.5A) ΔN-p63 (Patient 13: Airway changes & fibrosis) basal cell staining in COPD airway. Confirms basal cells identified by CK5/6 in 4.5A, indicating the stem cell nature of basal cells.

Figure 4.5B) ΔN-p63 (Patient 6: Goblet cell airway changes) This shows a small area of airway epithelium (A) that is positive for ΔN-p63, metaplastic cells (arrows) were all negative for ΔN-p63.

Figure 4.5C) CC10 (Patient 5: Airway changes & fibrosis) large airway, numerous positive cells.

Figure 4.5D) CC10 (Patient 6: Goblet cell airway changes) in a large airway, this is predominantly negative for CC10 cells. This picture along with Figure 4.7C demonstrates the range of positive cells within large airways.

Figure 4.5E) CC10 (Patient 6: Goblet cell airway changes) within a small airway. There are numerous positive epithelial cells. The acellular wash of staining is non specific staining within plasma leak in the sample.

Figure 4.5F) CC10 staining (Patient 8: Airway & pleural change) This photograph shows an area of negative metaplastic cells (arrows) in the sub pleura. The faint brown wash on the sample is also leak.

A CC10 antibody was used to identify Clara cells. COPD patients 1-10 were stained. CC10 staining was observed within the airways of all 10 patients examined, this is shown in Figure 4.5. Staining highlighted domed epithelial cells within the airways as expected (Figure 4.5C, D, E). There were numerous samples that contained airway fragments with evidence of underlying basement membrane, these areas contained high numbers of positive domed epithelial cells. No metaplastic cells showed positive signal in any sample (Figure 4.5F). Figure 4.6 summarises the staining intensity seen in airways with and without surrounding metaplastic cells.

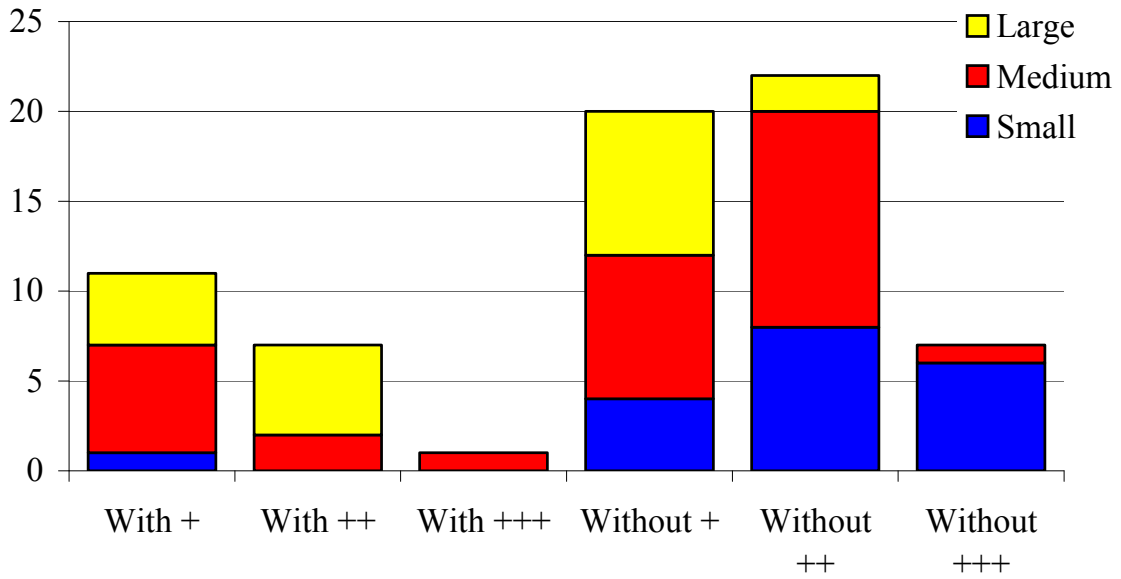


Figure 4.6 CC10 staining with & without metaplastic cells
Shows the airway numbers, divided into large (yellow), medium (red) and small (blue), showing the staining intensity of those airways with and without metaplastic cells.

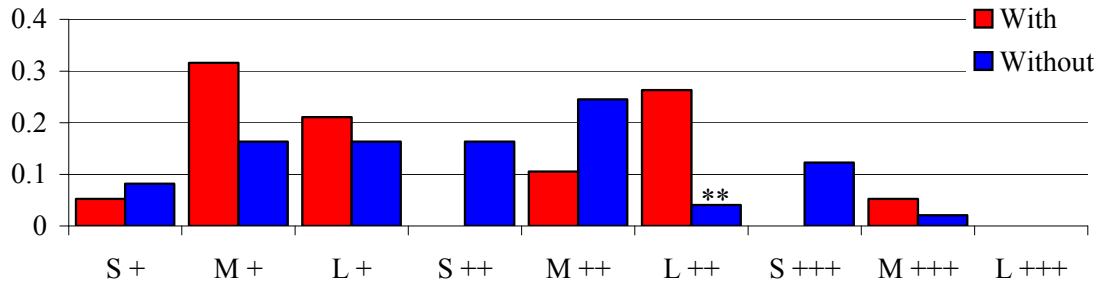


Figure 4.7 CC10 data comparing with and without population proportions
Shows the proportions of those with and without metaplastic cells for each staining and size category. ** $p<0.01$. L=large, M=medium, S=small

Chi-squared goodness of fit tests were performed within both with and without metaplastic cell populations, comparing the observed frequencies of airway size and staining intensity, against a null hypothesis of no difference. Both populations were

shown to be statistically different to a uniform distribution (with - 0.000432; without - 0.000116). The total number of airways with metaplastic cells differs significantly to that without (19:49), so further analysis to determine the underlying cause of the differences was performed using proportions and the z-statistic. This identified that only the large airways with moderate (++) staining were significantly different ($z = 2.707$, $p<0.01$) between the two populations. The values obtained in this analysis are plotted in Figure 4.7.

4.5.3 Results for Goblet cells: Mucin 5AC IHC and tinctorial stains

Goblet cells were identified with the use of a mucin 5AC antibody and tinctorial stains PAS and Alcian Blue. PAS staining highlights goblet and Clara cells, therefore mucin 5AC staining and cell morphology allowed identification of goblet cells. The subset panel with 28 COPD patients and 6 control samples were stained with mucin 5AC, examples of this stain are shown in Figure 4.10. COPD patients 1-10 were stained with PAS and Alcian Blue. The PAS stain should highlight all mucus whereas Alcian Blue highlights acidic mucus.

Mucin 5AC highlighted a range of airway cells within of all samples looked at (Figure 4.10A, B, C). Staining was cytoplasmic, showing the stored mucus within epithelial cells. The staining intensity and therefore the positive cells numbers varied between airways. Figure 4.9 summarises staining intensity seen according to airway size and the presence or absence of metaplastic cells. There was also positive staining in secreted mucus both within airways and parenchyma. There were no mucin 5AC positive metaplastic cells (Figure 4.10D). Patients 1-10 all showed some PAS and Alcian Blue (Figure 4.11) positive cells within the airways. There was no convincing PAS or Alcian Blue staining observed on any metaplastic cells (Figure 4.11B, D).

Airways were assessed for staining intensity and the presence of surrounding metaplastic cells. Mucin 5AC staining was determined as negative (-), low (+), moderate (++) or high (+++). The numbers of airways with and without surrounding metaplastic cells are shown in Figure 4.8 according to staining and airway size. The most striking difference is the number of negative small and medium airways without surrounding metaplastic cells. To further investigate these observations, chi-squared goodness of fit tests were performed within both with and without populations, comparing the observed frequencies of airway size and staining intensity, against a null hypothesis of no difference. Again, both populations were shown to be extremely

statistically different to a uniform distribution (with – 2.85E-12; without - 4.36E-27). For consistency, the z-statistic was used to compare the two populations. In this case, there were five categories classified as statistically significant; medium airways with no staining (M-, Z=-3.55, p<0.001), medium airways with strong staining (M+++, Z=3.22, p<0.01), small airways with no staining (S-, Z=-2.70, p<0.01), medium airways with moderate staining (M++, Z=2.36, p<0.05) and large airways also with moderate staining (L++, Z=2.05, p<0.05).

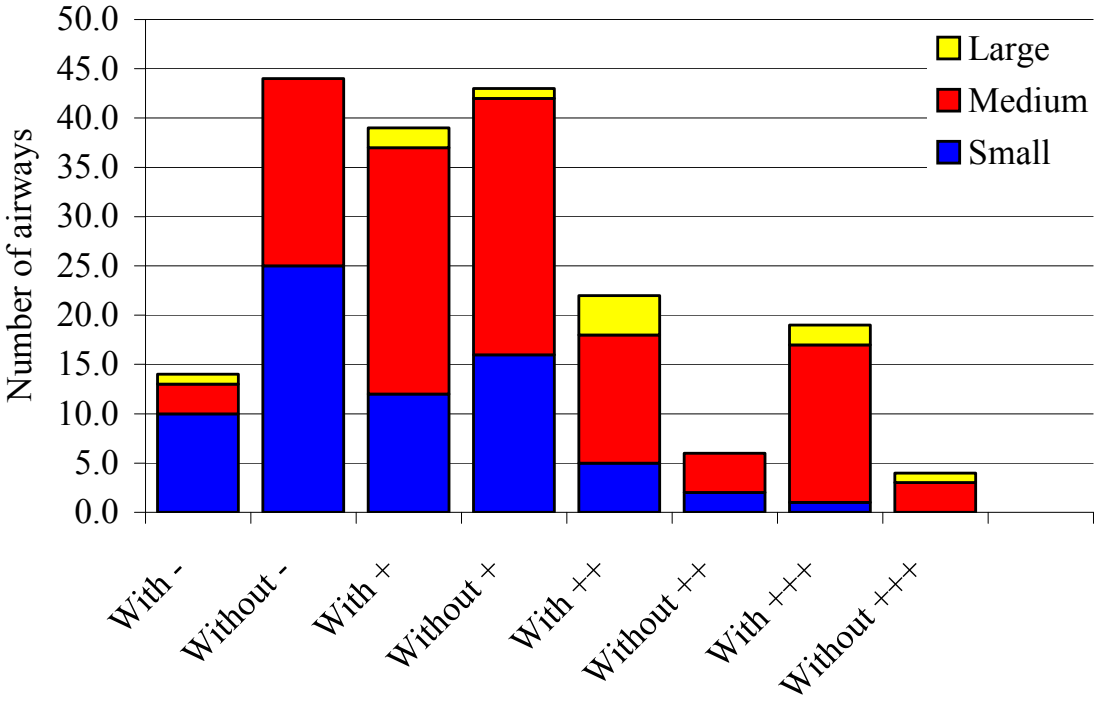


Figure 4.8 Mucin 5AC staining of airways with & without metaplastic cells
This graph shows the number of airways with and metaplastic cells surrounding the adventitia. These are grouped according to the intensity of staining, + low, ++ moderate and +++ high cell numbers. The airway size is represented by the three blocks of colour. The majority of +++ strong staining was found within the large airways. There are very few large airways present without any mucin 5AC staining. There are more small airways without metaplastic cells around the outside adventitia.

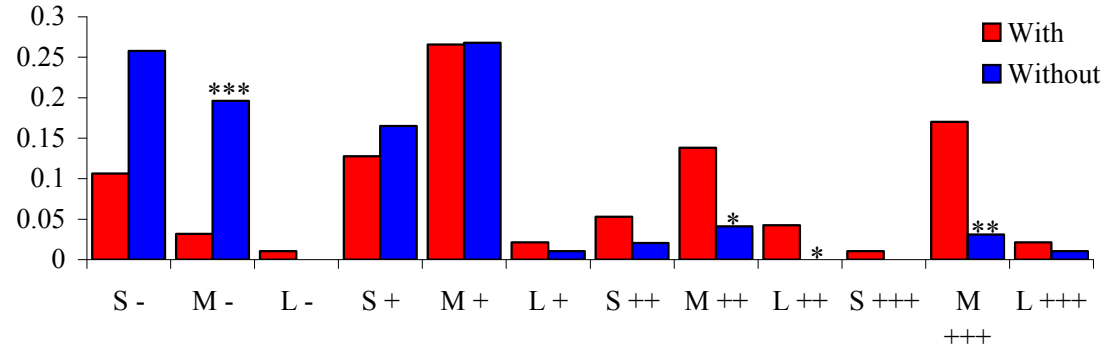


Figure 4.9 Mucin 5AC data comparing with and without metaplastic cell proportions
Shows the proportions of airways with and without airway metaplastic cells according to staining intensity and airway size.

4.5.4 Results for Submucosal gland progenitors: p40, mucin 5AC IHC and tinctorial stains

Seven samples in the sub panel had glands present, one showed positive mucin 5AC staining, the others were all either a weak wash or negative. This suggests that mucin 5AC is not the predominant mucin produced in sub mucosal glands.

Patient 5 (Airway changes & fibrosis) showed some positive and some negative glands for PAS and Alcian Blue. For patient 6 (Goblet cell airway changes) and patient 10 (Fibrosis & inflammation) all glands seen were positive for both PAS and Alcian Blue (Figure 4.11A, C). The other patients did not have any glands present.

As mentioned with the ΔN-p63 staining above there was evidence of ΔN-p63 positive basal cells within the mucus glands further highlighting that this progenitor population is not contributing to the metaplastic cells (Figure 4.10E, F).

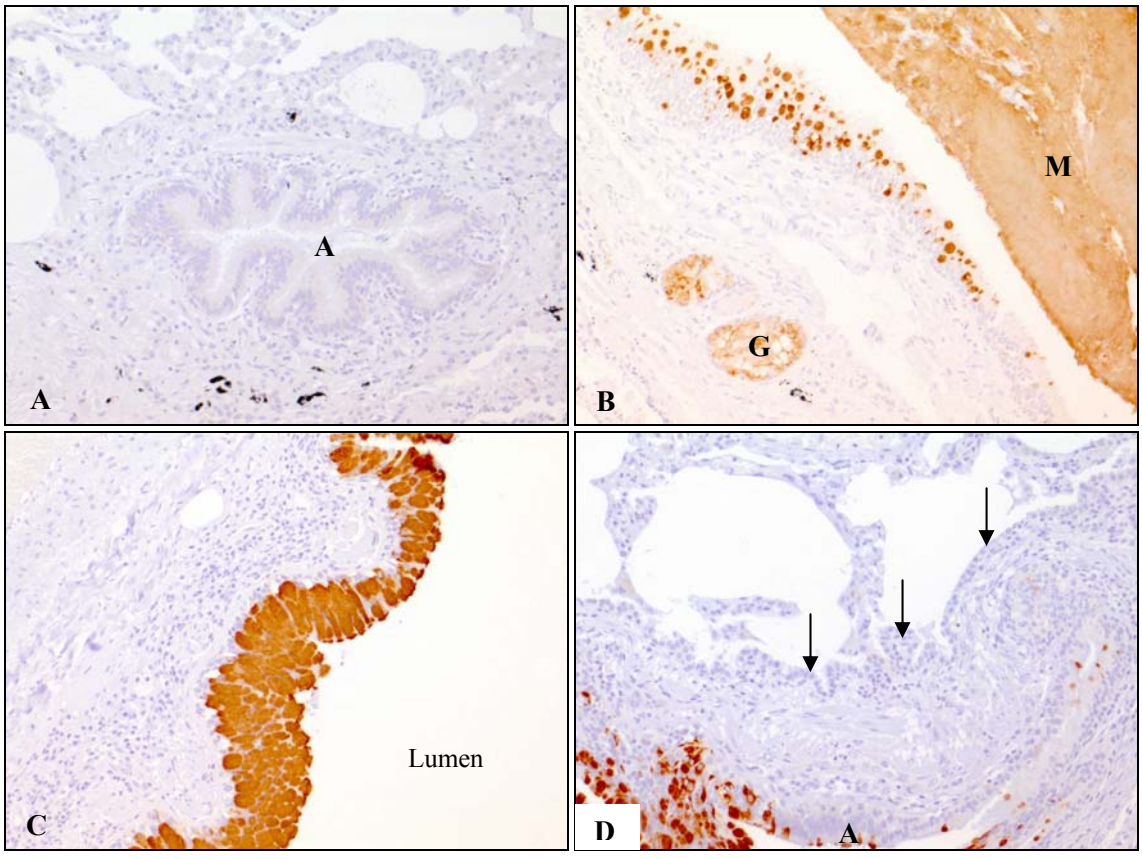


Figure 4.10 Goblet cells mucin 5AC IHC

Figure 4.10A) Mucin 5AC (Patient 20: Airway changes & fibrosis) negative within small airway (A).
Figure 4.10B) Mucin 5AC (Patient 25: Airway changes & fibrosis) positive goblet cells within epithelium and mucus plug (M). There is weak positive mucin 5AC staining on the submucosal glands (G).
Figure 4.10C) Mucin 5AC (Patient 21:Goblet cell airway changes) positive within an area of goblet cell hyperplasia, the only negative epithelial cells are the basal cells at the base of the epithelium, on the left edge of the epithelium.
Figure 4.10D) Mucin 5AC (Patient: 21: Goblet cell airway changes) positive in airway (A) at the bottom of the picture, the top of the picture shows as area of lots of metaplastic cells (arrows) all negative for mucin 5AC.

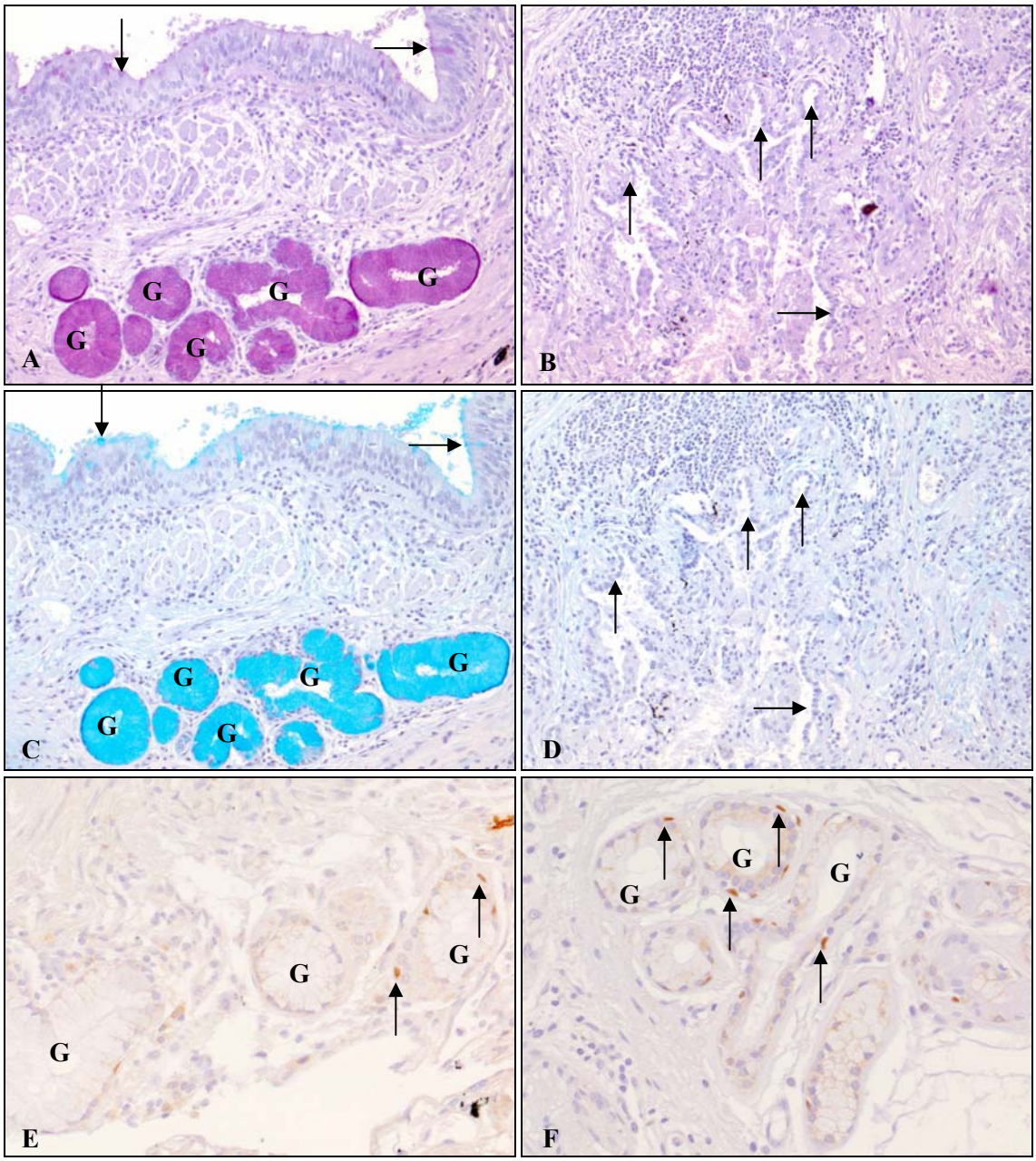


Figure 4.11 Mucus gland progenitors PAS, Alcian blue and ΔN-p63 IHC

Figure 4.11A) PAS (Patient 6: Goblet cell airway changes) shows positive mucus staining within the glands (G) and occasional positive cell staining within the airway epithelium (arrows).

Figure 4.11B) PAS (Patient 6: Goblet cell airway changes) An area of metaplastic cells (arrows), all of which were negative for PAS.

Figure 4.11C) Alcian blue (Patient 6: Goblet cell airway changes) shows positive mucus staining within the glands (G) and occasional positive cell staining within the airway epithelium (arrows). This Figure is a serial section to the PAS Figure 4.11A.

Figure 4.11D) Alcian blue. (Patient 6: Goblet cell airway changes) An area of metaplastic cells (arrows), all metaplastic cells were negative for Alcian blue. This picture is a serial sample to the PAS picture Figure 4.11B.

Figure 4.11E) x20 objective ΔN-p63 (Patient 23: Airway changes & fibrosis) shows positive basal cells within the mucus glands.

Figure 4.11F) x20 objective ΔN-p63 (Patient 34: Fibrosis) also shows positive basal cells within the mucus glands.

4.5.5 Results for Type II pneumocytes: Surfactant IHC

The aim of highlighting surfactant-producing cells was to examine the possible contribution of type II pneumocytes to the metaplastic population. COPD patients 1-10 were stained with a surfactant A antibody, staining is shown in Figure 4.12 below. Surfactant A was identified on inclusions within type II cells and also released surfactant that coated the lung surfaces as expected. Staining was not observed in the airway lumen or epithelial cells of any sample (Figure 4.12A). Strong staining was seen in some strings of parenchymal metaplastic cells and numerous other cells within the parenchyma (Figure 4.12C). Clara cells can also produce surfactant, so a second more specific surfactant antibody was also used.

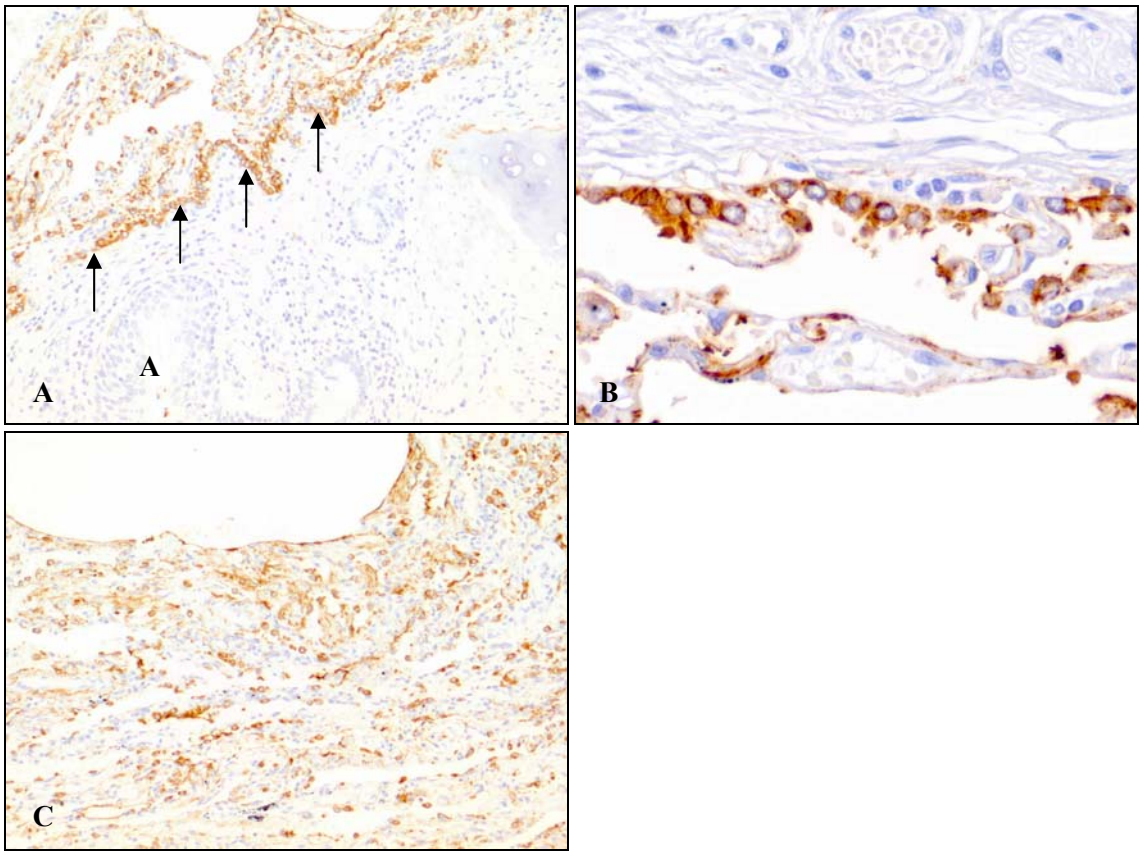


Figure 4.12 Surfactant A IHC staining

Figure 4.12A) Surfactant A staining positive on the metaplastic cells (arrows) and type II cells within the parenchyma. The airway (A) is negative

Figure 4.12B) x20 objective Surfactant A staining within some metaplastic cells, it can be seen here that the staining is cytoplasmic

Figure 4.12C) Surfactant A staining positive in the parenchyma, positive type II cells and large amounts of released surfactant can be seen within the parenchyma, this makes cell identification difficult

Prosurfactant C exclusively highlights type II pneumocytes. The precursor protein of surfactant is cleaved before release which gives a clean signal isolated to type II cells. Prosurfactant C antibody was applied to the full panel of 50 COPD patients and 6

control samples. This stain did not highlight any airway epithelial cells. All samples showed extensive parenchymal positive cells and highlighted some but not all metaplastic cells. Examples of staining can be seen in Figures 4.15 and 4.16.

Positive signal was seen in all metaplastic cell locations, adventitial, sub pleural, hilar, fibrosis and alveolar bed. (Figure 4.13B, C, D, F, F). All three phenotypes of metaplastic cell, uniform, macrophage-like and squamous showed some degree of staining. There was a tendency for squamous cells to exhibit fewer numbers of positive cells.

There were areas where the majority of metaplastic cells appeared to be positively stained for surfactant C, there were also areas where few cells showed positive staining. This distribution was reviewed to see if there were any common features between the areas of extensive staining. Strongly expressing areas had evidence of either inflammation or leak. Figure 4.14D shows an area of metaplastic cells with minimal staining, Figure 4.13B shows strong staining in areas containing leak and Figure 4.13C has strong staining associated with lymphocytic inflammation. It was observed that the tissue underlying extensive surfactant positive cells was muscularised or extensive areas of mesenchyme. This tissue was identified on H&E and then examined with the use of Desmin staining, there are areas of smooth muscle cell present, along with areas of mesenchyme.

The slides were examined to look at the amount of positive cells according to cell morphology and cell location. These findings are all represented in Figure 4.14 below. It should be remembered that the incidence is different in different locations and morphologies so when examining the graphs the percentage values should be considered within that location as opposed to comparing numbers between graphs. The graphs show that the majority of uniform cells in adventitia, hilar and sub pleural locations were 100% positive for surfactant C.

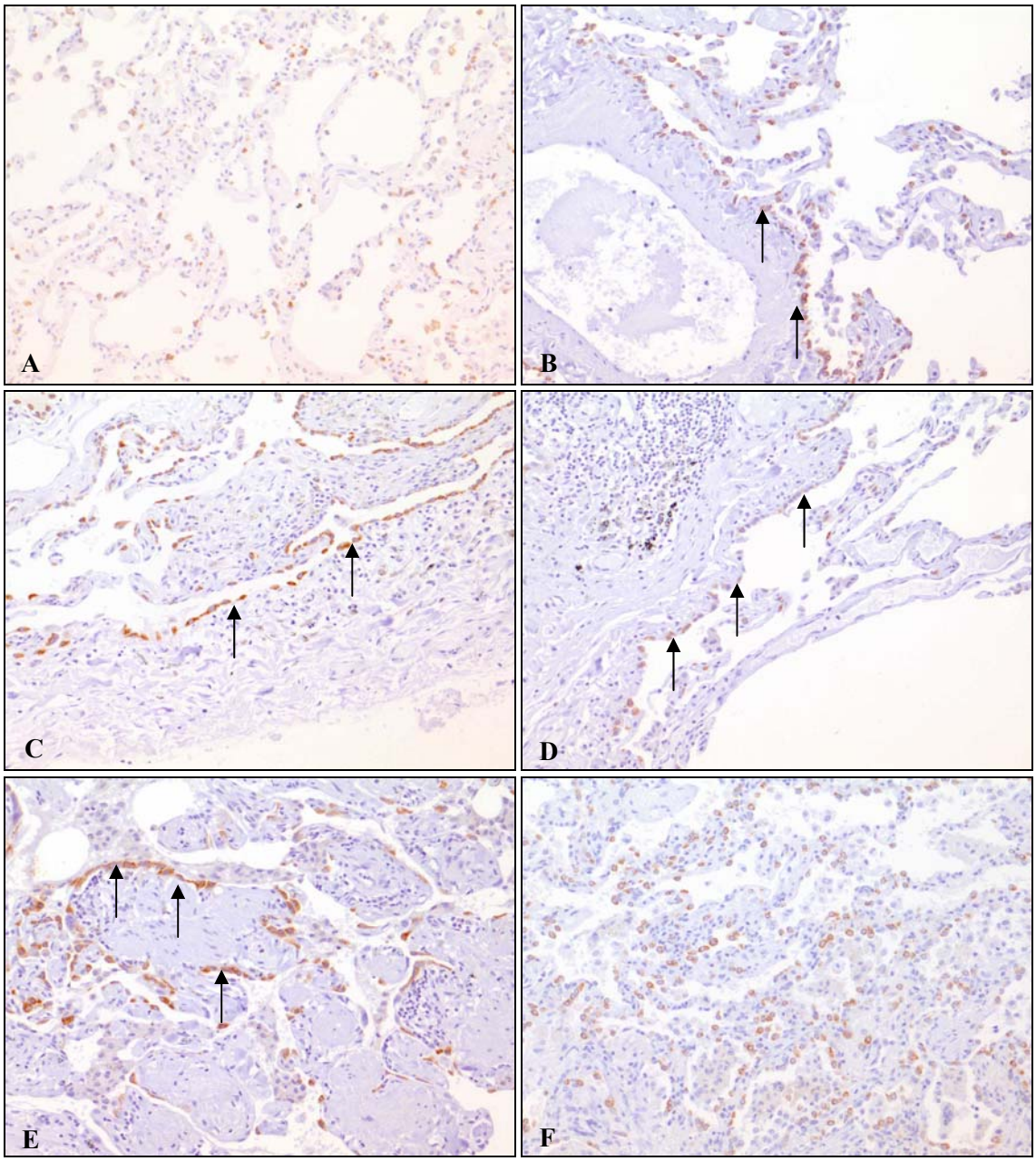


Figure 4.13 Surfactant C IHC in all metaplastic cell locations

Figure 4.13A) Surfactant C positive signal in control parenchyma

Figure 4.13B) Prosurfactant C (Patient 24: Airway changes & fibrosis) staining on adventitial metaplastic cells (arrows) and parenchymal type II cells

Figure 4.13C) Prosurfactant C (Patient 24: Airway changes & fibrosis) staining on sub pleural metaplastic cells (arrows).

Figure 4.13D) Prosurfactant C (Patient 24: Airway changes & fibrosis) staining on hilar metaplastic cells (arrows).

Figure 4.13E) Prosurfactant C (Patient 24: Airway changes & fibrosis) staining on metaplastic cells (arrows) adhered to areas of fibrosis

Figure 4.13F) Prosurfactant C (Patient 17: Airway changes & fibrosis) alveolar metaplastic cells.

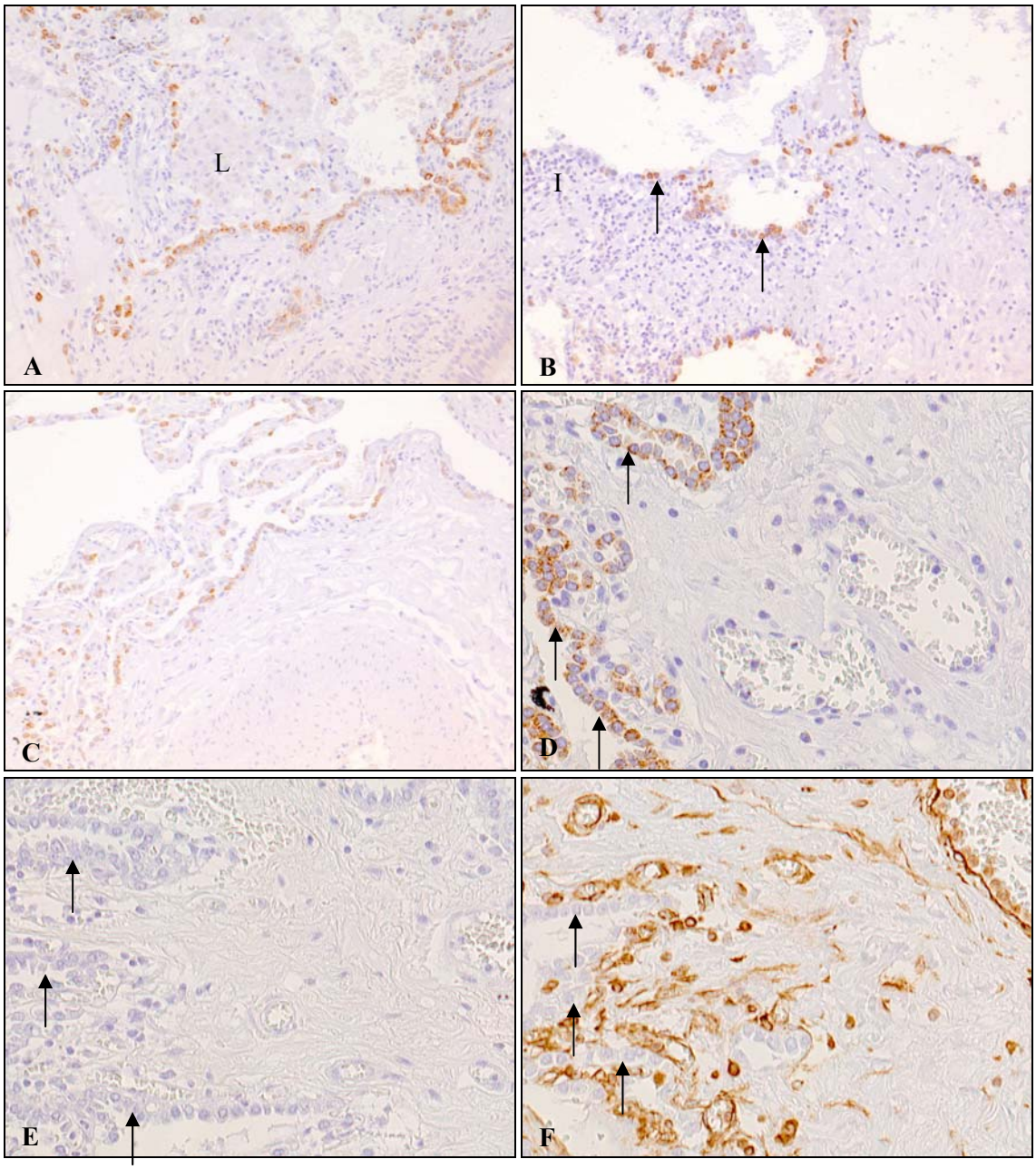


Figure 4.14 Surfactant C IHC signal according to local environment

Figure 4.14A) Prosurfactant C (Patient 20: Airway changes & fibrosis) shows the presence of strong surfactant stain where there is leak (L)

Figure 4.14B) Prosurfactant C stain (Patient 30: Fibrosis) shows the presence of surfactant stain where there is inflammation (I)

Figure 4.14C) Prosurfactant C stain (Patient 37: Goblet cell airway changes) shows minimal weak staining in the absence of leak or inflammation.

Figure 4.14D) x20 objective Prosurfactant C (Patient 5: Airway changes & fibrosis) shows positive stain around the external edge of a muscularised adventitia.

Figure 4.14E) x20 objective Desmin (Patient 5: Airway changes & fibrosis) shows no desmin positive cells within the expanded tissue of the airway adventitia. This suggests that the mesenchyme is not smooth muscle

Figure 4.14F) x20 objective Vimentin (Patient 5: Airway changes & fibrosis) shows positive stain within the expanded mesenchyme and underlying the metaplastic cells, this shows it is mesenchyme tissue, possibly containing fibroblasts

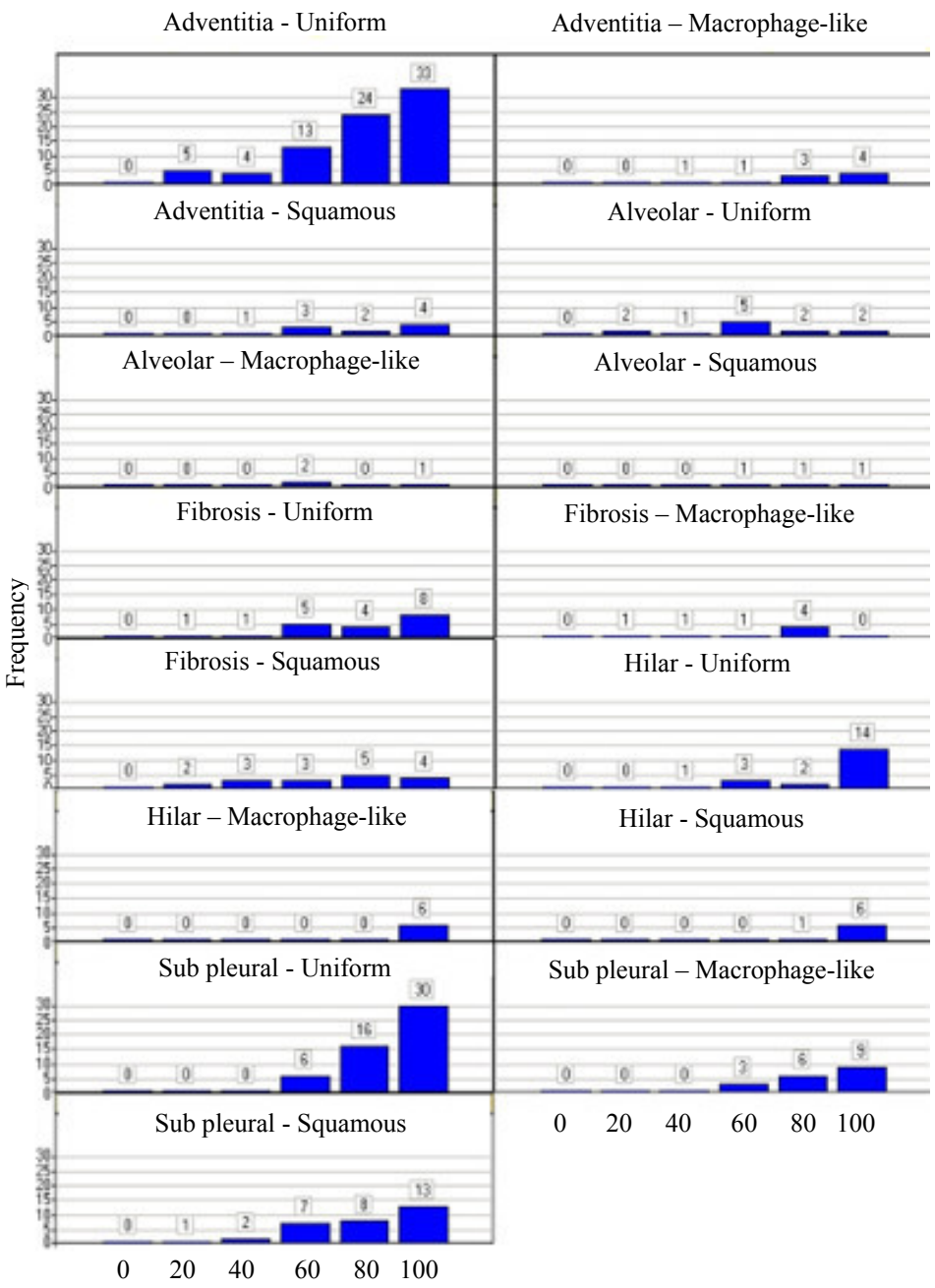


Figure 4.15 Percentage of surfactant C positive cells according to location and cell morphology

The percentage of positive cells based on the various cell locations and morphology. These are frequency plots so the most common lesions show the largest bars on the graphs. Squamous and macrophage-like morphologies were more often approximately 50% positive.

4.5.6 Results for Mesothelial cells: Mesothelin IHC

A mesothelin antibody was used to identify mesothelial cells, which cells are seen on the external surface of pleura. The aim of this marker was to exclude the mesothelial cell from the hypothesis that it contributes to the sub pleural metaplastic cell population. It was thought that if there were a breach of pleura, the cells could spread along the inside of the lining. This marker was run on a sub-panel of patients that showed clear

mesothelial cells on the pleural surface, including 22 blocks that represented 20 patients. 20/22 slides showed positive staining on mesothelial cells on the pleura (Figure 4.16). The two patients that did not show positive mesothelial cells were patient 6 (Goblet cell airway changes) and patient 25 (Airway changes & fibrosis). All 5 locations were represented within the slide set chosen and there was no staining observed on any of the metaplastic cells. The presence of mesothelial cells on a sample was reflective only of anatomical location, plane of cut and activation of the pleura.

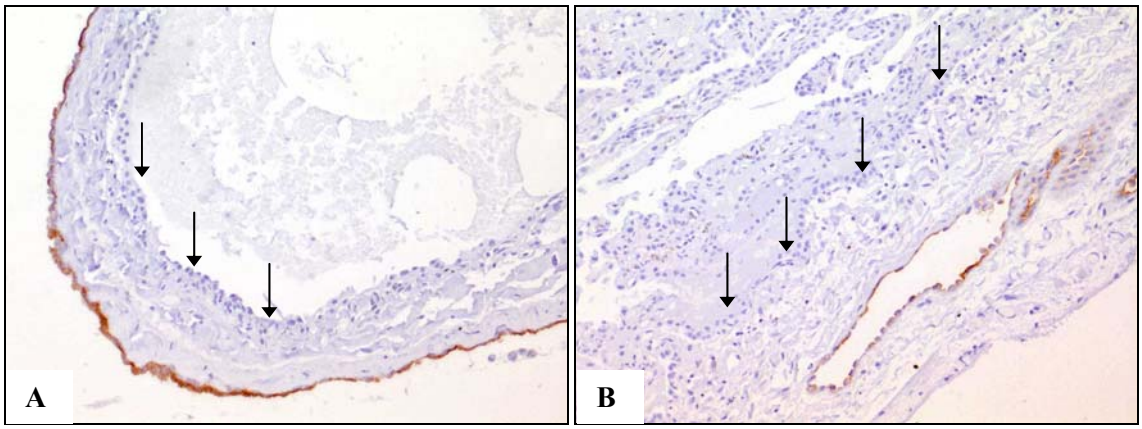


Figure 4.16 Mesothelin staining on the pleura
Figure 4.16A) Mesothelin (Patient 2: Goblet cell airway changes) positive staining on mesothelial cells, these are on the outer surface of pleura the sub pleural metaplastic cells are negative (arrows).
Figure 4.16B) Mesothelin (Patient 20: Airway changes & fibrosis) positive staining on mesothelial cells. These cells appear to be within a cystic inclusion of the pleura this is due to tissue plane of cut, catching the pleura outside edge, this stain is useful to confirm mesothelial cells that may be mis-interpreted as metaplastic cells. Sub pleural metaplastic cells are negative (arrows).

4.6 Results for Progenitor cells

The aim of the antibodies employed in this section was to address the question of whether or not the haematopoietic progenitor cells or bone marrow cells could be contributing to the metaplastic cell population. The cell surface markers used below are indicative of cells that derive from the haematopoietic or bone marrow pools.

4.6.1 Results for CD133 IHC

A CD133 antibody was used as a marker for progenitor cells. The full patient panel of 50 COPD patient and 6 controls were stained with CD133. Figure 4.17 below shows the cell types and number of patients exhibiting positive cells, positive staining examples are shown in Figure 4.18.

Table 4.4 Summary of the patients with positive staining for CD133

Pat	Pathology code	Cell Phenotype
2	Goblet cell airway change	Macs, parenchyma
3	Airway change & fibrosis	Vasc SM
5	Airway change & fibrosis	Epithelium
6	Goblet cell airway change	Epithelium
8	Airway & pleural change	Epithelium & plug
9	Goblet cell airway change	Epithelium
10	Fibrosis & inflammation	Epithelium
12	Airway change & fibrosis	Macs, parenchyma
13	Airway change & fibrosis	Parenchyma, vasc SM, microvasc
14	Fibrosis & inflammation	Endothelium, epithelium, macs
15	Airway change & fibrosis	Endothelium, epithelium, macs
16	Widespread ongoing damage	Vasc SM
17	Airway change & fibrosis	Macs
18	Airway change & fibrosis	Endothelium, epithelium, macs, vasc SM
19	Airway change & fibrosis	Endothelium, epithelium, macs, vasc SM
20	Airway change & fibrosis	Epithelium, macs, lymphocytes
21	Goblet cell airway change	Endothelium, epithelium, macs
22	Goblet cell airway change	Macs, vasc SM, parenchyma, fibroblasts
23	Airway change & fibrosis	Macs, submucosal cells, vasc SM
24	Airway change & fibrosis	Basal epithelium, vasc SM
25	Airway change & fibrosis	Epithelium, macs, parenchyma, vasc SM
27	Fibrosis	Macs, parenchyma
28	Airway change & fibrosis	Epithelium, macs, vasc SM
29	Goblet cell airway change	Macs, vasc SM
31	Fibrosis	Endothelium, macs, parenchyma, vasc SM
33	Airway change & fibrosis	Epithelium, basal
34	Fibrosis	Epithelium
36	Goblet cell airway change	Endothelium, vesicles in airway
37	Goblet cell airway change	Macs (wash)
38	Fibrosis	Macs, parenchyma
39	Airway & parenchymal change	Endothelium, parenchyma, Metaplastic cells
40	Goblet cell airway change	Epithelium, basal, suprabasal
41	Airway changes & fibrosis	Endoth^{elium}, macs, vasc SM, sub mucosal cells
42	Normal	Endothelium & glands
43	Airway & parenchymal change	Epith^{elium}, macs, vasc SM & + cells under mets
44	Airway & parenchymal change	Epithelium, cartilage, glands
45	Goblet cell airway change	Endothelium, macs, microvasc, cartilage
46	Airway & parenchymal change	Glands, cartilage, epithelium, basal
49	Airway & parenchymal change	Epithelium
50	Widespread ongoing damage	Epithelium, basal, cartilage
N2	Normal	Endothelium
N3	Normal	Endothelium, macs
N6	Normal	Macs

Occasional positive epithelial cells were seen in the basal, suprabasal and apical layers (Figure 4.18A). There was also positive cartilage, gland cells, fibroblasts, microvasculature and occasional sub mucosal cells within some samples (Figure 4.18B, C, D). Patient 32 had no positive cells within the sample but there were positive vesicles underlying the metaplastic cells surrounding the adventitia of an airway. These were not cell orientated and were very isolated. Patient 39 (Airway & parenchymal change) showed positive cells around a vessel which appeared to be bronchiolisation (Figure 4.18E). In all instances of positive staining there were only a few positive cells. Positive cells metaplastic cells were usually one or two within the population. All positive staining is summarised in Table 4.4.

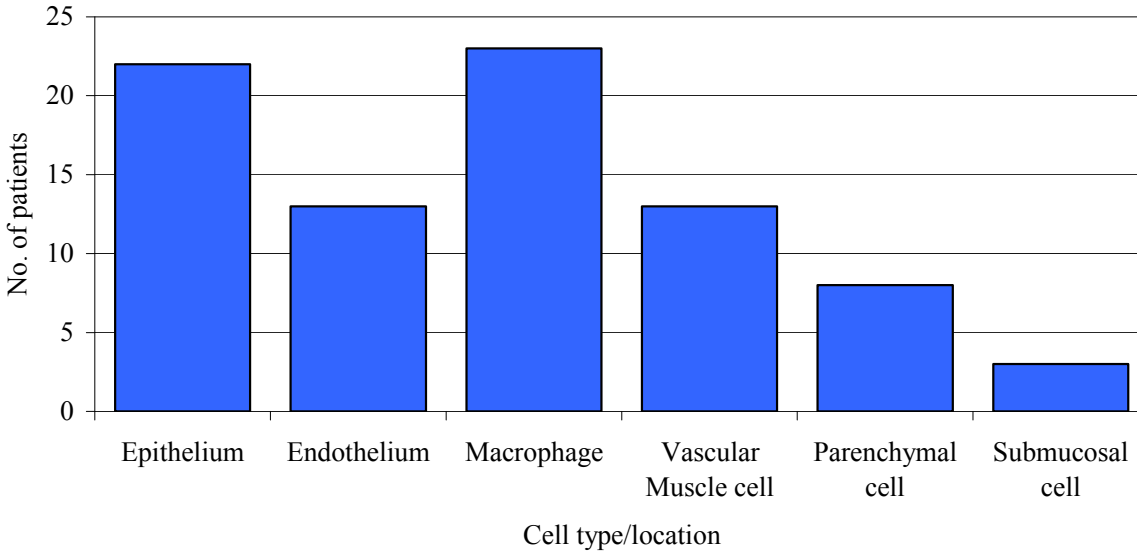


Figure 4.17 Cell phenotype & frequency of CD133 positive metaplastic cells
The number of patients who exhibit different cell phenotypes staining positive with CD133. Cells within the parenchyma and sub mucosa were difficult to identify as specific cell phenotypes, therefore the location of the cells was noted

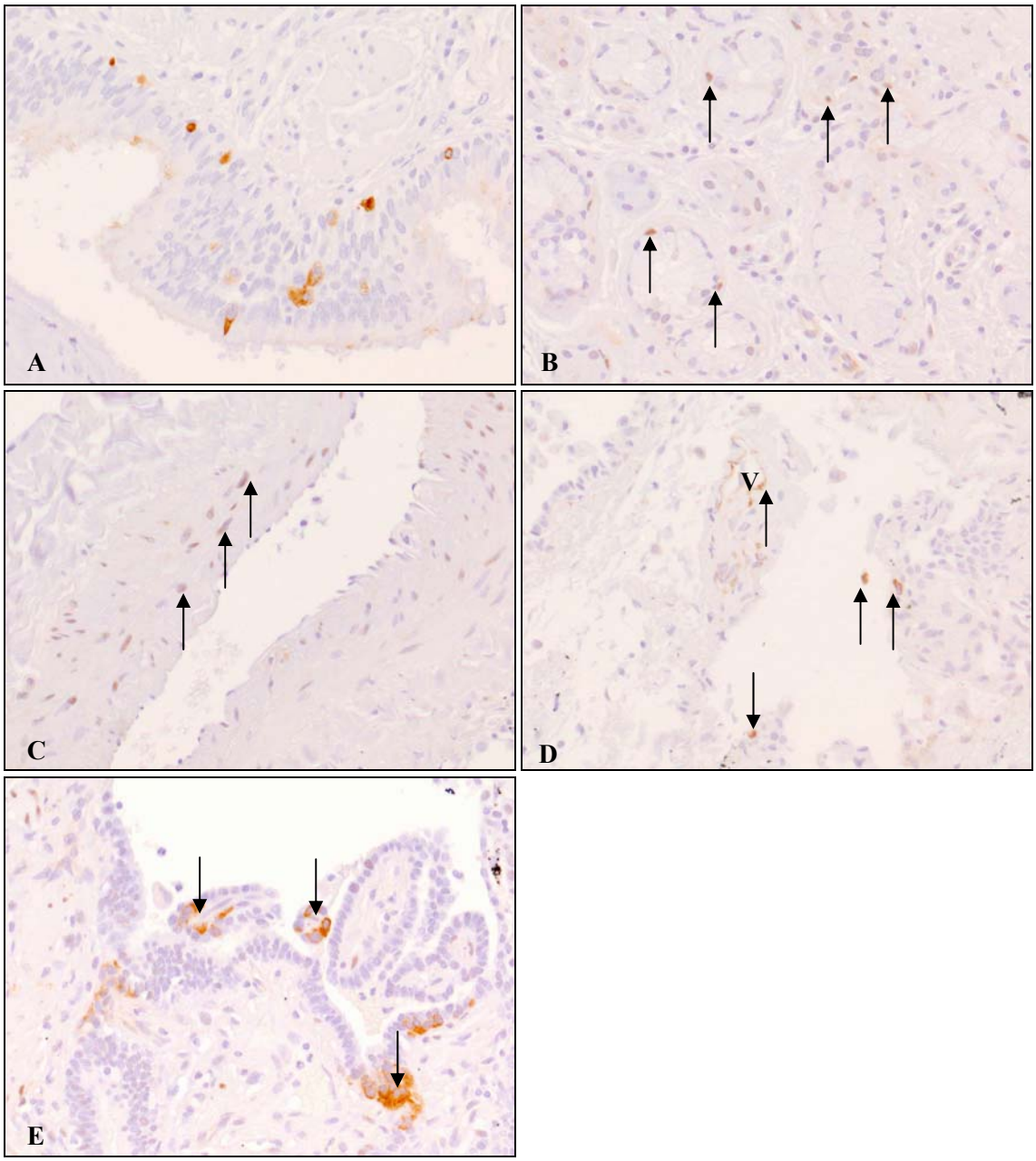


Figure 4.18 CD133 Progenitor cell IHC

Figure 4.18A) x20 objective CD133 (Patient 5: Airway changes & fibrosis) positive staining on a cluster of airway epithelial cells on COPD tissue. There are positive basal and apical cells.

Figure 4.18B) x20 objective CD133 (Patient 44: Airway & parenchymal change) positive staining on isolated cells within an area of mucus glands (arrows).

Figure 4.18C) x20 objective CD133 (Patient 22: Goblet cell airway changes) positive staining on smooth muscle cells within a large vessel (arrows).

Figure 4.18D) x20 objective CD133 (Patient 39: Airway & parenchymal change) positive staining on endothelial cells within a small vessel (V), positive metaplastic cells (arrows).

Figure 4.18E) x20 objective CD133 Patient 39 Airway & parenchymal change) positive staining on the only example found in the COPD cohort of positive bronchiolisation within the parenchyma.

4.6.2 Results for CD34 IHC

A CD34 antibody was used as an alternative marker for haematopoietic progenitor cells, representative staining is shown in Figure 4.20. This is a widespread cell surface molecule found on endothelial cells and lymphocytes within tissue as well as cell of

haematopoietic lineage. This marker was examined on the full panel of 50 COPD patients and 6 controls, staining may be seen in Figure 4.19. CD34 was found on all vessels and capillaries (Figure 4.20C). There was also some stain on matrix throughout most vessel walls, pleura and connective tissue (Figure 4.20D). The metaplastic cells in all locations showed some positive cell staining (Figure 4.20E, F) in all except Patient 23 (Airway change & fibrosis) and Patient 33 (Airway change & fibrosis). Both these patients had only a few changes noted within all lung compartments, with no mention of inflammation.

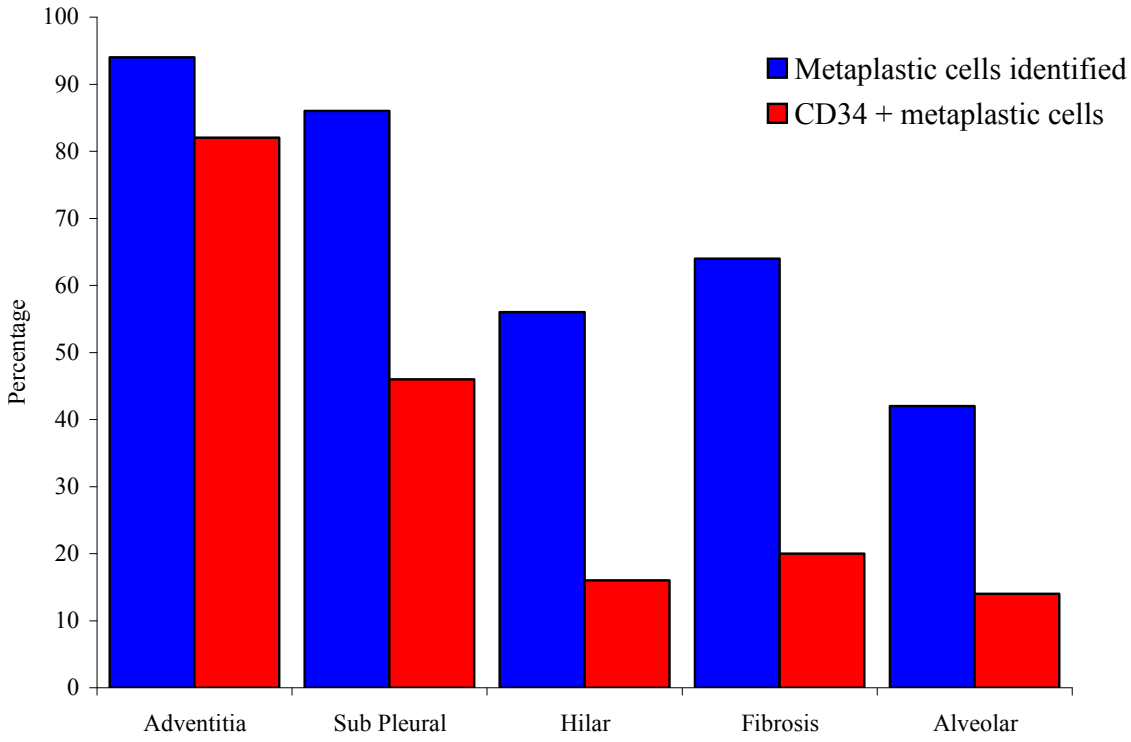


Figure 4.19 Distribution & frequency of CD34 positive metaplastic cells
The total percentage of samples with metaplastic cells in each of the locations (blue). The percentage of samples that contain some CD34 cells in each of the locations is plotted in purple. This shows that most of the adventitial metaplastic cells contained some positive CD34 cells, whereas numbers were much lower in the hilar, fibrosis and alveolar regions.

Positive cells were all either uniform metaplastic cells or appeared to be smaller cells sitting between metaplastic cells. These positive cells were very infrequent. All macrophage-like and squamous metaplastic cells were negative for CD34. 41/50 COPD samples had examples of positive adventitial metaplastic cells, 8/50 had hilar examples, 7/50 had fibrosis examples, 4/50 had alveolar examples and 20/50 had examples of sub pleural positive metaplastic cells. The distribution and frequency of metaplastic cells are represented in Figure 4.19.

The proportions of positive samples according to location may reflect the fact that the cells come in through the circulation. Details of those patients without any adventitial positive cells are summarised below in Table 4.5.

Table 4.5 Patients without CD34 positive adventitial metaplastic cells

Pat	Pathology Code – group	Uniform
4	Airway change & fibrosis	1/3 samples + sp
14	Fibrosis & inflammation	+ sp
16	Widespread ongoing damage	
19	Airway change & fibrosis	+ alv
35	Fibrosis & inflammation	+ sp, at ends
37	Goblet cell airway change	+ alv, + sp
38	Fibrosis	+ sp, + hilar, + fib

As CD34 highlights endothelial cells it is important to confirm that the CD34 positive cells are not endothelial cells. This may be achieved by comparison with an endothelial marker such as CD31 (Figure 72A).

CD31 marker has not been run on the specific blocks being used in this investigation, however numerous COPD samples have been examined within our lab for the presence of CD31 (PECAM-1). CD31 has been successfully highlighted on both the large and small vasculature of the lung. There are no instances of CD31 staining the metaplastic cell population (Figure 4.20B).

4.6.3 Results for c-kit IHC

An antibody for c-kit (CD117) was also used as a marker of progenitor cells. Staining was examined on COPD patients 1-10, examples are shown in Figure 4.21. Positive cells showed cytoplasmic staining. There were numerous single sporadic positive cells throughout the tissue in all samples, cell phenotype and distribution varied between patients (Figure 4.21A, B, C). Patient 3 (Airway changes & fibrosis) showed positive cells in an area of congestion and inflammation. Patient 4 (Airway changes & fibrosis) has some positive cells in a discontinuous area of sub pleural metaplastic cells. These may be metaplastic cells or part of the population directly underneath. Patient 5 (Airway changes & fibrosis) showed the highest prevalence of positive cells. These cells appear to be a mixture of inflammatory cells, epithelial cells, fibroblasts and muscle cells. To conclude, no definite positive metaplastic cells could be identified with c-kit staining.

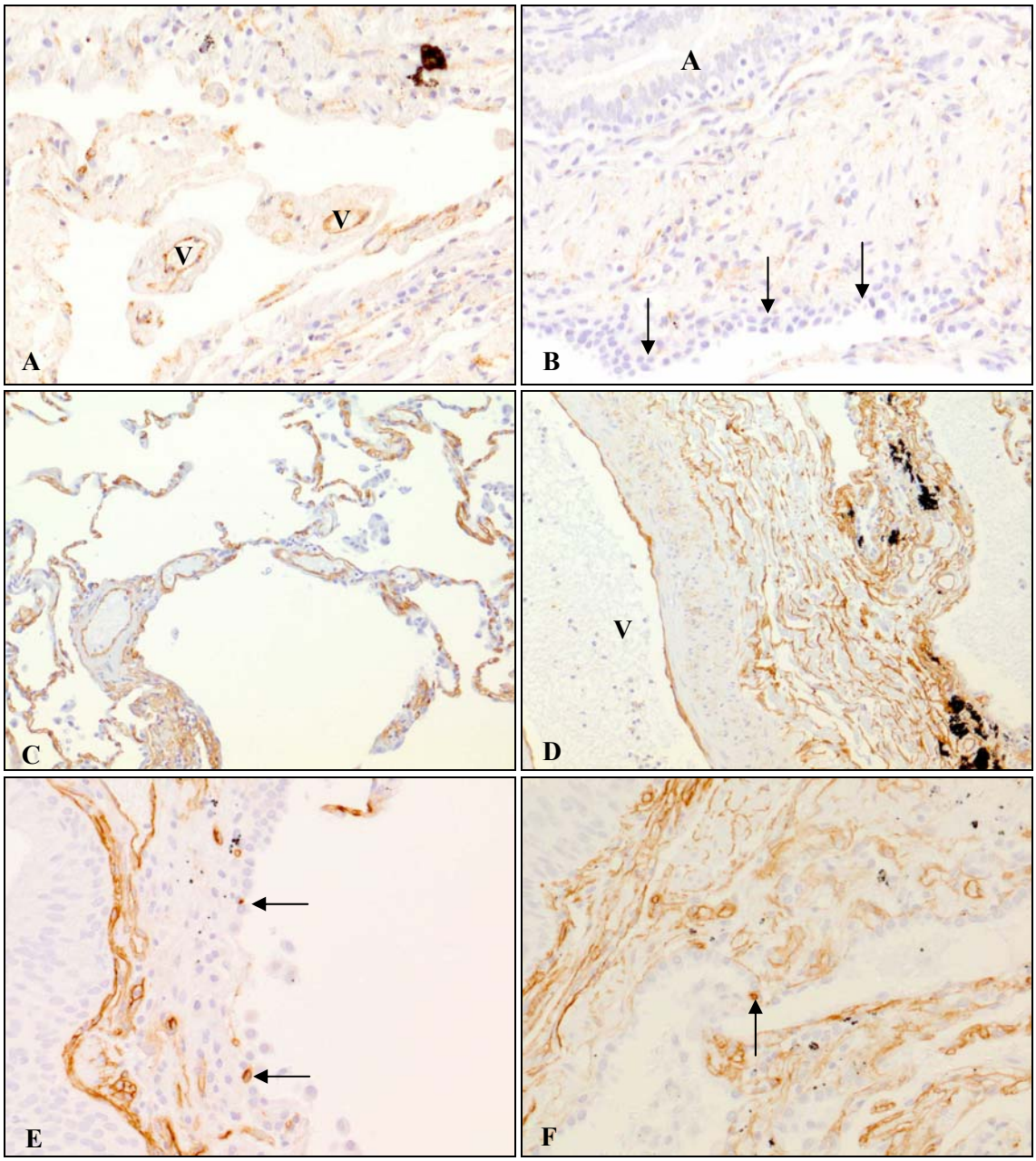


Figure 4.20 CD34 & CD31 IHC staining

Figure 4.20A) CD31 positive staining Patient 4: Airway changes & fibrosis) on small vessels (V) within one COPD patient. All intact endothelium including capillaries stain positive for CD31.

Figure 4.20B) CD31 staining (Patient 4: Airway changes & fibrosis) on an area of metaplastic cells. The metaplastic cells (arrows) surround the adventitia of an airway (A), these are all negative for CD31 staining.

Figure 4.20C) CD34 positive staining (Patient 3: Airway changes & fibrosis) showing all endothelial cells within vessels of all sizes.

Figure 4.20D) CD34 positive staining (Patient 1: Airway changes & fibrosis) showing endothelial staining on a large vessel (V), there is also extensive positive staining with the muscle matrix in the adventitia of the vessel and out into the parenchyma.

Figure 4.20E) x20 objective CD34 positive staining (Patient 46: Airway & parenchymal change) on isolated metaplastic cells (arrows).

Figure 4.20F) x20 objective CD34 positive staining (Patient 5: Airway changes & fibrosis) on isolated metaplastic cells the positive cells are highlighted with arrows.

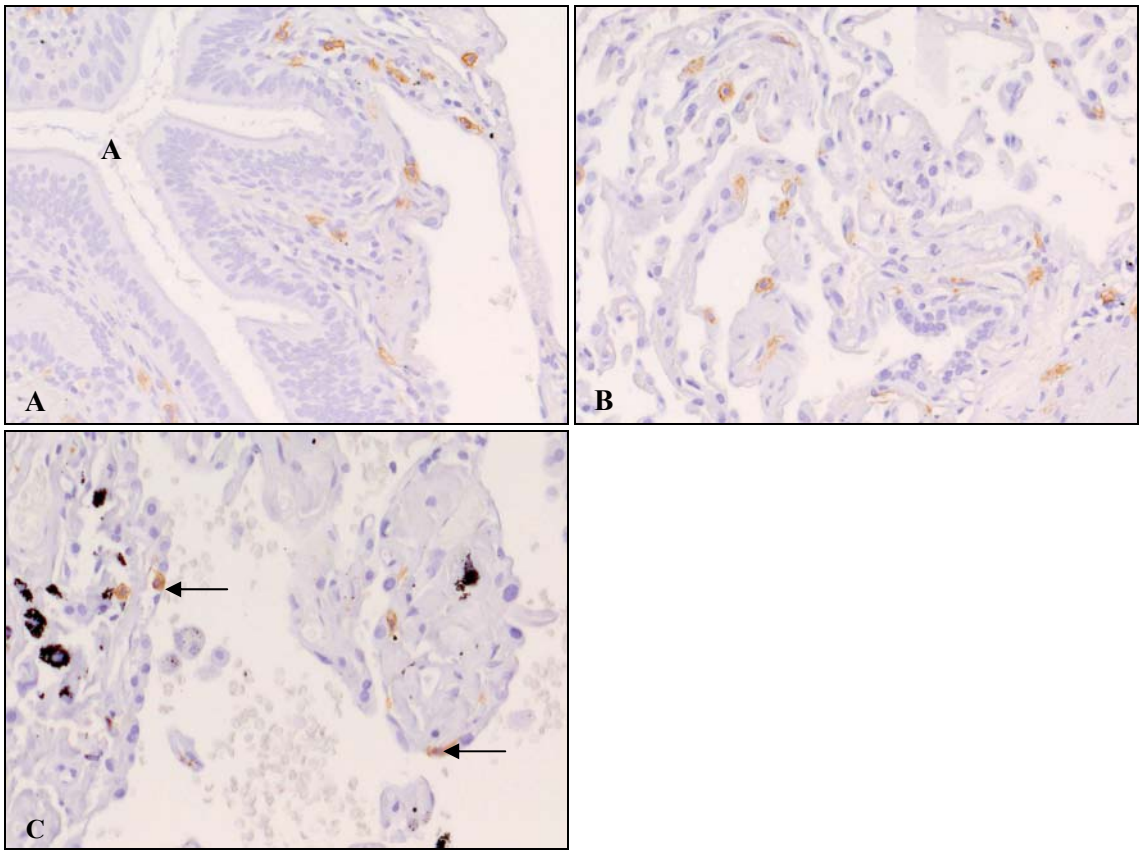


Figure 4.21 c-kit IHC staining

Figure 4.21A) x20 objective c-kit positive staining (Patient 4: Airway changes & fibrosis) on cells in the sub mucosa of a small airway of a COPD patient. These cells are probably inflammatory cells.
Figure 4.21B) x20 objective c-kit positive staining (Patient 4: Airway changes & fibrosis) in cells within the parenchyma of a COPD patient. These cells are probably inflammatory cells.
Figure 4.21C) x20 objective c-kit positive staining (Patient 4: Airway changes & fibrosis) within the sub pleura of a COPD sample, the cells highlighted with arrows appear to be metaplastic cells.

4.7 Results for ultrastructure analysis: electron microscopy

To further examine the characteristics of metaplastic cells in greater detail electron microscopy (EM) was used. The samples used were near to but not the actual blocks that were used for immunohistochemical analysis.

Airway epithelial cells and type II pneumocytes were used for comparison to the metaplastic cells, these are shown in Figure 4.22. Epithelial cells were mainly ciliated. A round cell nucleus was identifiable as were the internal structures such as golgi and rough endoplasmic reticulum. Tissue preservation was not optimal for EM so desmosomes or cell-cell junctions were not identifiable. Figures 4.22A and 4.22C contain ciliated cells and a secretory cell containing electron dense granules. Figure 4.22E has numerous secretory cells that contain large glycolytic packages; these are probably goblet cells.

Type II cells were identified within the samples. Lamellar bodies have been washed out during paraffin processing, however, there was a clear space where this lipid based structure had been. There were also microvilli visible on the cell surfaces. The nuclei were a slightly irregular rounded shape. A narrow basement membrane can be seen under some type II cells.

Metaplastic cell examples are shown in Figure 4.23. Metaplastic cells appear to be sitting on a membrane. There are spaces within the cells that resemble the spaces of the lamellar bodies of type II cells. This would suggest the cells produce surfactant, which has been demonstrated in section 4.5.5 by immunohistochemistry. The nucleus size was similar to that of epithelial cells, however nuclei were irregular shapes with grooves and convolutions. Nuclear colour ranged from grey to intense black in areas, some contained dark nucleoli. The metaplastic cells have small microvilli, not cilia. These projections are similar to those seen on type II cells.

Figure 4.22 Electron microscopy of Airway epithelium and type II cells.
All scale bars in the bottom right of the pictures show 10μm.
Figure 4.22A) This shows an area of airway epithelial cells, the airway lumen is at the top of the picture. There are cilia protruding into the lumen on most cells (arrow). There are two cells containing granules, these are secretory epithelial cells, possibly goblet or Clara cells (S).
Figure 4.22B) Shows a type II cell (II) adhered to a membrane within the parenchyma. There are small microvilli present on the external surface of the cell. The round spaces within the cell represent where lamellar bodies have been lost from the cell during processing.
Figure 4.22C) Shows an area of epithelial cells with the luminal surface on the right of the picture, the full length of cilia is visible on these cells. There is a large secretory cell in the centre of the picture (S) with the nucleus visible at the base of the cell. Other epithelial cells show quite open cytoplasm.
Figure 4.22D) Another example of a type II cell (II).
Figure 4.22E) Shows epithelium with the luminal surface at the top of the picture, there are secretory goblet cells showing pale packages of mucus within the cytoplasm. The layer underneath these cells contains much smaller cells, probably basal cells.
Figure 4.22F) Another example of a type II cell (II).

Figure 4.23 Electron microscopy of Metaplastic cells
Figure 4.23A) Shows two metaplastic cells (M) adhered to an electron dense matrix. The cells have microvilli on the apical surfaces. The nuclei are quite pale and have an irregular shape.
Figure 4.23B) Shows metaplastic cells, possibly forming two layers; this may be the result of plane of cut on the sample. These cells also show surface microvilli and an irregular nucleus although the nuclei here are darker. There are occasional spaces in some of the cells these may represent where lamellar bodies were. There is a visible matrix at the base of the cells.
4.35C) Shows metaplastic cells that are similar to those seen in Figure 4.23B, the multi-layer nature of these cells appears to be plane of cut as there is no clear attachment. The cells in this example seem to have smaller paler nuclei and there is little evidence of lamellar bodies. The right of the picture shows the cells are on basement membrane (arrows).
Figure 4.23D) Shows metaplastic cells in s single layer, with large dark nuclei, evident microvilli and a definite basement membrane (arrows).
Figure 4.23E) Shows a metaplastic cell with microvilli, dark irregular nucleus on a clear basement membrane.
Figure 4.23F) Shows metaplastic cells in a string across the picture, there are numerous holes suggestive of lamellar bodies and a range of cell shapes and sizes. The nuclei appear quite large but are open and perhaps lacking in contents, this may be in part due to processing.

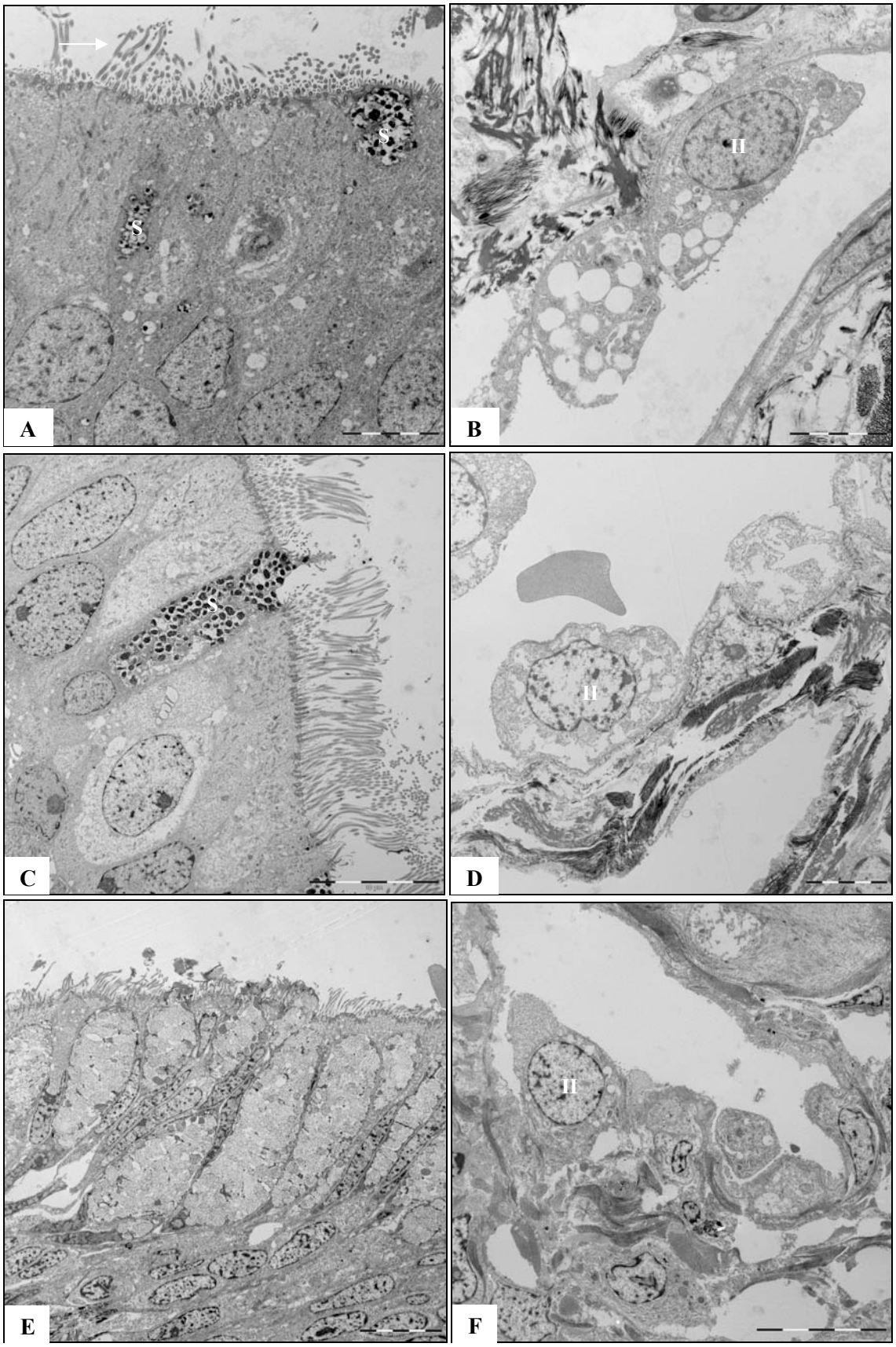


Figure 4.22 Electron microscopy of airway & type II cells

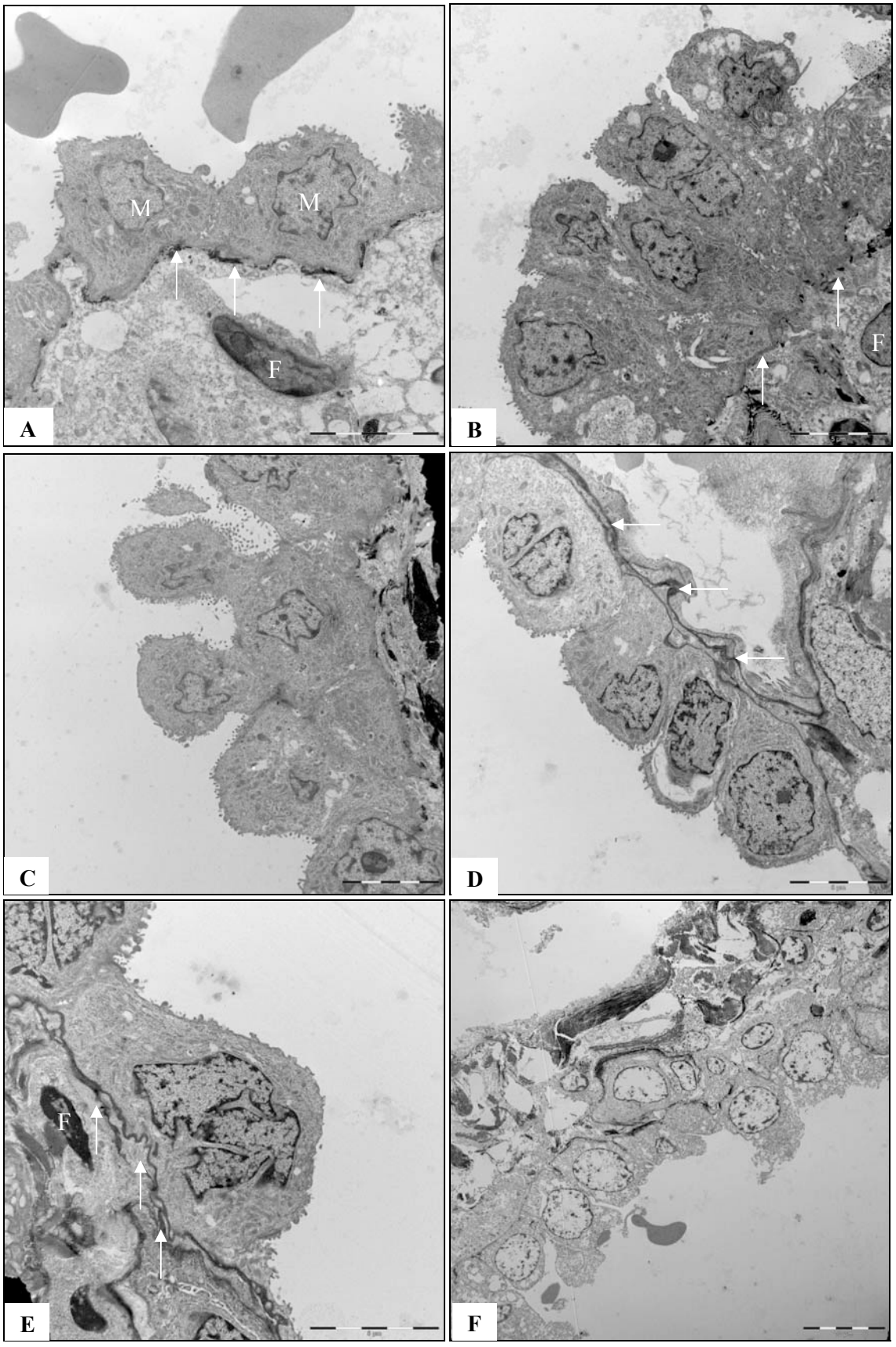


Figure 4.23 Electron microscopy of metaplastic cells

4.8 Discussion of the phenotype of diffuse metaplasia in COPD

Pan cytokeratin staining on the COPD cohort and resection control samples provided confirmation that the metaplastic cells were all of an epithelial phenotype. These occur in all five locations; adventitia, sub pleural, hilar, fibrosis and alveolar. Pan cytokeratin staining was observed in squamous flattened, uniform round and irregular macrophage-like metaplastic cells. Cytokeratin staining was shown on airway epithelium, type II cells and mesothelial cells, which has been previously reported (Broers *et al.* 1989). Cytokeratins are found exclusively on epithelial cells suggesting the origin of metaplastic cells is airway epithelium, mucus gland progenitors, type II cells or mesothelium, unless the cells originate outside the lung.

4.8.1 Resident progenitor cell populations

CK5/6 was used to examine basal cell phenotype, as this marker shows some overlap with squamous cells, ΔN-p63 was also used to confirm basal cell characteristics. ΔN-p63 was consistently observed in airway basal epithelium. It was also seen within mucus gland basal cells. Both findings were expected and have been shown previously (Wang *et al.* 2002, Chilosi & Doglioni 2001). Occasionally there were positive cells within the parenchyma. These localisations were examined and determined to be either airway remnants adjacent to a vessel with some evidence of airway basement membrane or bronchiolisation that showed CK5/6 staining along with CK14 areas of squamous change. Figure 4.24 is a diagram of the bronchiolisation. There were no metaplastic cells that demonstrated either CK5/6 or ΔN-p63 basal cell markers. It may be concluded that basal cells are not contributing to the metaplastic cell population. There did not seem to be any correlation with airway basal cells number and the presence or absence of adventitial metaplastic cells.

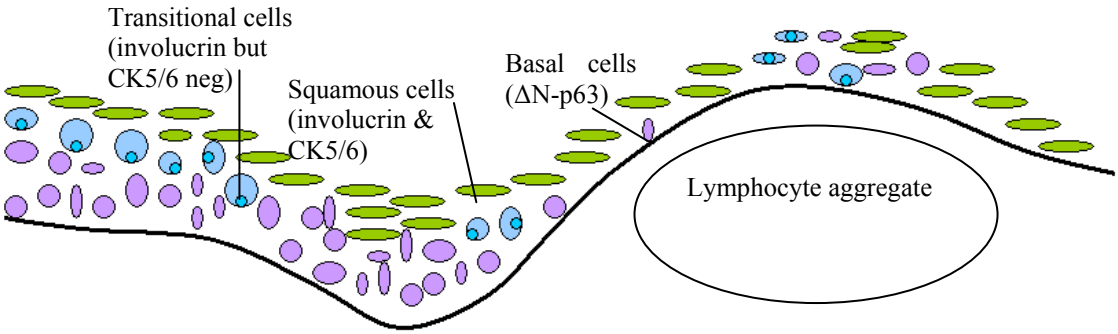


Figure 4.24 Diagrammatic representation of bronchiolisation
Pictorial representation of the area of bronchiolisation. Purple represents the basal cells, blue represents the mucinous transitional cells and green represents the squamous cells. The transitional cells will mature to ciliated cells if the stress and damage signals are removed. The lymphocyte aggregate may be contributing to the maintenance of the squamous immature cell phenotype.

The next progenitor cell considered was the Clara cell. This may have been a more logical candidate as these are thought to have more influence in smaller airways. CC10 was used to highlight Clara cells and successful staining was achieved in all samples. There was some vessel and tissue plasma leak staining but this was easily identified and discounted, as it is acellular. Airway Clara cell number varied between patients and according to airway diameter. Smaller airways tended to have a higher proportion of positive cells, with mid-sized airways showing a greater range of stain and the large airways usually had quite low levels of staining. This corroborates findings that have been reported previously (Boers, Ambergen & Thunnissen 1999). The large airways with moderate (++) staining was the only staining intensity that showed a significant difference between the airways with and without surrounding metaplastic cells. The large airways with strong moderate staining were more likely to have surrounding metaplastic cells.

As CC10 staining has not been co-localised with progenitor cell or type II cell markers the three possible sub populations of Clara cell previously identified by Snyder, Zemke & Stripp (2009) cannot be considered. Although there was airway staining throughout the samples, there were no instances of CC10 positive metaplastic cells. Airway remnants with clear basement membrane and in some cases ciliated cells also contained some CC10 positive cells. There might have also been some positive staining within some of the areas of bronchiolisation. It may therefore be concluded that the Clara cell, a key peripheral progenitor is also not contributing to the metaplastic cell population and its presence within the airways does not appear to influence adventitial metaplastic cell presence. If there were any influence it was only suggested in the larger of the airways examined.

Goblet cells are not true resident progenitor cells, these cells act as transit amplifiers. It was important to consider this cell population as the metaplastic cell role and phenotype is unclear and the secretory and immunomodulatory features of goblet cells may be beneficial in the lung periphery. In normal airways the predominant goblet cell mucin is mucin 5AC. This was employed along with tinctorial stains to highlight mucus and identify this cell population. Goblet cell hyperplasia is thought by some groups to be present in COPD airways (Saetta *et al.* 2000). Goblet cells were readily identifiable in the airways as expected. The metaplastic cells were negative for mucin 5AC, Alcian blue and PAS in all samples. Alcian Blue staining highlights both sulphated and carboxylated mucopolysaccharides. The dye has a positive charge and will bind to low density negatively charged mucus. The PAS stain highlighted all mucus containing cells, including goblet cells and Clara cells. The distribution of goblet cells alters through the respiratory tree. Goblet cells are absent from airways less than 1mm in diameter becoming more frequent moving up into the bronchi. Normal airways have around 8 goblet cells/mm² in airways with 1-3mm diameter (Mercer *et al.* 1994). Many airways observed in this study seemed to have greater goblet cell numbers. The work carried out in chapter 3 suggests that an increased number of goblet cells in the airways was predicative of surrounding metaplastic cells, so although goblet cells do not directly contribute to metaplastic cells, the airway goblet cells may influence metaplastic cell presence. Statistical analysis carried out on the goblet cell staining suggests that the airways with metaplastic cells were significantly different to proportions of airways without surrounding metaplastic cells in the following categories, small negative, medium negative, medium ++ and medium +++, also large ++. This suggested that as the airways increase in size and have larger proportions of cells positive, there was increased likelihood of surrounding metaplastic cells.

The tinctorial stains Alcian Blue and PAS, along with immunohistochemistry for mucin 5AC also highlight both mucus and serous cells, all gland cells. PAS highlights cells that are producing neutral mucus with a magenta stain and Alcian Blue highlights serous cells with a pale blue stain (Lamb & Reid 1970). The absence of metaplastic cell staining as mentioned above suggests that mucus gland basal cells are not contributing to the metaplastic cell. This is further confirmed by the lack of ΔN-p63 basal cell staining within the metaplastic cells as this marker was also seen within the mucus glands as expected (Wang *et al.* 2002). This is perhaps unsurprising as metaplastic cells

are observed right into the lung periphery even lining the pleural internal surfaces. Airway mucus glands are found adjacent to bronchi (first eight lung generations) whilst cartilage is still present. Smaller bronchioles (remaining 15 lung generations) do not have mucus glands or cartilage associated. The smallest bronchi seen have a diameter of approximately 1.86mm.

Surfactant markers were used to examine the metaplastic cell population for type II cell characteristics. In normal tissue surfactant A seen in type II cells, Clara cells and serous cells in sub mucosal glands, gives an indication that the phenotype is one of these three cell types (Soerensen *et al.* 2005, Saitoh *et al.* 1998). In the samples examined, surfactant A was observed in a significant sub-population of metaplastic cells in all locations. Lack of staining with CC10 has discounted Clara cell contribution and the absence of Alcian Blue, PAS or ΔN-p63 discounts mucus gland progenitors. Type II cells are the only remaining cells that produce surfactant A. If the metaplastic cells are type II cells, the population may be capable of self-renewal.

To further investigate possible pneumocyte phenotype, prosurfactant C was used. It is only produced in the lung by type II pneumocytes. Prosurfactant C is the precursor of surfactant C, cleaved released surfactant is not detected by the prosurfactant specific antibody, making staining cleaner to interpret. Staining in a sub-population of metaplastic cells gives weight to the theory that type II cells contribution to metaplastic cells. Not all cells stain positive for either surfactant A or prosurfactant C, co-localisation was not carried out to determine if there was a surfactant negative population. The distribution of surfactant positive cells was examined according to location and cell morphology. An overview of cell characteristics associated with areas of extensive cell staining was gained, more muscularised locations typically adventitial and sub pleural showed the largest numbers of positive cells. Positive staining also seemed to be associated with the uniform metaplastic cell morphology, however, this may simply reflect that these cells are the predominant phenotype observed. Type II cells phenotype is in part determined by basement membrane collagen and matrix components. Laminin and fibronectin ratio in culture has been shown to drive the cell towards a more type I or type II cell phenotype (Rannels *et al.* 1987). Adhesion to muscle would mean a lack of these phenotypic signals and may produce a ‘default’ epithelium.

Surfactant production may reflect cell maturation, mixed origin or a divergent mature phenotype. cAMP and other hormonal triggers are responsible for controlling the maturation of type II cells *in utero*. These are necessary for lamellar body formation and surfactant production. Type II cell storage vesicles for surfactant are depleted or even disappear when cell division is about to occur, however type II cells still possess some lamellar bodies so progeny should be capable of rapidly producing surfactant. Therefore negative cells would support a theory of maturation of either a primitive or bone marrow progenitor cell (Shannon, Gebb & Nielsen 1999). There are also growth factors that can illicit hyperplasia in type II cells. Ulich *et al.* (1994) have shown that a single tracheal dose of KGF in mice can lead to type II cell hyperplasia in mice. Sauleda *et al.* (2008) have examined levels of KGF and HGF in COPD patients, showing that serum levels of HGF increased and this in turn may stimulate type II hyperplasia.

Increased numbers of surfactant positive cells correlated to areas of leak. Surfactant is comprised of surfactant proteins and phospholipids. Surfactant within the alveolar bed helps lower surface tension; improving lung compliance as it makes inspiration and expiration easier. Surfactant may also play a role in preventing airway collapse in airways smaller than 0.5mm when breathing out (Macklem, Proctor & Hogg 1970). Lowering alveolar surface tension helps to clear fluid. Surface tension forces should draw fluid out of capillaries into the alveolar bed, by lowering surface tension it prevents this fluid influx (Hohlfeld, Fabel & Hamm 1997).

Surfactant A is also associated with innate defence modulation. It can regulate dendritic cell maturation, control T cell proliferation, increase phagocytosis of microbes and apoptotic cells as well as aggregating pathogens into clumps (Brinker *et al.* 2001). These immuno-modulatory roles may be beneficial in COPD individuals who are at higher risk for infection: it may also explain why surfactant levels are increased in areas of inflammation. Mutti (2006) has shown increased surfactant D in COPD patient's serum, surfactant A and surfactant D both have similar immuno-modulatory functions. Increased defence by surfactants may help slow infection spread and protect lung structure. Metaplastic cells may be a source of increased surfactant. Surfactant lowers the release of inflammatory cell cytokines and suppresses transcription factor activation. This helps to attenuate inflammation, so is important in regulating inflammatory resolution. Macrophages and type II cells are responsible for surfactant clearance so it may be expected that surfactant levels would be lower in the presence of macrophages.

It may only be released surfactant that is cleared by macrophages, therefore it is more likely that macrophages would be associated with lymphocytic inflammation or leak (Wang *et al.* 2006b).

As mesothelial cells line the external surface of pleura and metaplastic cells are often observed lining the internal surface, the possibility of mesothelial cells colonising the internal surface was investigated. Mesothelin is produced exclusively by mesothelial cells so was used as a specific indicator. Mesothelin has been used previously to identify mesothelial derived tumours. This marker was found exclusively on mesothelial cells of the pleura within all samples.

The above staining accounts for all the resident lung progenitor populations and strongly suggests that metaplastic cells are type II cells. Whilst prosurfactant C identifies a sub population, there is a further population of metaplastic cells clearly marked with generic epithelial markers which has not, as yet, had any specific population markers identified.

Diffuse metaplastic cells in COPD do not demonstrate cilia however, there are instances of bronchiolisation. These often extend from damaged airways and show some remnants of basement membrane underlying the cells. This escaped airway population has sporadic CK5/6 positive basal cells, CC10 positive Clara cells and some ciliated epithelial cells. The look and morphology of these areas is more similar to airway epithelium than the metaplastic cells. These are not areas of metaplasia but may also result from airway damage.

The EM work has allowed confirmation of cell phenotypes that can be excluded from possible contributors of metaplastic cells. Nettesheim *et al.* (1990) used EM to examine airway progenitors. This included demonstration of Clara cells with numerous dark osmiophillic and filamentous granules and were without microvilli. Metaplastic cells do not contain dark or filamentous granules, but do have surface microvilli. Immunohistochemistry for CC10 supports the conclusion that metaplastic cells are not contributed to by Clara cells.

Key EM features of neuroendocrine cells are cell surface microvilli, small solitary halo granules and a generally darker cell appearance. Reznik-Schuller (1977) looked at drug-induced damage of hamster lung and used EM to examine some of the epithelial phenotypes; neuroendocrine cells, Clara cells and tumour cells. The features of

neuroendocrine cells do not share any characteristics of the COPD metaplastic cell. Hamster tumour cells appeared similar to the uniform metaplastic cells in the COPD lung, this supports the need to examine the possibility of a pre-neoplastic lesion.

Stockstill *et al.* (1995) used an ozone exposure model to elicit extensive alveolar compartment metaplasia, comprising of ciliated, Clara and cuboidal cells. EM demonstrated Clara cells similar to those in terminal bronchioles. Ciliated cells appeared similar to airways but the cilia were more swollen in the metaplastic cell population. There was also another cell type that had fibre bundles, basal bodies, granules and glycogen deposits. From the photographs there was no evidence of lamellar bodies and none of these cells resembled the COPD metaplastic cells. This highlights that the lung can produce a range of metaplastic responses.

EM also allows the diagnosis of type II cell to be further interrogated. *In vitro* work showed type II culture produced a single layer of cells. EM showed lamellar bodies packed around the nucleus that were similar to those seen in adult type II cells treated with hormone (Ballard *et al.* 1986). Other work has looked at rat type II cells contrasting neonatal, juvenile and adult tissue. The day old rat had fewer lamellar bodies (Farioli-Vecchioli *et al.* 2001). Clegg *et al.* (2005) have used a *S aureus* infection model to examine alveolar response to damage. Flattened cells with small lamellae bodies were thought to be type II cells differentiating into type I cells. The *S aureus* infected lungs also had parenchymal cuboidal epithelial cells that contained few lamellae bodies, a reduced cytoplasm and surface microvilli. These cuboidal intermediate cells share many features of COPD metaplastic cells although metaplastic cells in COPD have much more variable cytoplasm ratios. Matsui *et al.* (2000) examined parenchymal samples from individuals with lymphangioleiomyomatosis. Type II cells, type I cells and intermediate cells were identified. The intermediate cells were thought to be transitional cells changing from type II to type I cells. Some had lamellar bodies, others did not. The appearance of the intermediate cells was less rounded, with a paler, rounder more open nucleus than the metaplastic cells seen in the COPD cohort.

Nakashima *et al.* (1991) used a model of repeat oxidant exposure to produce type II hyperplasia and fibrosis in hamsters. This generated alveolar bed epithelial hyperplasia. A type II cell phenotype was assumed to be attributable to the presence of lamellar bodies, further examination of these cells was limited. Miller & Hook (1990) used a

silica challenge to produce type II hypertrophy. Hypertrophic type II cells showed increased numbers of lamellar bodies when compared to normal type II cells. The number of lamellar bodies in the COPD metaplastic cells does not seem to be greater than that seen in normal type II cells, therefore the metaplastic cells are probably not acute hypertrophic type II cells. Lee, Trochimowicz & Reinhardt (1985) have used a chronic titanium dioxide exposure model to produce an alveolar hyperplasia and bronchiolisation. EM of the bronchiolising cells demonstrated similar characteristics to the COPD metaplastic cells.

Rhodes *et al.* (1990) have used a rat model of pneumonia to examine epithelial response to damage. This suggested that in times of damage, airway Clara cells, might migrate into the alveolar bed and contribute to the reparative cell population. It has been suggested that reparative cells may migrate through the canals of Lambert and spread into the alveolar bed from bronchioles. Rhodes *et al* noted lamellar bodies and surfactant staining in some cells but not those with a cuboidal morphology. Surfactant expression in the pneumonia rat model is similar to the cells in COPD. Rhodes suggested these cells arise through expansion of a common progenitor for Clara cells and alveolar type II epithelial cells with the development of intermediate cells resulting in surfactant producing lamellae in both parenchyma and airways

Kasper & Fehrenbach (2000) have previously used EM to compare the type II cell response in irradiated rat lungs with foetal rat type II cells. The group used EM to show flattened elongated cells that had the basic morphology of a type I cell that contained lamellar bodies. This supports the idea of type II cells replenishing the type I cell population in times of damage.

In conclusion, the metaplastic cells within the COPD cohort appear to be altered type II cells that are not hypertrophic or differentiating into type I cells.

4.8.2 Summary of epithelial markers

Many of the metaplastic cell localisations correspond to normal type II distribution. Surfactant production is one key type II cell characteristic, surface pseudopodia on metaplastic cells when examined using EM are comparable to type II cells. The most plausible option for the metaplastic cell phenotype is a de-differentiated type II phenotype.

The cytokeratins found to be positive on metaplastic cells are also found on both type II cells and airway epithelial cells. There are, however, markers exclusive to type II cells (surfactants A and C) and epithelial cells (mucins 2 and 4). This demonstrates that metaplastic cells have characteristics of both alveolar epithelium and airway epithelium suggesting a de-differentiated form of epithelial cell.

Developing mouse lung epithelial cells co-express markers for neuroendocrine cells, Clara cells and type II cells in the distal airways (Wuenschell *et al.* 1996). Airway maturation sees the retention of expression patterns consistent with differentiated type II cells, Clara cells and neuroendocrine cells suggesting a common progenitor. Considering this, it may be expected that metaplastic cells observed here would express CC10, a neuroendocrine cell marker and type II marker. The metaplastic cells lacked CC10 staining or neuroendocrine cell characteristics on EM.

Airway epithelial cells can alter cytokeratin profile in response to injury rapidly becoming squamous. It has also been shown these altered phenotypes can persist if the cause of irritation or damage is maintained for example, continued smoking. It has also been shown that Clara cells can de-differentiate to produce other epithelial cell types (Evans, Cabral-Anderson & Freeman 1978) and that type I cells may be able to de-differentiate back into type II cells (Shannon, Jennings & Nielsen 1992). In COPD continual lung injury from smoking or other damage may elicit a repair process that causes the proliferation and distribution of a type II hyperplasia or a more probable type II metaplasia. Type II hyperplasia may be seen in acute respiratory distress syndrome, acute interstitial pneumonia and diffuse alveolar damage as well as other more chronic conditions. Proliferation rates range from around 5% to 40% depending on the condition. This type II overgrowth is partially cleared by apoptosis (Bardales *et al.* 1996). These proliferating plastic type II cells coat lung surfaces. Deposition on the alveolar surface matrix may lead to normal differentiated type II or even type I cells. Type II cells adhere to a number of matrix proteins including laminin. If these cells are deposited on adventitial surfaces, then perhaps underlying matrix or micro-environmental cues are not sufficient to drive the cells to any recognised differentiated state and therefore these cells remain in a de-differentiated state.

It is now well recognised that type II pneumocytes can give rise to type I pneumocytes. Trans-differentiation of type II cells occurs via TGFβ signalling through the Smad pathway. TGFβ alters the levels of p15 and p21 that are cyclin-dependent kinases. This

inhibition in turn downregulates the transcription of genes needed for cell cycle progression. Research has identified Smad4 and TGFβ in type II pneumocytes that have just proliferated and are about to undergo trans-differentiation. As cells underwent trans-differentiation to type I cells, TGFβ was secreted. Released TGFβ may exert autocrine regulation on surrounding cells, preventing progression to type I cells (Bhaskaran *et al.* 2007). There is an abundance of TGFβ in the COPD lung, perhaps ectopic TGFβ is stopping proliferation and/or trans-differentiation of type II cells (Takizawa *et al.* 2001). Araya *et al.* (2007) have demonstrated that a myriad of changes occur in the peripheral squamous metaplastic airways of COPD patients, many of these result from TGFβ upregulation of proteins and signals. This altered proliferation and differentiation behaviour may mean that type II cells or other epithelial cells produced in the parenchyma are prevented from maturing and are held in this primitive metaplastic phenotype.

4.8.3 Summary of progenitor cell markers

CD34 is a common progenitor cell marker that also highlights endothelium. It is important to exclude the endothelial population when examining this marker. CD31 also called PECAM-1 is an endothelial cell marker. CD31 staining allowed the distinction of endothelial cells and possible haematopoietic progenitor cells. CD31 was found on vascular endothelial cells of vessels, both systemic and pulmonary, in all parenchymal capillaries and lymphatic endothelium. There is no evidence of any metaplastic cells staining positive for CD31, which is consistent with cell morphology. Endothelial cells have less cytoplasm and are smaller and flatter than metaplastic cells unless activated. Endothelial phenotype can be discounted by cytokeratin presence and the lack of CD31 positive staining.

During foetal development capillary network spread into the lung is one of the triggers for overlying epithelium to differentiate and become type I cells (Hislop 2002). CD31 highlights the absence of capillaries underlying the majority of metaplastic cells. This is perhaps one reason why cells are not maturing to become type I cells.

CD31 was also seen on macrophage surface membranes, although this is not thought to be significant. Macrophage staining may have been a result of the innate tendency for immunoglobulins to bind to macrophages. It is also possible that vessels had undergone damage and macrophages had engulfed cell debris, isotype staining was negative, this excluded non-specific staining. Alexander-Sefre *et al.* (2003) used CD31 and pan CK to

examine tumour lymphovascular space invasion to show no co-localisation. Now endothelial cells have been excluded, it is possible to consider the presence of CD34 positive progenitor cells staining in metaplastic cell population. CD34 positive cells were identified in numerous locations throughout the lung including within the metaplastic cell population. Recent work suggests that CD34 positive cells do not contribute to an epithelial phenotype (Teisanu *et al.* 2009). Further examination of these findings is necessary dual staining for cytokeratin and CD34 would help to confirm that the CD34 positive cells are metaplastic cells and not lymphocytes or fibroblasts that appear to be in the same location.

Staining here has demonstrated that CD133 is capable of producing a number of lung phenotypes. These include basal and ciliated epithelium, smooth muscle cells, fibroblasts, endothelium and inflammatory cells, although the occurrence is sporadic. CD133 positive epithelium has been demonstrated within the prostate (Shepherd *et al.* 2008). The CD133 positive cells were much less frequent than CD34 positive cells. CD133 positive cells only contributed to metaplastic cells in patient 39 and underlying the metaplastic cells in patient 32. CD133 should highlight CD34 positive cells and a further sub population of progenitor cells. Peinado *et al.* (2006) inspected COPD patients for the presence of progenitor cells within the pulmonary arteries using a number of progenitor cell markers CD34, CD45 and CD133. CD34 positive cells appeared to colocalise with CD133 positive cells and were found in areas of denuded epithelium. CD45 positive inflammatory cells also appeared to be CD133 positive. This suggests that progenitor cells are capable of migrating to the lung when damage occurs.

This is in contrast with 49 of the 50 patients having CD34 positive cells and not in accordance with the literature that suggests that CD133 should highlight similar numbers to CD34. Paczkowska *et al's* (2009) work shows that there is a small population of cells that express both CD34 and CD133. Peinado and Paczkowska appear to have conflicting data on the overlap of expression of CD34 and CD133. The work carried out here suggests a divergence of markers in certain cell types, for example epithelial cells seemed to be CD133 positive, CD34 negative whereas metaplastic cells were more likely to be CD34 positive CD133 negative. This suggestion is based on the general distribution of these markers within the samples. Co-localisation with dual staining would be necessary to confirm this.

Fadini *et al.* (2006) looked at circulating progenitor cells in COPD and showed lowered numbers, perhaps inferring a reduced reparative potential in COPD patients. This may be the case or the lack of cells in the blood may suggest migration into the tissue.

Jiang *et al.* (2002) examined mouse enriched HSC and bone marrow progenitor cells, showing cells can give rise to skeletal myoblasts, cardiac myoblasts, endothelium, hepatic and bile duct epithelium, lung, gut and skin epithelia and neuroectodermal cells. Once cultured, these cells were negative for CD34, CD44, CD45, c-kit and MHC class I and II negative. Without β-gal modified mice to track these cells, investigation of progenitor cell potential is difficult. This loss of marker upon maturation may explain the infrequency of positive cells within the COPD lung.

The c-kit bone marrow population is a subpopulation of CD34 positive cells. C-kit staining in this study showed a large number of patients with positive apical epithelial cells and positive epithelium. Fibroblasts and muscle cells showed positive staining within the COPD samples. In mice normal c-kit expression is on mast cells, some leukaemic cells and some bone marrow cells. Lammie *et al.* (1994) examined human tissue and found scattered c-kit inflammatory cells in many organs including lung. Toludine blue staining confirmed these were mast cells. C-kit was also expressed on some epithelial cells, however this was not within lung epithelium. The work carried out by Lammie *et al* contrasts with the results here, as there was no mention of fibroblasts or muscle positive cells or airway epithelium staining.

Overall, CD34 positive progenitor cells are contributing to the metaplastic cell population. This is at odds with work by Teisanu *et al.* (2009) who suggested that epithelial cells do not derive from CD34 positive cells. This marker is shed as cell mature into another phenotype making it difficult to determine the overall influence that progenitor cells have to the metaplastic cell population. There may be other local progenitors giving rise to this colonising cell. As the metaplastic cell population has not been widely studied it is difficult to determine if this progenitor cell contribution should be expected. Further work co-localising CD34 and CD133 would be necessary to interrogate the population further. Also although CD34 and CD133 are often thought of as bone marrow progenitor cells, these cells can exist as resident cells in organs, for example, CD133 positive resident cells have been shown in adipose tissue (Poteser *et al.* 2008). Further investigation with progenitor markers and co-localisation with

cytokeratin would be necessary to confirm bone marrow or haematopoietic progenitor cell origin and the contribution to epithelial cells.

Metaplastic cells in COPD peripheral lung were shown to be an epithelial phenotype as demonstrated by the presence of pan cytokeratin on the whole population. There is no contribution of basal cells of the airways or mucus glands to the metaplastic cell population. This was confirmed by the absence of staining for CK5/6, p40, mucin 5AC, PAS and Alcian blue. The lack of mucin 5AC, PAS and Alcian blue stain also confirmed that there was no evidence of a goblet cell phenotype within the metaplastic cells. Neither was there evidence for a contribution of Clara cells as there was no staining of CC10 within the metaplastic cell population.

There was evidence of a type II cell phenotype within a significant subpopulation of metaplastic cells. This was confirmed by demonstration of surfactant A and C within this sub population suggesting that some metaplastic cells type II characteristics.

The absence of mesothelin staining in the metaplastic cell population confirmed that mesothelial cells do not act as a progenitor for metaplastic cells.

There was occasional evidence for multipotent progenitor cell contribution to the metaplastic cell population. Small numbers of cells showed CD34 positive staining and even smaller numbers of cells in some patients showed either CD133 or c-kit positive staining. This suggests that there may be a pluripotent cell population (possibly bone marrow derived) capable of contributing to the metaplastic cell population, however further work would be necessary to confirm this.

The features highlighted on electron microscopy were similar but not identical to type II pneumocytes.

This data, coupled with surfactant data, suggests that some metaplastic cells have similarities to a type II phenotype.

Chapter 5. Epithelial and secretory characteristics of the metaplastic lesion in COPD

5.1 Epithelial markers

This section provides a summary of the epithelial markers that may be employed to gain more information about epithelial phenotype and cell state, i.e. immature, mature or damaged.

5.1.1 Cytokeratin

Cytokeratins (CK) are found exclusively in epithelial cells. The 20 known cytokeratins are divided into two subgroups, acidic class 1 and basic class 2. Cytokeratin strands are made up of numerous soluble subunits that pair up to form helical strands. Strands start from the cell membrane at an anchor point, subunits join from the apical end, elongation stops when the strand reaches the cell membrane. Cytokeratins anchor to desmosomes, which link one cell to another, and hemidesmosomes which adhere cells to the basement membrane. The intermediate filaments form a mesh around the nucleus, holding it in position. Subunits are produced and released to diffuse through the cytoplasm (Portet *et al.* 2003). Cytokeratins comprise of an amino-acid terminal head, a central rod and a carboxy-terminal tail. Phosphorylation sites are usually in the head domain. When these sites are phosphorylated, cytokeratin breakdown can be initiated. Phosphorylation is also thought to control the dramatic rearrangement of cytokeratins necessary during cell division. Protein kinase C has been shown to be capable of phosphorylating cytokeratin (Yano *et al.* 1991).

Cytokeratins have many functions including the ability to aid mechanical cell stability and cell movement. This is possible by rapidly forming and reforming filaments (Chou, Goldman 2000, Coulombe *et al.* 2000). Cytokeratins also help cells adapt to the extracellular environment (Ma *et al.* 1999) and may help transmit extracellular signals to nucleus (Ingber 1997).

Normal cytokeratin distribution within epithelial cells has been examined previously. CK5 and CK6 pair with CK14 and have been demonstrated on bronchiolar basal cells. However CK5/6 antibody also highlights squamous cells and has been used to distinguish various tumour types. Mesotheliomas and squamous cell carcinomas are CK5/6 positive and adenocarcinomas are negative (Camilo *et al.* 2006, Harnden,

Southgate 1997). Basal cell or mucinous hyperplasia showed single or multiple layers positive for CK5/6. In squamous metaplasia basal cells tend to be hyperplastic but remain CK5/6 positive. Overlying layers were also positive for CK5/6 (Blobel *et al.* 1984).

Previous work has shown normal CK14 expression varies between individuals. Where present CK14 was preferentially expressed on basal cells and squamous metaplasia. Myoepithelial cells of sero-mucus glands were positive and alveolar cells were negative (Broers *et al.* 1989). In adult lung CK13 demonstrated a slightly different distribution and pairing to CK14 as it paired with CK4, it usually demonstrated squamous epithelial cells with the occasional columnar cell. CK13 is an accepted indicator of abnormal squamous epithelium. Squamous metaplasia shows loss of CK7 and CK18 positive cells, replaced by CK13. There was no expression of CK13 or CK20 within normal airway (Broers *et al.* 1989).

CK7 has been shown on all bronchial epithelial cells except the basal layer (Broers *et al.* 1989). Type II pneumocytes have been shown to express the simple CK7, CK8, CK18 and CK19 (Blobel *et al.* 1984, Iyonaga *et al.* 1997). CK7 is a simple adult epithelial marker that pairs with CK18 or CK19. Broers *et al.* (1989) found that CK19 showed a similar distribution to CK18. The only cytokeratin pairing capable of forming in the absence of cell-cell adhesion, is CK8 and CK18. Cytokeratin distribution is often altered in tumours, generally tumour markers are simple cytokeratins. CK8 and CK18 are a common pairing but CK17, CK18, CK19 and CK20 are also used to examine tumours. Investigation of tumours cytokeratins led to the identification of tissue polypeptide antigen (TPA). Comprised of fragments from CK8, CK18 and CK19 (Cyfra 21-1 subunit), tissue polypeptide-specific antigen (TPS) is used in tumour diagnosis. CK7, CK8, CK18 and CK19 isoforms have all been shown within adenocarcinoma cells, the surrounding tissue did not have altered forms (Gharib *et al.* 2002). The majority of adenocarcinomas and basal cell carcinomas are positive for CK18, whereas, most squamous cell carcinomas are negative (Barth *et al.* 2000). These simple cytokeratins typical of cancer cells overlap with fetal keratins, normal simple adult cells and those found in cells that have no cell-cell contact (Paine *et al.* 1995). Clearly cells surviving without cell-cell contacts allow cell movement contributing to foetal growth and repair but also tumour progression. Cytokeratin mutations have been associated with diseases such as liver cirrhosis and irritable bowel disease (Omary, Coulombe &

McLean 2004). CK18 has been shown to interact with TNFR1-associated death domain protein, sequestering the protein thereby preventing it from acting on TNFR1 and avoiding apoptosis through the TNF-activated pathway (Inada *et al.* 2001). This characteristic is of benefit to tumour cells and may contribute to the avoidance of cell death seen in cancer. There are other mechanisms that cytokeratins may be involved in, which avoid cell death. The CK8-CK18 pairing may alter the cell surface targeting of the Fas ligand preventing cell death (Gilbert *et al.* 2008).

Whilst cytokeratins are found in pairs, Stosiek, Kasper & Moll (1992) have shown epithelial cells are capable of demonstrating more than one cytokeratin pairing at one time. Airway epithelial cells were positive for usual adult respiratory epithelial cytokeratin filaments, namely CK7, CK8 and CK18. There was also weak staining squamous type CK5, CK14 and CK17. These two groups of cytokeratins were colocalised within one cell at both an mRNA and protein level. Caution is needed when interpreting cytokeratin profile as it must be remembered that damage and repair mechanisms may alter the cytokeratin profile. Table 5.1 summarises the distribution of cytokeratins in foetal and adult tissue.

5.1.2 E-cadherin

E-cadherin is an epithelial specific cell adhesion molecule that mediates cell-cell contact. In tumours a loss of E-cadherin was determined to be suggestive of a more metastatic tumour with a poorer prognosis (Miyanaga *et al.* 2008). E-cadherin is found on lung epithelial cells including cells of bronchus and bronchioles, with several N-cadherin positive cells throughout the bronchioles (Tsuchiya *et al.* 2006). There is little literature to suggest that type II cells express E-cadherin (Kasper 1995).

Liu *et al.* (2001) examined the E-cadherin profile in NSCLC patients and found that around half the tumours had cell membrane staining. Another third showed weak staining and about one-tenth were negative for E-cadherin. In most samples with reduced staining there was a positive cytoplasmic signal, usually diffuse and in some cases, granular. This demonstrated a range of E-cadherin distribution although other research suggests that diminished tyrosine phosphorylation of E-cadherin leads to internalization. It becomes cytoplasmic and non-functioning (Nawrocki *et al.* 1998).

Table 5.1 Summary of Normal & Foetal distribution of cytokeratin filaments

CK	Type	CK Pair	Normal Distribution	Foetal distribution in the lung
1	II	9, 10, 11	Keratinizing epidermis i.e. skin Suprabasal cells	
2	II	9, 10, 11	Keratinizing epidermis i.e. skin	
3	II	12	Cornea	
4	II	13	Non-keratinizing stratified sq. epi^{m} Basal cells	Alveolar stage, scattered + in peripheral epi^{m}
5	II	14, 17	Non-keratinizing stratified sq. epi^{m} Basal, glandular, myoepithelia, mesothelium	
6	II	14, 17	Sq. epi^{m}	
7	II	19	Simple epi^{m}, inc lung pseudostratified respiratory & transitional epi^{m}	Pseudoglandular period Weak +. Canalicular period sub pop^{n} + cells. Alv stage + A
8	II	18	Simple epi^{m} or sq., lung pseudo^{stratified} resp., transitional epi^{m}, secretory	Pseudoglandular period Alveolar stage Airway & glands +
9	II	1, 2	Keratinizing epidermis i.e. skin	
10	I	1, 2	Keratinizing epidermis i.e. skin	
11	I	1	Keratinizing epidermis i.e. skin	
12	I	3	Cornea	
13	I	4	Non-keratinizing stratified sq. epi^{m}*	Alveolar stage, scattered + in peripheral epi^{m}
14	I	5, 6	Non-keratinizing stratified sq. epi^{m}, basal cells, glandular	Alveolar stage some + basal cells; upper A
15	I		Non-keratinizing stratified sq. epi^{m}	
16	I	6	Sq. epi^{m}	
17	I	5, 6	Sq. epi^{m}	
18	I	8	Simple epi^{m}, inc lung pseudo^{stratified} respiratory & transitional epi^{m}	Pseudoglandular period Alveolar stage Airway & glands +
19	I	7	Simple epi^{m}, inc lung transitional epi^{m}, basal & myoepithelial cells	Pseudoglandular period Alveolar stage Airway & glands +
20	I		Simple epi^{m} mainly gastric epi^{m}	

Eidelman *et al.* (1989) identified two different E-cadherin distributions, polarized and non-polarized. Nonpolarized expression was observed in skin, upper GI tract, respiratory tract, vagina, cervix and transitional urinary tract epithelium. Polarized expression was observed on simple and columnar epithelium of the stomach, intestines, gall bladder, bile ducts, pancreas and areas of male and female reproductive systems.

These areas all had more intense staining on the cell's apical pole. This work suggests lung epithelium should have a uniform distribution of surface E-cadherin. Differing E-cadherin distribution may indicate an alternate origin, for example an apical distribution may suggest a gut cell origin. Distribution within the cell may also suggest altered function due to internalization.

E-cadherin is also important during development, it has been demonstrated on the surface of the bronchus buds and is later restricted to the airways (Kasper *et al.* 1995). The position of E-cadherin expressing cells in development gives cues to the rest of the tissue determining the phenotype of overlying cells (Takeichi 1988).

5.1.3 Mucins

Mucins are produced mainly by epithelial cells but also, under certain circumstances endothelial cells (Jung *et al.* 2002), possibly fibroblasts and other cell types (Volin *et al.* 2008). Mucins have been mentioned with regard to mucin profile changes in COPD (section 1.5.5). Mucins normally found in lung are, mucins 1, 2, 4, 5AC, 5B and 8. These mucins are not all found in all individuals, and some, like mucin 4 are usually seen when induced such as during infection (Lopez-Ferrer *et al.* 2001). One epithelial cell that produces mucus is the goblet cell, which has been mentioned in section 4.1.3. Lung mucins form part of muco-ciliary clearance helping to remove cell debris and particulates from the airways. Mucin may also play an immunomodulatory role. The presence of different mucins may suggest recent infection, an altered mucin profile or characteristics of epithelium from another compartment.

All cell phenotypes discussed are local progenitor or transit amplifying epithelial cells occurring somewhere within the lung. These cell types have all been examined as potential contributors to the metaplastic cell population. As well as lung originating progenitor cells, there are also cells from the bone marrow that may be released into the blood and sequestered to many organs, such as the lung.

5.2 Aims and objectives

The aim of the work in this chapter was to compare the metaplastic cell lesion with reparative epithelial responses.

> Does the cytokeratin profile of the metaplastic cell population resemble that of epithelial repair (Figure 5.1) or airway epithelial spread like Lambertosis (Figure 5.2)?

CK5/6 was used to highlight basal epithelium, CK7 and CK18 show simple epithelium and CK14 shows upper airway basal or squamous epithelium.

> Are the metaplastic cells producing mucins and are these similar to the airway mucins?

Samples were stained with PAS and Alcian blue to give an overview of acidic and neutral mucins. Immunohistochemistry was used to demonstrate mucins 5AC, 2 and 4 for more in depth investigation.

Two possible ways in which a repair phenotype could be identified.

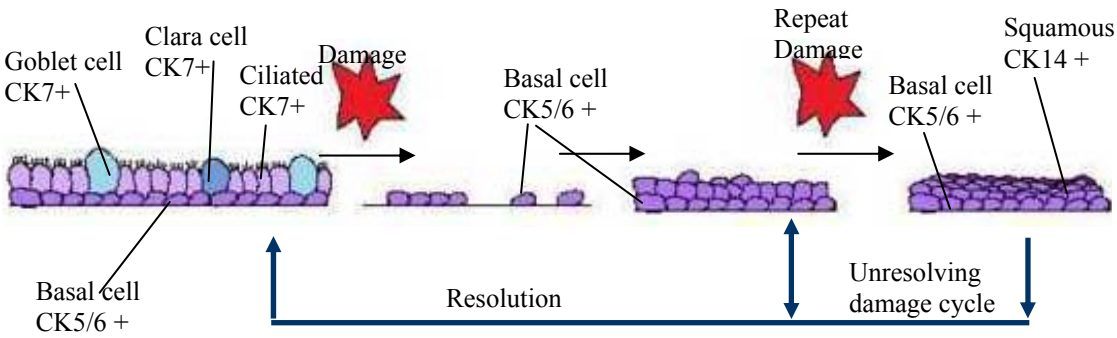


Figure 5.1 Repair & cytokeratin profile
This diagram shows that ciliated, goblet and Clara cells are replaced by squamous epithelium when damaged or lost completely. If then unstimulated or damaged further the epithelium reverts to a normal structure. If there is sustained or repeated damage or insult the epithelium may remain squamous.

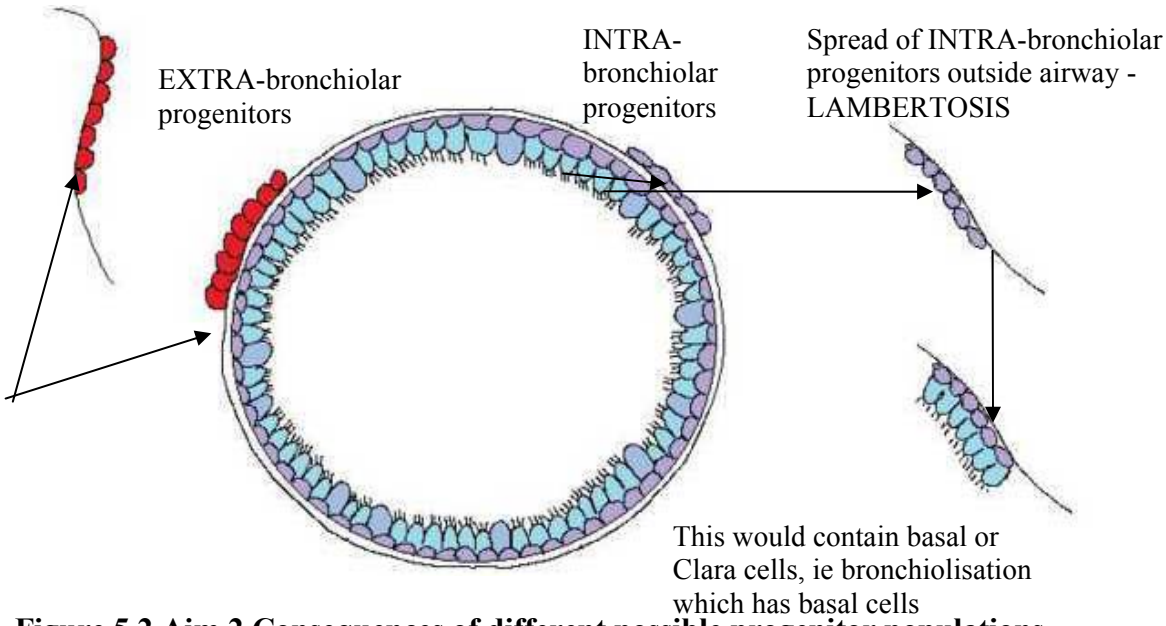


Figure 5.2 Aim 2 Consequences of different possible progenitor populations
Red cells represent extra-bronchiolar progenitor cells from outside the airways. These may be epithelium phenotypically similar to other organs, foetal epithelium, pre-neoplastic cells, or bone marrow progenitor cells Blue cells represent cells airway cells; this spread would be termed Lambertosis if connected to the airways or bronchiolisation if the cells were more ectopically located within the parenchyma.

5.3 Results for Epithelial profile examination

It was demonstrated in chapter 4 that all metaplastic cells were cytokeratin positive. This was confirmed using a pan cytokeratin antibody, more specific cytokeratin antibodies have been employed in this chapter.

5.3.1 Results for Cytokeratin 5/6 IHC

A CK5/6 antibody was used to identify cells containing CK5 or CK6 usually found on basal cells and squamous cells. The aim of this stain was two fold; to examine the basal airway cell population and to gain an insight into whether some or all of the metaplastic cells were of a squamous nature. The subset panel of samples that included blocks from 28 COPD patients and 6 controls was stained. This consisted of 44 slides. This staining was discussed in the section regarding basal cells in section 4.6.1 (Figure 4.5). The only squamous positive cells within the parenchyma were determined to be areas of bronchiolisation. These were also discussed in section 4.6.1.

5.3.2 Results for Cytokeratin 7 IHC

A CK7 antibody was used to identify normal simple epithelium. Simple epithelium is typically found on ciliated cells, goblet cells, Clara cells, gland cells and type II cells. The question addressed with this marker was whether the metaplastic epithelial cells were simple epithelium. COPD patients 1-10 were stained, staining examples may be

found in Figures 5.3 and 5.4. CK7 positive staining was seen on a sub-set of airway pan CK positive cells, morphologies included ciliated, goblet and domed cells (Figure 5.3A, C). Airway basal cells were negative. Positive metaplastic cells showed a range of morphologies squamous, macrophage-like and uniform cells were all positive. The proportion of metaplastic cells in any location highlighted by CK7 varied from approximately 70% to almost all metaplastic cells (Figure 5.3E, 5.4A). CK7 negative metaplastic cells appear to be sporadically distributed throughout the locations, adventitial, hilar, sub-pleural, fibrotic and alveolar and are all three morphologies. Patient 5 (Airway change & fibrosis) showed much more variability with areas of metaplastic cells showing as few as 10% positive cells. This variation was regional across the slide and may suggest staining artefact. Patient 4 (Airway change & fibrosis) seemed to have fewer CK7 positive cells adjacent to inflammation. A summary of the overall approximate percentage of cells staining within the airways and metaplastic cells of patients 1-10 is summarised in Table 5.2.

Table 5.2 Summary of CK7 staining in airways & metaplastic cells

Pat	Pathology Code	CK7 positive metaplastic cells	CK7 positive airways (except basal)
1	Airway changes & fibrosis	80-90%	100%
2	Goblet cell airway changes	80-90%	100%
3	Airway changes & fibrosis	90-100%	100%
4	Airway changes & fibrosis	<90%	100%
5	Airway changes & fibrosis	10-80%	100%
6	Goblet cell airway changes	70-100%	100%
7	Normal	80%	100%
8	Airway & pleural change	90-100%	100%
9	Goblet cell airway changes	90-100%	100%
10	Fibrosis & inflammation	90%	100%

In addition to highlighting perinuclear cytoplasm of epithelial cells CK7 was also seen on the matrix adjacent to positive cells in the parenchyma. This gave the impression of a ‘snail trail’ possibly suggesting cell migration (Figure 5.40C). This was not observed with any of the other cytokeratins used here. To further investigate this ‘snail trail’ stain, wheat germ agglutinin (WGA) lectin staining was obtained from colleagues (Sarah Bolton and Daniella Kostic). WGA is found on type I pneumocytes and was used to investigate whether the CK7 ‘snail trail’ staining may be type I cells (Figure 5.4E, F). This staining was contrasted with the parenchymal staining shown in Figure 5.4D.

To summarise, airways showed positive staining of all cells except basal cells. The metaplastic cells were around 70% positive, although this was variable, there was no pattern of positive cell distribution associated with location or morphology of the metaplastic cells.

5.3.3 Results for Cytokeratins 18 IHC

A CK18 antibody was also used to identify simple epithelial cells and is known to pair with CK7. This marker was examined to investigate the cytokeratin profile metaplastic cell population and perhaps provide information on whether this is a reparative cell. CK19 may also pair with CK7 so a sub-set if not all CK7 positive cells would be expected with CK18. COPD patients 1-10 were stained with CK18 antibody. Where positive, CK18 was perinuclear, the proportion of positive airway epithelial cells was much more variable than the CK7 staining (Figure 5.4B, D). Basal cells were negative but numerous other epithelial cells were also negative. There were also fewer positive metaplastic cells (Figure 5.4F). The proportion of cells staining positive for CK7 and CK18 was similar in patient 5 (Airway changes and fibrosis), patient 9 (Goblet cell airway changes) and patient 10 (Fibrosis & inflammation).

Table 5.3 Summary of CK18 staining within the metaplastic cells & airways

Pat no.	Pathology Code – group	Mets CK18 + (%)	Airway CK18 + (%)
1	Airway changes & fibrosis	50-70	10-90
2	Goblet cell airway changes	0-50	30-60
3	Airway changes & fibrosis	10-70	10-50
4	Airway changes & fibrosis	10-70	10-30
5	Airway changes & fibrosis	70-90	70-90
6	Goblet cell airway changes	70-90	30-70
7	Normal	0	0-30
8	Airway & pleural change	0-80	10-30
9	Goblet cell airway changes	70-90	30-90
10	Fibrosis & inflammation	10-90	0-90

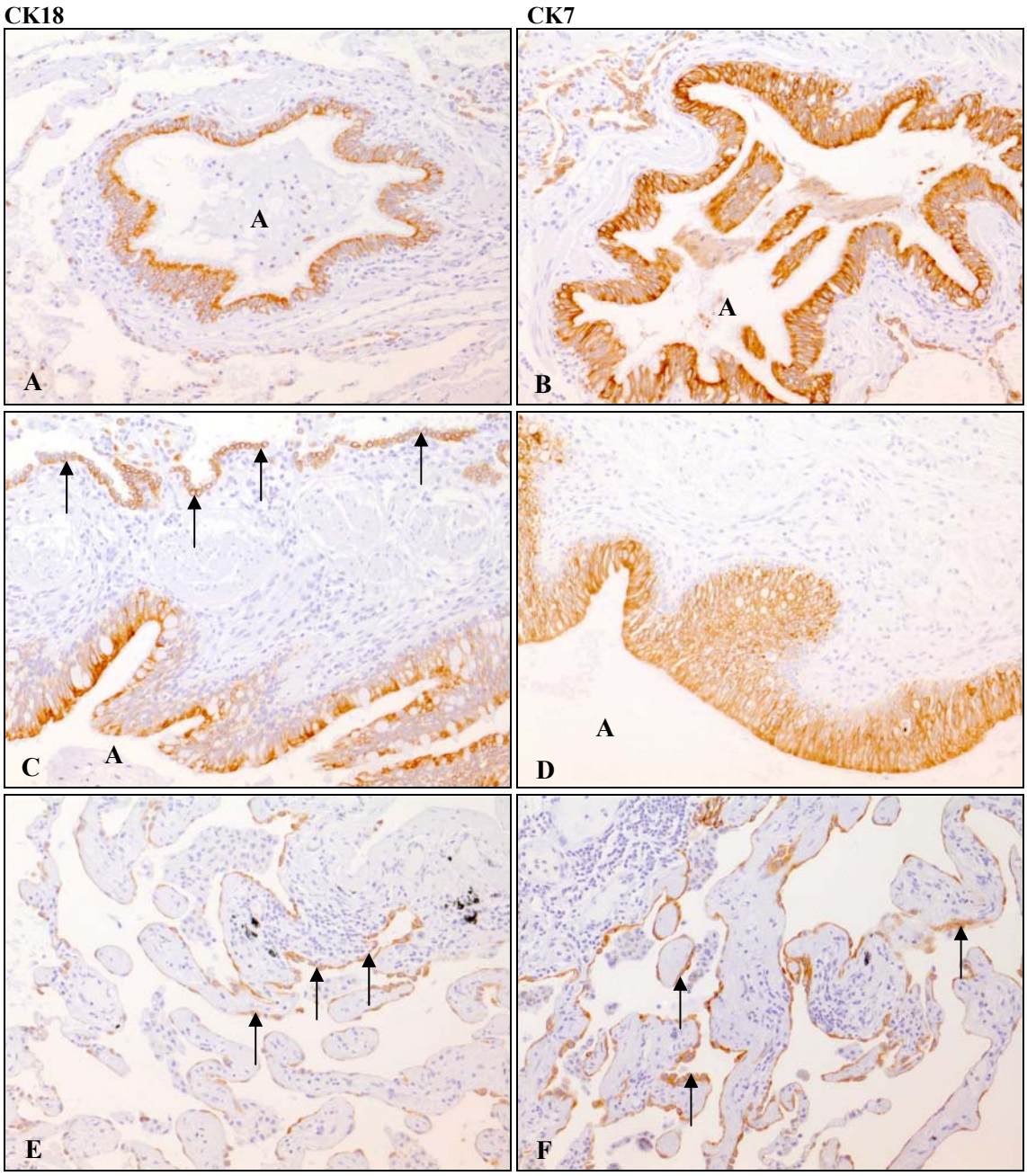


Figure 5.3 Simple cytokeratins, CK7 and CK18 IHC

Figure 5.3A) CK18 staining representative distribution in the small airway (A) is in the centre of the airway lumen, numerous strong and weak positive cells throughout the epithelium, negative towards the basal layer.

Figure 5.3B) CK7 representative distribution in the small airways (A) shows airway lumen. There is strong positive staining on the majority of cells within the airway.

Figure 5.3C) CK18 representative staining in the large airways (A), what appears to be holes in the epithelium of this airway is mucus filled goblet cells. There was a mixture of positive/negative cells within airway epithelium. There are adventitial metaplastic cells (arrows) at top of picture, the majority of these are positive for CK18.

Figure 5.3D) CK7 representative staining in the large airways (A). The majority of airway epithelium is positive with the exception of the basal cells, these are negative.

Figure 5.3E) CK18 staining on a squamous metaplastic population (arrows) that is lining an area of sub pleural fibrosis.

Figure 5.3F) CK7 staining on a squamous metaplastic cell population (arrows) that is lining an area of sub pleural fibrosis. Sections A-F are all from Patient 5: Airway changes & fibrosis.

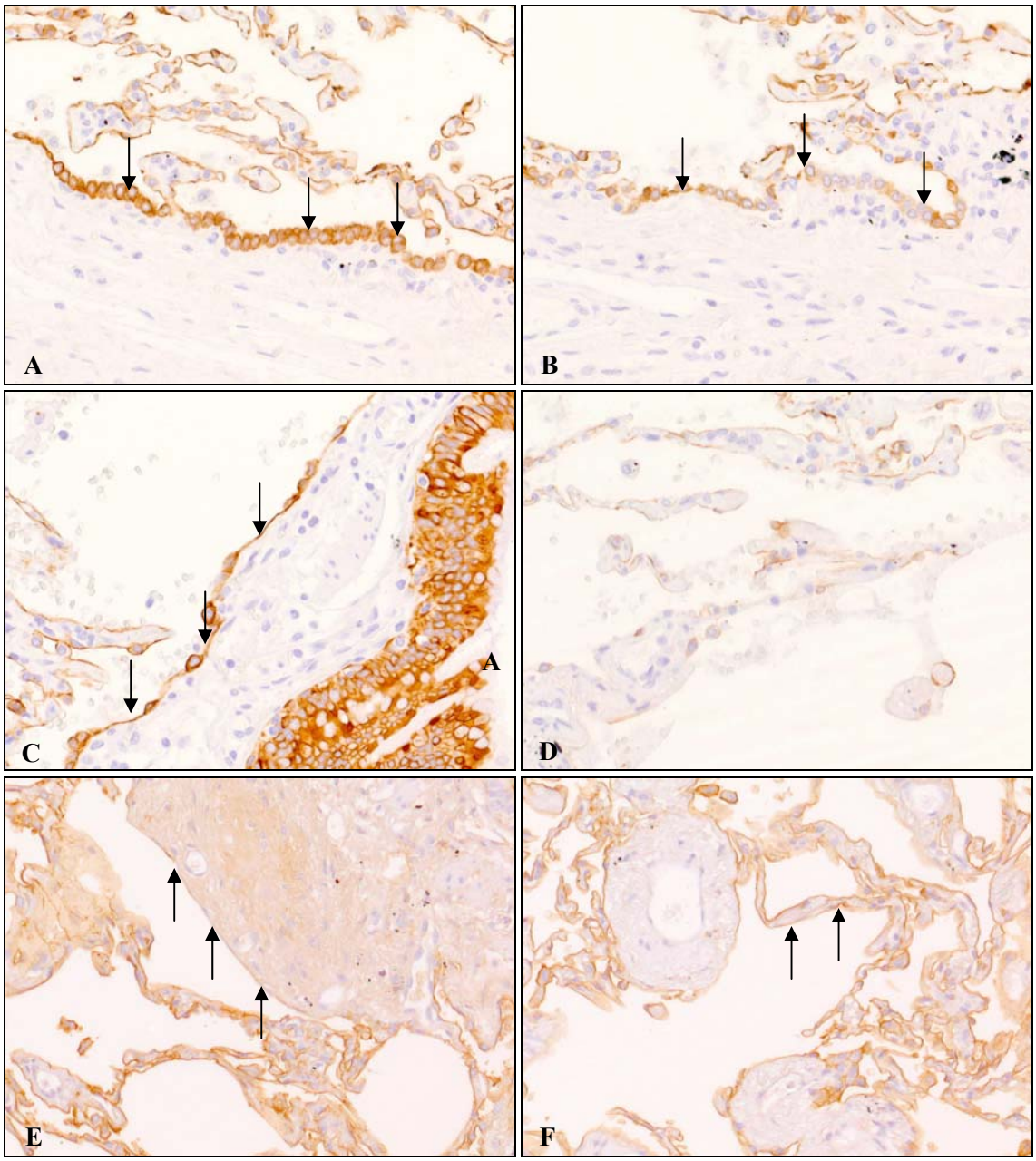


Figure 5.4 CK7 and Wheat germ agglutinin staining – type I cells

Figure 5.4A) x20 objective CK7 staining showing the majority of metaplastic cells positive (arrows).
Figure 5.4B) x20 objective CK7 staining showing only a few metaplastic cells staining positive (arrows), taken with 60A this shows the variability of cell staining with CK7.
Figure 5.4C) x20 objective CK7 staining showing surface slick (arrows) along external adventitia surrounding airways (A). There are numerous positive cells within the airway epithelium.
Figure 5.4D) x20 objective CK7 staining showing minimal staining in the parenchyma, there is also a macrophage with membrane positive staining present (arrows). Sections A-D are all from Patient 5: Airway changes & fibrosis.
Figure 5.4E) x20 objective Wheat Germ Agglutinin highlighting type I cells, note lack of stain on muscle surrounding vessel (arrows). (E and F are from Patient 34: Fibrosis)
Figure 5.4F) x20 objective Wheat Germ Agglutinin highlighting type I cells, note lack of stain around small vessel (arrows), the extent of parenchymal staining is much greater with WGA than with CK7 suggesting that if it is type I cells staining positive with CK7, then it is only a subpopulation, not all type I cells.

There was no evidence of any matrix surface staining in any sample. The lack of 'snail trail' staining and generally fewer positive cells may suggest the CK18 antibody is less

sensitive than the CK7 antibody as both cytokeratins are found in simple epithelium. CK7 can also pair with CK19 which would account for the lack of CK18 positive staining.

To summarise the CK18 staining, positive cells were seen within the airways, except basal cells and metaplastic cells of all patients examined. The proportion of positive cells was variable on both airways and metaplastic cells, within and between patients. To summarise there was no pattern of CK18 distribution associated with metaplastic cell location or morphology.

5.3.4 Results for Cytokeratin 14 IHC

A CK14 antibody was used to identify squamous epithelial cells, it may also highlight basal cells. The subset panel of samples that included blocks from 28 COPD patients and 6 controls was stained.

Table 5.4 Details of individuals with CK14 airway staining

Pat	Pathology Code – group	Airway	Gland
5	Airway changes & fibrosis	+++	None
6	Goblet cell airway change	+	None
11	Goblet cell airway change	+	+
12	Airway changes & fibrosis	+	+++
18	Airway changes & fibrosis	++	++
19	Airway changes & fibrosis	++	None
20	Airway changes & fibrosis	++	None
23	Airway changes & fibrosis	++	+++
24	Airway changes & fibrosis	++	None
25	Airway changes & fibrosis	++	++
33	Airway changes & fibrosis	+	+++
34	Fibrosis	+++	+++
41	Airway changes & fibrosis	++	++
46	Airway & parenchymal change	++	++
48	Widespread ongoing damage	++	++
49	Airway & parenchymal change	++	None
N2	Normal	++	++
N3	Normal	+	None

The above table uses the following symbols +, there are a few positive cells present, ++ there are some positive cells present, +++ there are a lot of positive cells present.

Positive cells were identified on 16/28 COPD and on 2/5 control samples, representative staining is shown in Figure 5.6. The airways showed areas with positive basal cells and in some cases basal and overlying epithelial cells were positive (Figure 5.6F). Positive airway cells were clustered together (Figure 5.6B). There were occasional clusters of positive cells within the glands. See Table 5.4 above for individual patient data. 3/28

COPD patients had parenchymal positive cells, however these were very small numbers of cells. Patient 4 (Airway changes & fibrosis) has a few positive cells in a cluster of what appears to be an infolding of pleura. Patient 32 (Airway change & pleural change) has a few isolated positive metaplastic cells within a sub pleural area of fibrosis. Patient 49 (Airway & parenchymal change) has positive squamous cells on several blocks of fibrosis. These were all determined to be areas of bronchiolisation. Figure 5.6C and Figure 5.6D shows an area with a few positive CK14 cells and a corresponding area stained with CK5/6. This is the only example of co-localisation. Metaplastic cells were not usually CK14 positive (Figure 5.6E). To summarise the airways showed areas of positive basal cells, areas of positive basal cells with overlying positive cells and positive basal cells within the glands. Not all basal cells were CK14 positive. This staining distribution according to location is summarised in Figure 5.5.

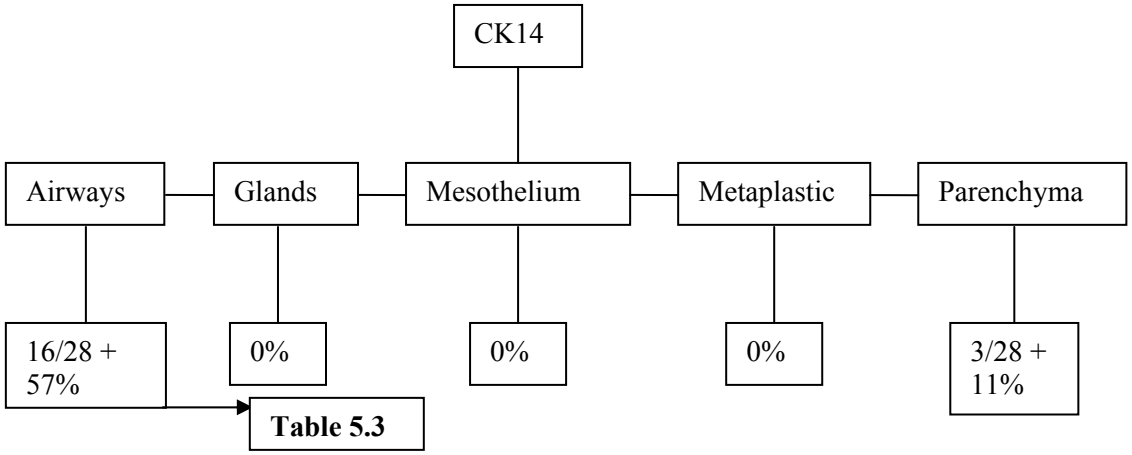


Figure 5.5 Diagrammatic overview of CK14 distribution
The areas of epithelium in the lung and the number and percentage of patients demonstrating positive CK14 staining. Table 4.10 contains a summary of the patients with positive airway staining

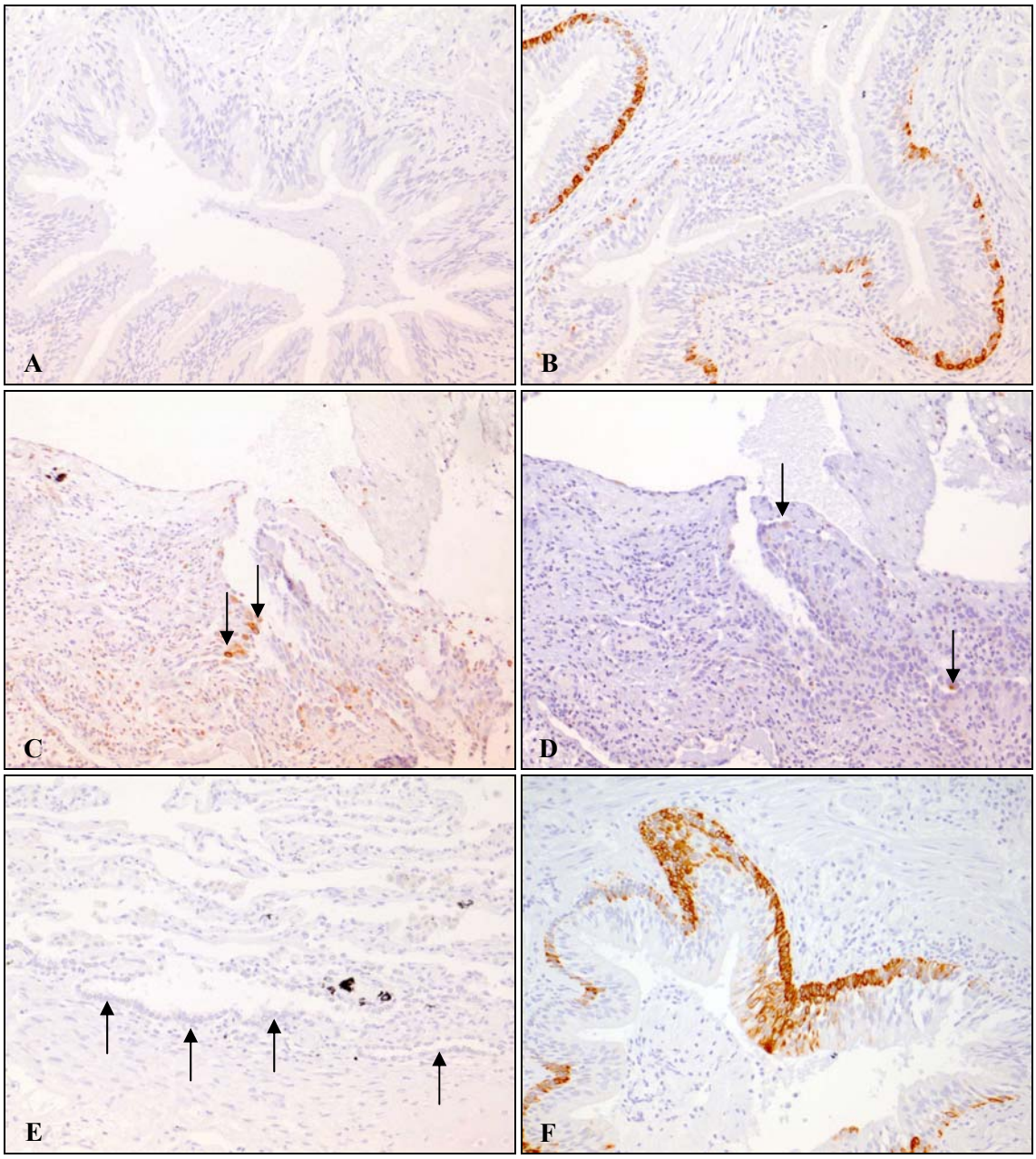


Figure 5.6 CK 14 IHC

Figure 5.6A) CK14 positive staining was not observed in all airways. This picture shows a small airway (A) that is negative for CK14.(Patient 5: Airway changes & fibrosis)

Figure 5.6B) CK14 positive staining in clusters within 2 large airways (A). The CK14 positive cells are all on the basolateral surface of the epithelium

Figure 5.6C) CK14 positive staining in a few cells in this area of consolidated parenchyma. This is the only example of CK14 positive staining (arrows) that had CK5/6 co-localising to a similar area.

Figure 5.6D) CK5/6 staining on a similar section to the CK14 staining in Figure 5.6C. There are a few CK5/6 positive cells present (arrows). (C and D Patient 30: Fibrosis)

Figure 5.6E) CK14 positive staining was not seen on any metaplastic cells. The picture here shows a string of negative cells (arrows)on the surface of fibrosed tissue.

Figure 5.6) CK14 positive in the upper layers of epithelium as well as basal layer. (A, B, E & F are Patient 5: Airway changes & fibrosis)

To summarise, there was a population of metaplastic cells that were highlighted with pan-cytokeratin antibody but were not clearly identified to be positive for any other cytokeratin. Other metaplastic cells were positive for CK7 or CK18. Dual staining was not carried out so it is not possible to confirm if the two cytokeratins were co-localising.

5.3.5 Results for E-Cadherin IHC

An E-cadherin antibody was used to identify cell adhesion molecules usually found on epithelial cells. This stain was used to examine the staining distribution between airways, type II cells and metaplastic cells to gain an insight into phenotype or maturation. The subset panel of samples including samples from 28 COPD patients and 6 controls was stained. This is a complex stain that has a range of distributions within a cell. These are important as membrane orientated signal suggests functional adhesion molecules whereas cytoplasmic signal indicates internalised non-functioning E-cadherin. These different cellular locations are outlined in Figure 5.7. Positive staining was seen on all samples, staining is shown in Figure 5.9.

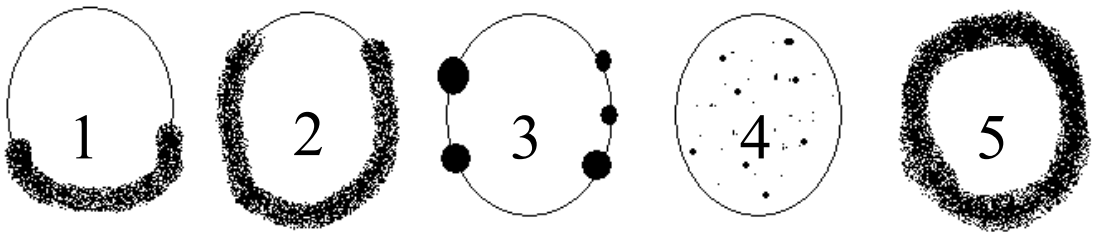


Figure 5.7 Diagrammatic representation of E-cadherin staining

This is a diagrammatic representation of the possible cellular staining patterns seen on epithelial cells with an E-cadherin antibody. 1) shows staining at the basal cell membrane, 2) shows basal and side membranes positive, 3) shows vesicular staining along cell sides, 4) shows a granular wash across the cells and 5) shows strong staining around the whole membrane of the cell, more intense than the staining of 1 and 2.

The airways showed variable staining patterns, epithelial cells were often a speckled type 4 distribution. 7/26 COPD patients and 1/6 controls had glands present. These tended to be either mainly type 4 staining pattern (Figure 5.9E) with very little evidence of stain, or type 5 staining pattern with all cells showing intense staining (Figure 5.9D). Focal lesions and the numerous single cells within tissue were usually type 2 staining pattern (Figure 5.9A), whilst there was also often an occasional type 5 staining in these areas.

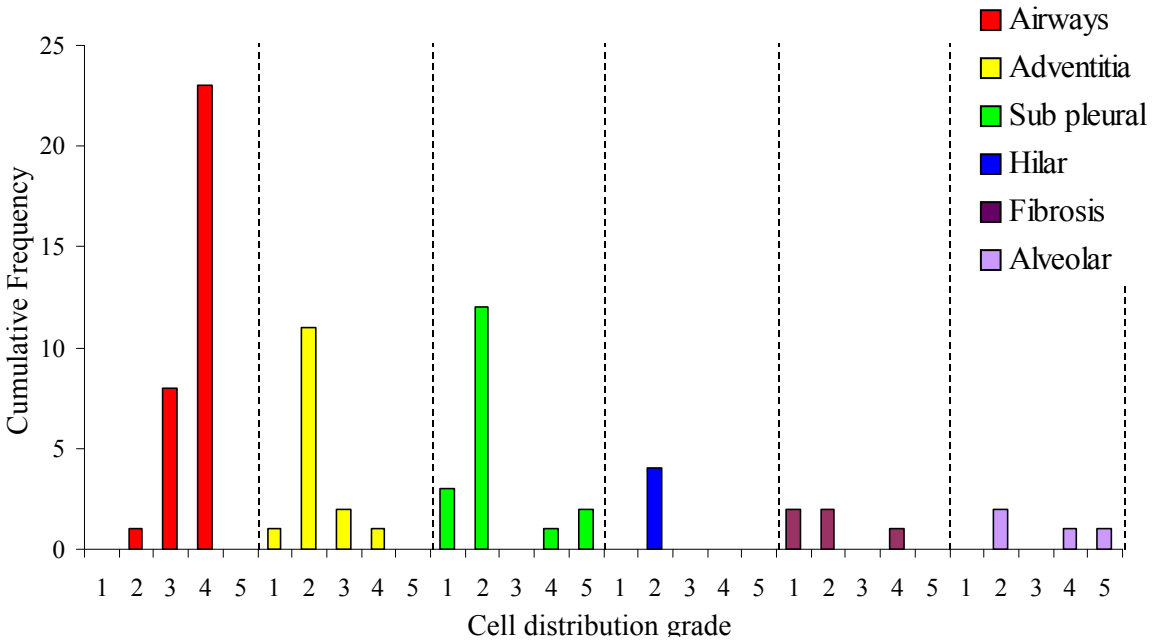


Figure 5.8 Summary of the E-cadherin staining pattern according to location
The above data is based on 28 COPD samples and 6 control samples. Staining pattern grades relate to those illustrated in Figure 5.7. The dominant cellular distribution in adventitial, sub plural and hilar metaplastic cells was grade 2, membrane orientated stain at the base and sides of the cell. The majority of the airways showed grade 4 staining pattern, speckled cytoplasmic staining, indicative of internalised adhesion molecules. Distribution was noted for the airways of 28 COPD and 6 controls. Adventitia was noted on 15 occasions, sub pleural 18, hilar 4, fibrosis 5 and alveolar 4.

To summarise, airways were variable, the dominant signal was punctuate minimal staining (grade 4). Within adventitia, sub pleural and fibrosis metaplastic cells the dominant signal was clear membrane staining on the cell base and sides (grade 2). Many of the instances of metaplastic cells that showed grade 5 staining although this was rarely the dominant cellular distribution.

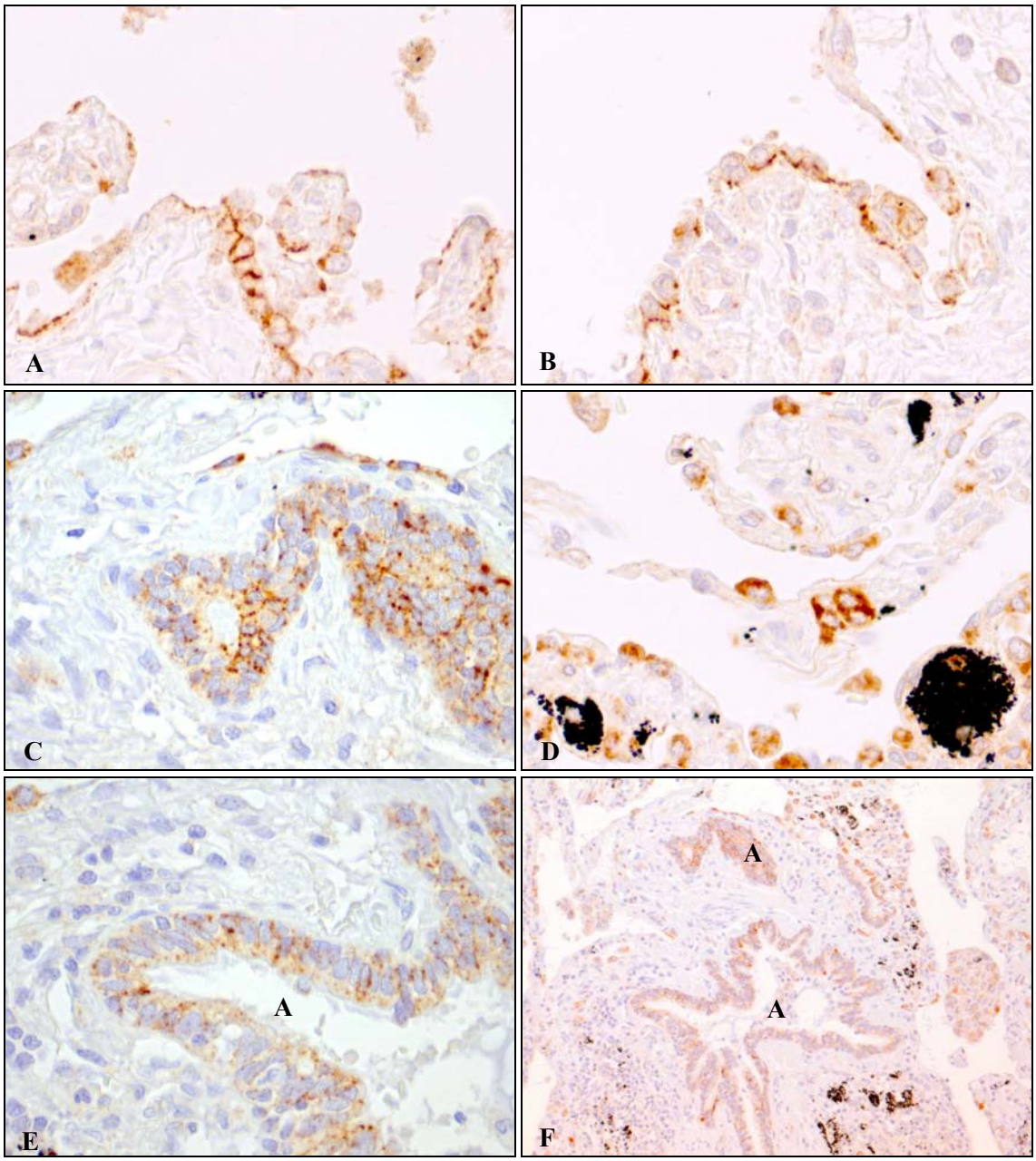


Figure 5.9 E-cadherin IHC staining

Figure 5.9A) x40 objective E-cadherin showing staining on the base and sides of the cell (Type 2 staining pattern).
Figure 5.9B) x40 objective E-cadherin showing staining at the base of the cells (Type 1 staining pattern).
Figure 5.9C) x40 objective E-cadherin showing staining vesicles on cell sides (Type 3 staining pattern).
Figure 5.9D) x40 objective E-cadherin showing staining in the whole of the cell (Type 5 staining pattern).
Figure 5.9E) x40 objective E-cadherin showing punctate faint stain across whole of cell (Type 4 staining pattern).
Figure 5.9F) E-cadherin staining on small airways

5.4 Results for mucin examination

Mucin 5AC has already been discussed in the goblet cell section 4.11.3. There was no metaplastic cell staining observed for mucin 5AC.

5.4.1 Results for mucin 2 IHC

A mucin 2 antibody was run to examine the mucin 2 secretory profile on both airway and metaplastic lung epithelial cells. The staining is shown in Figures 5.11 and 5.12. Mucin 2 is found within upper respiratory tract and gut. This antibody was examined on the COPD sub set of 28 patients and 6 control samples. All samples showed some positive signal, that was either positive airways cells, mesothelium, macrophages or occasional positive cells in the parenchyma (Figure 5.11B, D, F 5.12B). 17 of 28 patients examined showed some positive metaplastic cells and cell location is shown in Table 5.5 below. With the exception of patients 33 (Airway changes & fibrosis) and 49 (Airway & parenchymal change) there were only a few positive cells in the COPD samples. There was only one occurrence of weakly positive cells in the hilar region and there were no instances of mucin 2 production in the alveolar metaplastic cells. This may be due to the fewer number of occurrences of metaplastic cells in these locations. Control sample 2 was the only control block with any convincing positive stain and this was seen within the glands. This marker was found on some metaplastic cells but was an infrequent occurrence.

Table 5.5 Summary of Mucin 2 staining within the COPD cohort
The location alveolar has been excluded from the table, as there were no instances of alveolar metaplastic cells staining positive for mucin 2.

Pat	Pathology Code	Adv	SP	Hilar	Fib
5	Airway changes & fibrosis	-ve	+		
6	Goblet cell airway changes	+ + Nuclei			
19	Airway changes & fibrosis				+
20	Airway changes & fibrosis		+		+
21	Goblet cell airway changes		+		
23	Airway changes & fibrosis		+		+
24	Airway changes & fibrosis	+			
25	Airway changes & fibrosis	+			
30	Fibrosis	Wash	+		
31	Fibrosis				+
32	Airway & pleural change			+ Wash	+ Wash
33	Airway changes & fibrosis		Lots +		
34	Fibrosis	Gran +			
41	Airway changes & fibrosis		+		
46	Airway & parenchymal change	+	+		
48	Widespread ongoing damage	+	+		
49	Airway & parenchymal change	Lots +	Lots +		Lots +

5.4.2 Results for mucin 4 IHC

A mucin 4 antibody was employed to examine the distribution of this mucus, normally found in some upper airway epithelium and upon stimulation by inflammation. The whole panel of 50 COPD patients and 6 controls were stained. Mucin 4 showed apical staining on some airway epithelium of all samples. Some airway epithelium had strong cytoplasmic staining, with a wash in many goblet cells (Figure 5.11A, C, E, 5.12A, C). The strong airways epithelial cell signal appears to be from damaged cells and squamous metaplasia. This was further examined by looking at squamous cellular morphology on H&E (Figure 5.12C, D). There was strong staining in many glands. All samples showed positive endothelial staining on the majority of large vasculature, but not the capillaries. This endothelial staining was not expected. There was also a wash in congested plasma within the vessel lumen. 9/50 patients did not show any positive signal on metaplastic cells, 2/6 control samples showed minimal positive signal on metaplastic cells present. The positive metaplastic cell staining was noted to sometimes be more prevalent near areas of inflammation.

Table 5.6 Summary of Patients without metaplastic cell mucin 4 staining

Pat	Pathology Code – group	Metaplastic cells
1	Airway change & fibrosis	Negative
2	Goblet cell airway changes	Negative
3	Airway change & fibrosis	Negative
4	Airway change & fibrosis	Negative
7	Normal	Negative
24	Airway change & fibrosis	Negative
33	Airway change & fibrosis	Negative
45	Goblet cell airway changes	Negative
48	Widespread ongoing damage	Negative
N1	Normal	Few cytoplasm adv
N2	Normal	No obvious mets
N3	Normal	No obvious mets
N4	Normal	Few + adv mets
N5	Normal	Negative
N6	Normal	No obvious mets

Patient 3 (Airway changes & fibrosis) is the only COPD patient without positive metaplastic cells that demonstrated airway inflammation. Several COPD patients without mucin 4 metaplastic cells have inflammation or alveolitis within the parenchyma.

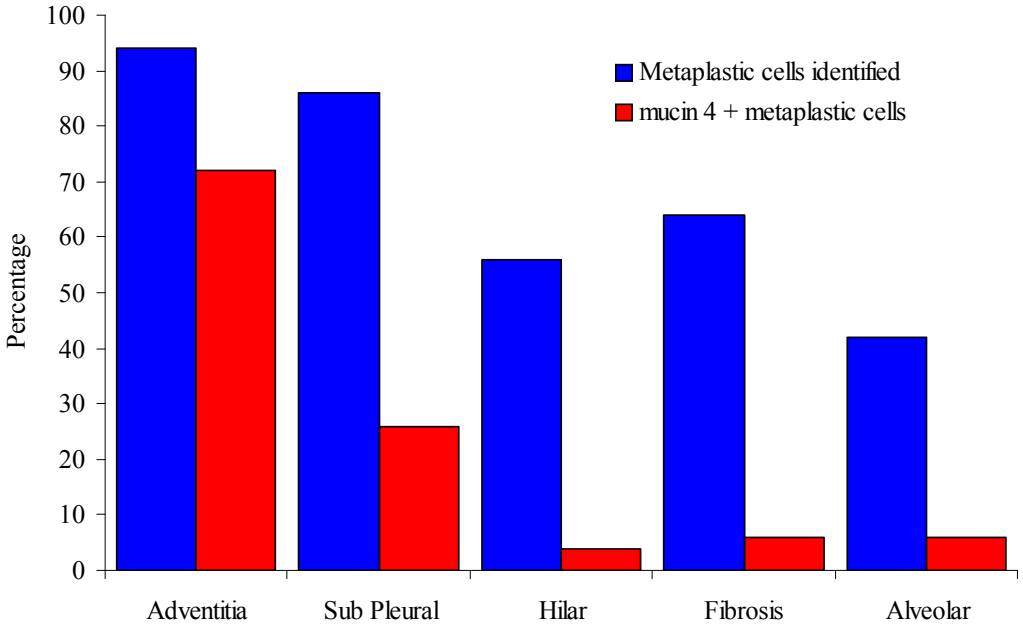


Figure 5.10 Frequency & distribution of mucin 4 positive metaplastic cells
Shows in the blue column the percentage of samples that have metaplastic cells present within all of the COPD samples. The red column shows the number of mucin 4 positive samples within all of the COPD patients. This provides an indication of the frequency of mucin 4 positive cells in the different locations of the parenchyma. The majority of adventitial metaplastic cells are mucin 4 positive whereas only a small proportion of those found on the hilar, fibrosis or alveolar surfaces are demonstrating positive mucin 4 staining.

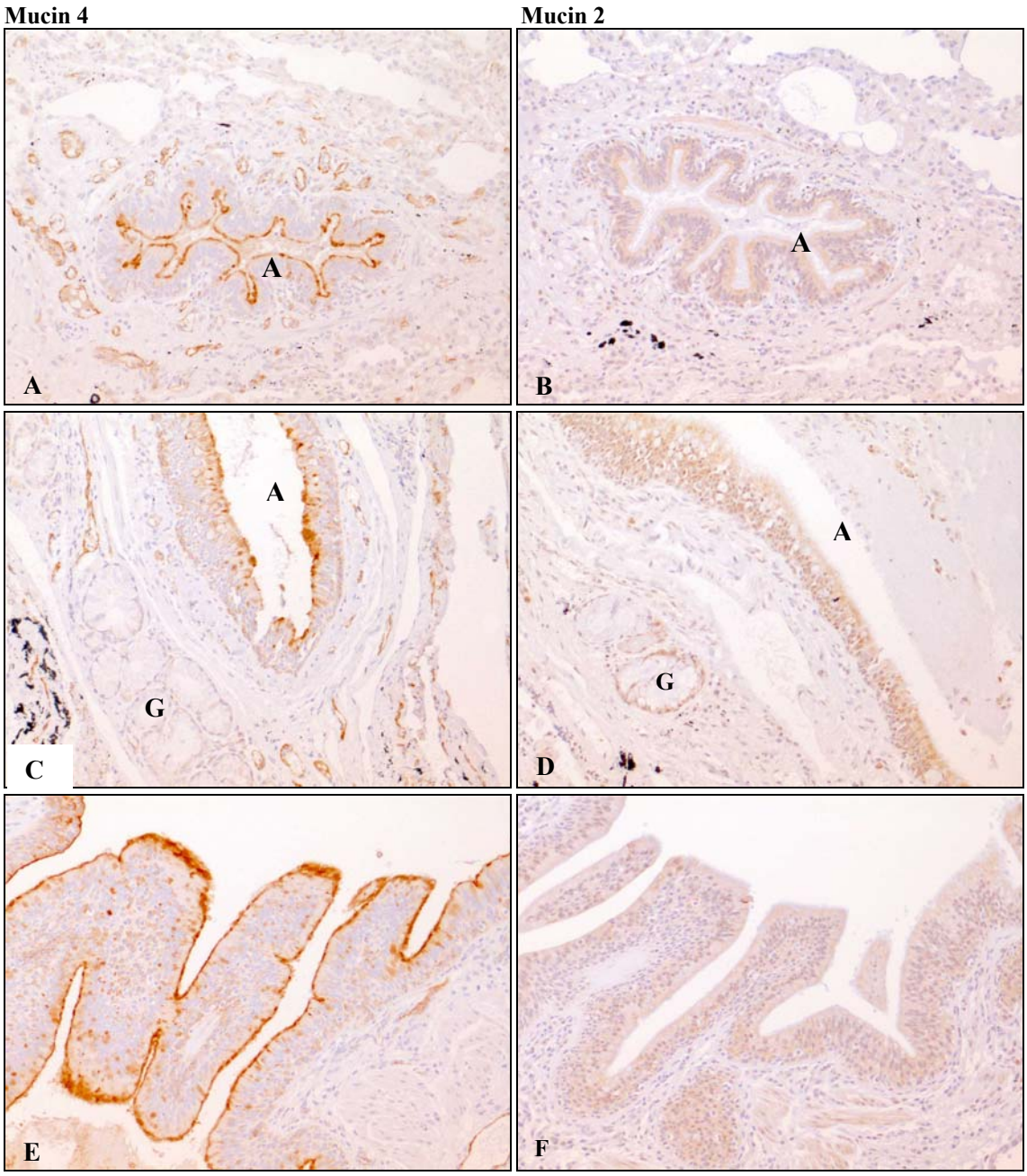


Figure 5.11 Mucin IHC, mucin 2 & mucin 4

Figure 5.11A) Mucin 4 staining (Patient 20: Airway changes & fibrosis) on apical surface of ciliated cells within a small airway (A). Occasional cytoplasmic positive epithelial cells. All endothelium was mucin 4 positive.

Figure 5.11B) Mucin 2 staining (Patient 20: Airway changes & fibrosis) small airway (A) epithelium, brown wash - no positive stain. This sample also showed occasional sub mucosal positive cells, probably macrophages. 66B shows positive cells as opposed to a wash seen in all 68B, D, F mucin 2.

Figure 5.11C) Mucin 4 staining (Patient 25: Airway changes & fibrosis) within cells and on cilia surface of a large airway (A), the glands (G) (bottom left of picture) are negative.

Figure 5.11D) Mucin 2 staining (Patient 25: Airway changes & fibrosis) large airway (A) epithelial and glandular (G) wash negative, no positive stain.

Figure 5.11E) Mucin 4 staining (Patient 20: Airway changes & fibrosis) Large airway. Apical surface positive stain, a few positive cells.

Figure 5.11F) Mucin 2 staining (Patient 20: Airway changes & fibrosis) Large airway. Epithelium, brown negative no positive stain

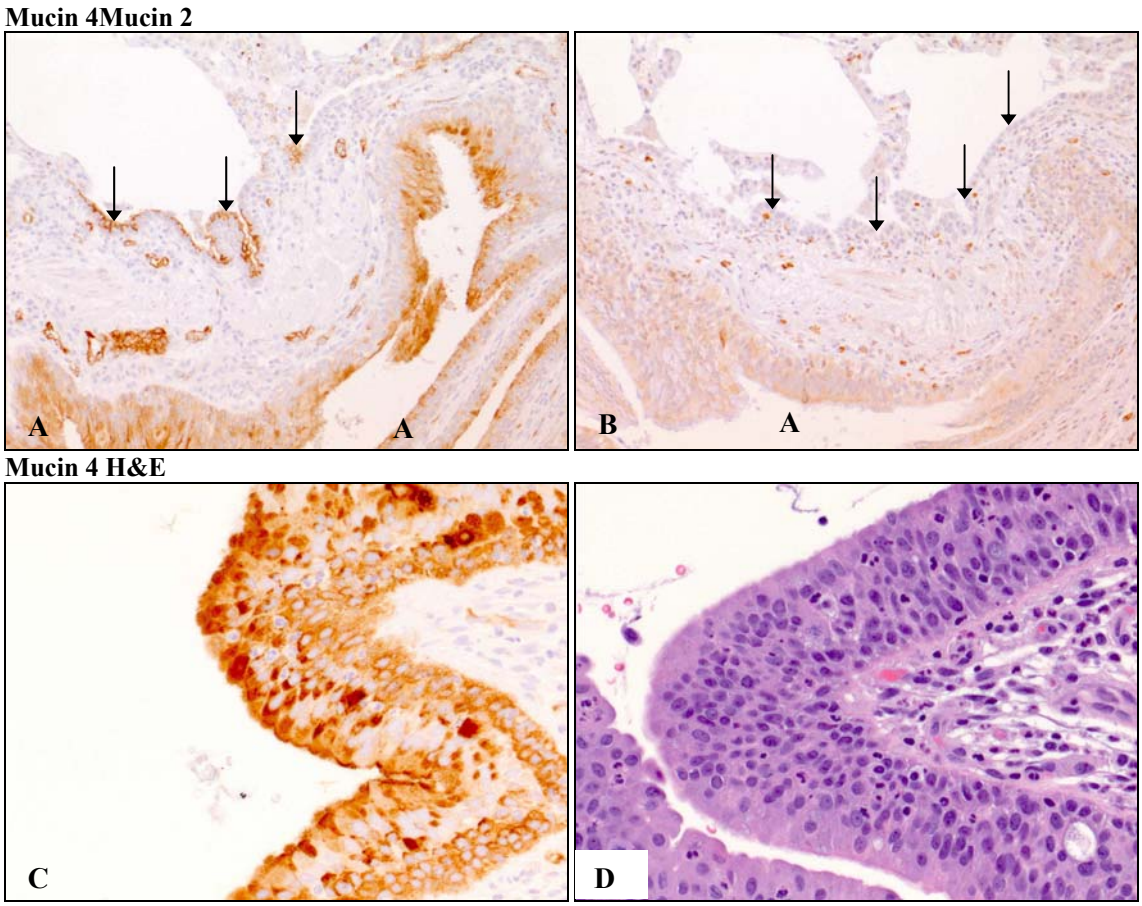


Figure 5.12 Mucin IHC continued, mucin 2 & mucin 4

Figure 5.12A) Mucin 4 staining (Patient 21: Goblet cell airway changes) is positive on cilia and the apical surface of some airway epithelial cells (A) and on the apical surface of some metaplastic cells (arrows).
Figure 5.12B) Mucin 2 staining (Patient 21: Goblet cell airway changes) is positive on some tissue macrophages and there is a faint wash in the airway epithelium, the metaplastic cells (arrows) are negative.
Figure 5.12C) x20 objective Mucin 4 staining (Patient 50: Widespread ongoing damage) showing an area of positive staining on squamous epithelium. There are positive apical surfaces and positive cells through the whole epithelial layer including the basal cells.
Figure 5.12D) x20 objective H&E staining of the same airway as Figure 5.12C to demonstrate squamous epithelial phenotype

5.5 Discussion of epithelial profile of diffuse metaplasia in COPD

Pan cytokeratin staining on the COPD cohort and resection control samples provided confirmation that the metaplastic cells were all of an epithelial phenotype. These occur in all five locations; adventitia, sub pleural, hilar, fibrosis and alveolar. Pan cytokeratin staining was observed in squamous flattened, uniform round and irregular macrophage-like metaplastic cells. Cytokeratin staining was shown on airway epithelium, type II cells and mesothelial cells, which has been previously reported (Broers *et al.* 1989). Cytokeratins are found exclusively on epithelial cells suggesting the origin of metaplastic cells is airway epithelium, mucus gland progenitors, type II cells or mesothelium, unless the cells originate outside the lung.

5.5.1 Epithelial profile

The canals of Lambert are thought to be outgrowths of airway epithelium that form ducts allowing cell and gaseous movement between the airways and parenchyma.

CK5/6 was clearly seen in basal epithelium of at least some bronchioles on all patients as expected and this has been published previously (Broers *et al.* 1989). Also expected the larger airways tended to exhibit more basal cells compared to smaller airways (Boers, Ambergen & Thunnissen 1998). This is the normal distribution of basal cells within the airways and any increase of suprabasal CK5/6 positive cells may be a reflection of an increase in proliferation that has not yet matured (Barth *et al.* 2000). CK5/6 staining on airways and metaplastic cells are summarised in Figure 5.13. Areas of bronchiolisation often had positive basal cells as well as positive squamous cells present.

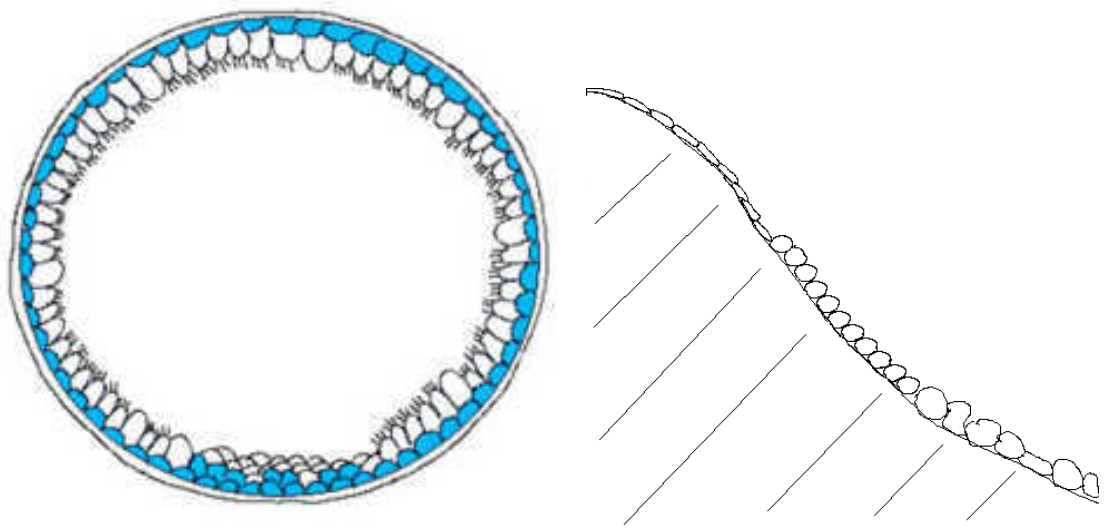


Figure 5.13 Diagram representing CK5/6 airway & metaplastic cell staining
Gives an overview of the distribution of staining found within the airways, basal and suprabasal staining and absent within all metaplastic cells regardless of location or morphology.

Cytokeratin staining is usually observed as a strong nuclear halo. Several patients also showed a very strong discrete perinuclear vesicular stain. This is not normal cytokeratin distribution. It was thought that vesicular packaging might be a consequence during cell replication and division, however this requires further consideration.

Cytokeratins are present in all adult epithelial cells and are therefore found in cells about to undergo mitosis. Cytokeratin rearrangement during cell proliferation has been examined and it was found that during interphase cytokeratins are unchanged. During prophase, metaphase and anaphase the filaments adjust to surround mitotic apparatus but are still present. During telophase the filaments reform around two new nuclei. Once

cell separation has occurred, the filaments re-adjust but are always present throughout mitosis and there is no packaging of cytokeratin at any phase (Aubin *et al.* 1980). Although cytokeratin filaments may be depolymerising and rebuilding this dynamic activity is not visible with antibody staining. Other work looking at 40 cell lines showed some cell lines behaving in the manner described above however, nearly half (17/40) exhibited a fine speckling across the cytoplasm during mitosis. From the examples shown, these speckles were very small and did not constitute vesicles (Lane, Goodman & Trejdosiewicz 1982). This cytokeratin behaviour differs from some other cytoskeletal elements where microfilaments are largely absent during mitosis. This suggests that cytokeratins play a role in preserving the cytoskeletal structure throughout mitosis.

Cells with CK5/6 vesicular stain were of goblet cell, columnar cell or type II cell morphology. These cells appear mature, large goblet cells were producing mucus, whilst the nucleus was not condensed. Type II cell morphology was also unremarkable.

It could be argued that if cytokeratins are not seen packaged in normal epithelial cells or during proliferation, that the cells are about to enter cell death. Cell death can occur via programmed cell death, apoptosis, or the damage related cell death, necrosis. Necrosis is unlikely to result in cytoskeletal packaging, as the whole cell disintegrates releasing cell contents. It has been shown previously that cytokeratins become phosphorylated during apoptosis, leading to filament breakdown and soluble subunit release into the cytosol (Schutte *et al.* 2004). Subunits would not be visible by IHC.

The antibody used in the work carried out here was raised against CK5. CK5 has a very similar structure to CK6 and the antibody is named anti CK5/6 as it recognises both cytokeratins. There are other antibodies available that detect CK5 and CK8. There is no scientific evidence published that any cytokeratin filaments are packaged in epithelial cells therefore it is unlikely the antibody is incorrectly labelling any other keratins. This information suggests that the vesicles being highlighted do not stain cytokeratin but are detecting another protein. Within our research department IL-13 has been observed in a vesicular peri-nuclear pattern of epithelial cells. These vesicles appear to be storing a protein that cross-reacts with the cytokeratin antibody used here.

To summarise, all patients examined show a CK5/6 positive basal cell population in airways and some mucus glands. In addition, obliterated airway remnants and in bronchiolisation there were CK5/6 cells. There are no instances of positive staining of

metaplastic cells with CK5/6 antibody. There were 6 patients, including 2 controls with inappropriate vesicular staining. This is not cytokeratin specific staining. Clarification of what the vesicular protein is would require further work, however it can be considered not to alter the overall CK5/6 interpretation.

Cytokeratin 7 distribution was consistent across all 10 patients examined. Airways were highlighted throughout the epithelial layers with the exception of basal cells. There was positive staining within glands and type II cells. This is in keeping with findings by Broers *et al.* (1989) CK7 is a simple cytokeratin and should be seen on all epithelium except the airway basal layer, therefore it should highlight ciliated columnar cells, Clara cells, goblet cells and neuroendocrine cells. Overall cytokeratin 7 distribution on airways and metaplastic cells is summarised in Figure 5.14.

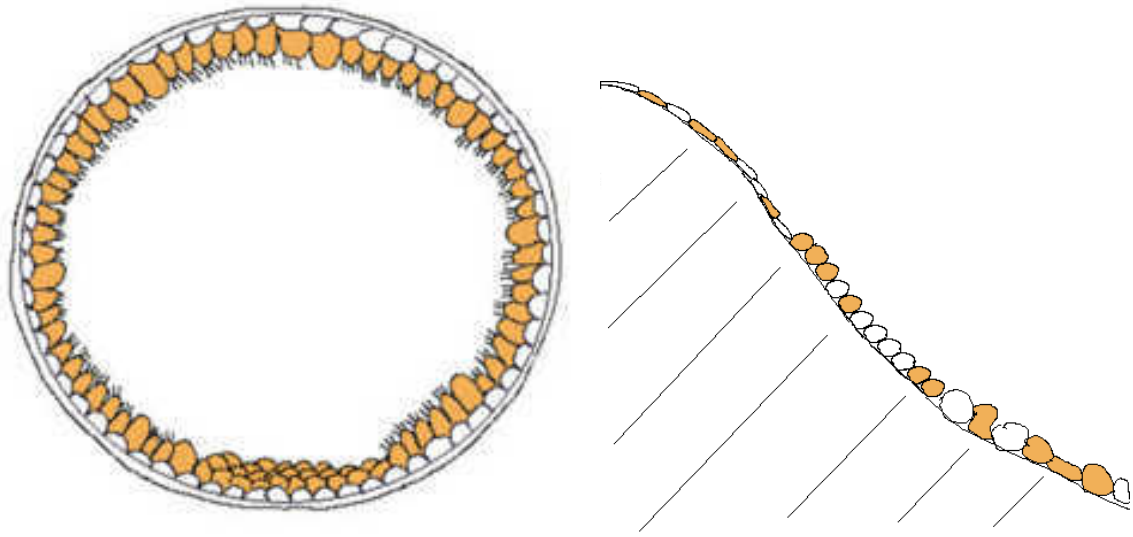


Figure 5.14 Diagram representing CK7 airway & metaplastic cell staining
Diagrammatic representation of the airway (left) and metaplastic cells (right) and the CK profile and distribution generally observed on epithelial cells. All cells in the upper layers of airway epithelium stained positive. A range of metaplastic cells stained positive for CK7, this staining did not seem to follow any pattern associated with the metaplastic cell morphology there were positive and negative cells observed in all three morphologies.

Cytokeratin 18 was stained across patients 1-10, using the same slide set as was used for CK7 staining, however this did not give uniform results across all patients. Airway staining ranged from around one-tenth of cells positive to almost all airway epithelium being positive, again with the exception of the basal cell population. A summary of CK18 staining is shown for airways and metaplastic cells in Figure 5.15. The lack of

basal cell staining is consistent with findings in the literature (Broers *et al.* 1989). Broers stated that CK18 was found on all airway epithelial cells and type II cells (Broers *et al.* 1988). Wang *et al*. (2006a) found in normal tissue that all columnar cells were CK18 positive and there was no mention of this being a variable signal. This would suggest that this mixed positive signal is unexpected. The CK18 staining was variable between patients, with a range of positive staining varying from around 10% to 90% of the metaplastic cell population.

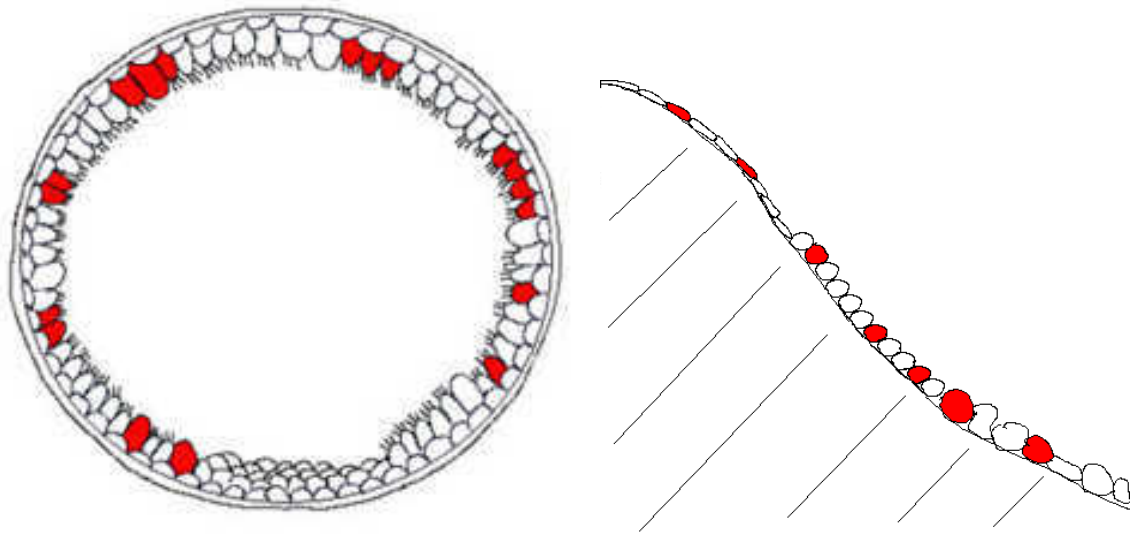


Figure 5.15 Diagram representing CK18 airway & metaplastic cell staining
Diagram to represent the positive staining observed with CK18. There was variable staining 10-80% of epithelial cells in the upper layers of the airways. There is also variable staining seen in the metaplastic cells.

CK7 also demonstrated a 'snail trail' effect within all samples examined. There were what appeared to be acellular areas with a very fine surface line of CK7 staining within the parenchyma and on some adventitial surfaces. This distribution of staining was not seen with CK18 staining. CK18 pairs with CK7 so was expected to show the same distribution. Pan CK antibody should also have highlighted all CK7 positive areas but pan CK did not show any 'snail trail' staining. Pan cytokeratin antibody was produced by adding several specific antibodies to create a cocktail. This resulted in lower levels of each individual antibody. This lower concentration may explain the lack of surface staining with the cocktail pan cytokeratin antibody. The CK7 single antibody was applied to tissue at 2.6µg/ml and the pan cytokeratin cocktail was applied at an overall concentration of 1.7µg/ml, indicating that contributing CK7 antibody in the cocktail was significantly lower than the single antibody protocol.

Brandsma *et al.* (1994) used a developmental model to examine retardation of lung maturation upon drug administration causing diaphragmatic hernia. Research showed cuboidal cells throughout the parenchyma as a result of type II cell maturation and lung development being slowed. Surfactant staining was employed to monitor type II cell maturation to type I cells, the change of plump type II cell to flattened type I cell. Surfactant staining was strong in plump cuboidal cells, weaker in thin cells and absent in very thin cells. This demonstrates gradual loss of surfactant as type II cells matured. Although surfactant was employed to visualise this cell population, the same maturation events would be visible if examining a simple cytokeratin. The cytokeratin structure would need to be degraded for the drastic shape change required to go from round type II cells to the flat thin type I cell that is virtually invisible on H&E. This gradual loss may explain the apparent surface staining in some areas of parenchyma. This may reflect recent repair and ongoing maturation of type II cells to type I cells in the lung parenchyma. Previous work carried out within our department has examined distribution wheat germ agglutinin, which is a lectin marker of type I cells. The lectin showed much greater staining within the alveolar bed and perhaps less staining on vascular adventitia. The distribution of CK7 was not consistent with the presence of type I cells. Perhaps it was intermediate filament deposition as a result of cell movement. This has not been published in the scientific literature and seems somewhat improbable, as cytokeratins are intracellular structural support not extracellular.

The mis-match between CK7 and CK18 staining may have been due to differing antibody sensitivity or alternative cytokeratin pairing with CK19. It has been shown that both CK18 and CK19 are present in airway epithelium. CK18 is more often associated with a migratory or reparative phenotype whereas the 'normal' cells have CK19, however expression of these two cytokeratins seemed to be readily interchangeable within lung epithelium (Paine *et al.* 1995).

CK14 was observed within 12/28 COPD airway samples examined. This positive staining was within basal, suprabasal and upper epithelial layers. It was not found on all basal cells or all other epithelium. CK14 was not seen on metaplastic cells. CK14 staining is summarised in Figure 5.16. Previous work suggests that CK14 is found within basal cells (Hong *et al.* 2004, Reis-Filho *et al.* 2003). CK5 and CK6 can also pair with CK17 which is also a recognised basal cell cytokeratin, the lack of CK14 may indicate CK17 expression instead (Dabbs *et al.* 2006). Broers *et al.* (1989) found a

similar distribution of CK14 in the airways to those observed here. The production of squamous epithelium which may be highlighted by CK14 has been shown to be a reversible part of normal repair processes (Park *et al.* 2006).

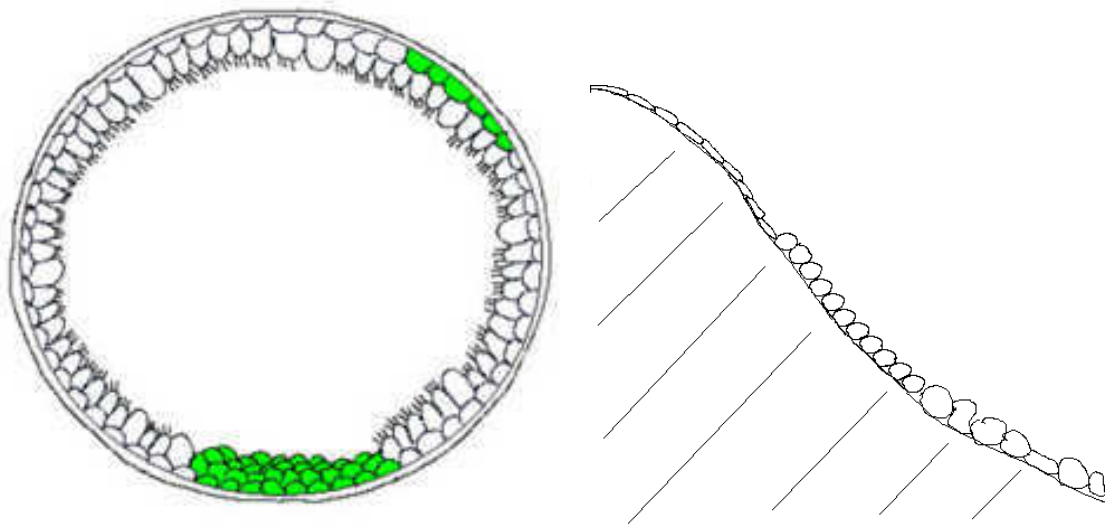


Figure 5.16 Diagram representing CK14 airway & metaplastic cell staining
Diagram of an airway and area of metaplastic cells the green cells showing that CK14 positive staining was observed on some occasions in the basal layer and other occasions basal and suprabasal layers. There was no positive CK14 staining seen within the metaplastic cell population.

Broadly the cytokeratin profile on metaplastic cells showed no CK5/6 or CK14 staining on the metaplastic population with around 70% positive for CK7 and less than positive for CK18.

The metaplastic population was devoid of any CK5/6 staining. This suggests that basal cells are not playing a progenitor role. All other airway epithelial cells CK7 positive; even those that are squamous or damaged and are showing squamous markers such as CK14 are also CK7 positive. A significant sub population of metaplastic cells were CK7 positive, however a variable number were also negative. Further work and dual staining are necessary to further examine the metaplastic cell profile, there are still cytokeratin positive cells that have not had the cytokeratin profile clearly defined.

If the metaplastic response was a normal repair process then it may be expected to show a similar cytokeratin profile to airways. The lack of basal cell staining perhaps indicates an alternative progenitor however; all other airway progenitors are CK7 positive. There are CK7 negative cells within metaplastic cells that are CK5/6 negative. The cytokeratin profile does not match any airway cell. Although Lambertosis has been defined histologically as being the same as epithelial cells that line the adjacent airway, there

does not seem to have been any immunohistochemical analysis of these cells (Lambert 1955). In the airways there was abundant staining of CK7 and CK18 with the occasional area of more squamous epithelium that demonstrates CK14 staining and CK5/6 within the basal layer. This pattern is clearly not reflected in the metaplastic cells, so refutes the diagnosis of Lambertosis. If the colonising cells were an extension of airway and were Lambertosis, ciliated cells would be present. Ciliated cells are the most abundant cell type within normal airways (Mercer *et al.* 1994). Based on the differences in cytokeratin profile highlighted above it may be concluded that the metaplastic cells seen within COPD were not proliferations of the crypts of Lambert defined by Lambert in 1955 (Lambert 1955).

Continuing this definition of Lambertosis epithelium, further ectopic epithelial proliferations within other lung locations would be expected to adopt a normal epithelial organisation. This would include the presence of basal cells and ciliated cells. This would be called bronchiolisation (Jensen-Taubman, Steinberg & Linnoila 1998). There are instances of this occurring in a few of the COPD patients of the cohort examined here but, these lesions are clearly distinct from the metaplastic cell population. The definition of metaplasia is an abnormal epithelial cell type. The cytokeratin profile has demonstrated that the metaplastic cell profile is not the same as in any other lung epithelial compartments. The locations of metaplastic cells are also abnormal further supporting the diagnosis of metaplasia.

The other epithelial marker employed to further examine the metaplastic cell population was the cell adhesion marker E-cadherin. Previous literature has mentioned on occasion that type II pneumocytes express E-cadherin at the cell base however, several of these papers are looking at tumour or diseased cells (Pardo *et al.* 2007, Dai *et al.* 2007). The E-cadherin staining within the COPD patients was not consistent or strong across the airway epithelium. The staining on metaplastic cells was variable with some showing a membrane stain and others showing strong staining across the cell. Blanco *et al.* (2004) used E-cadherin and other cell-cell markers to examine epithelial changes occurring in a rat model of silica induced carcinoma development. Strong membrane staining was seen in normal and hyperplastic bronchiolar epithelium. Normal isolated type II cells showed low E-cadherin expression and hyperplastic type II cells showed greatly increased expression. Adenomatoid lesions and all tumour cells showed significantly lower levels of E-cadherin. These findings perhaps highlight that the airway epithelium of severe

COPD patients is not normal as there were very few areas with strong membranous staining. The increased staining in many of the metaplastic cells indicates type II hyperplasia or normal epithelial characteristics as opposed to a pre-neoplastic lesion.

To summarise, the metaplastic cells are not the same cytokeratin profile as the airways. This rejects the diagnosis of Lambertosis or further into the periphery of the lung, bronchiolisation. The lack of basal cell markers and squamous markers also excludes a normal airway repair type response. This may be a chronic repeat damage response but it does not show normal damage repair behaviour. Alongside further progenitor and secretory information the cytokeratin pattern will allow comparison to other metaplastic lesions occurring both within the lung and on other mucosal surfaces.

5.5.2 Secretory profile

Mucin 5AC is produced by airway goblet cells. Goblet cells have an altered mucin profile in COPD patients. This is possibly an imbalance of mucin 5AC and mucin 5B being production. The mucin 5AC stain allows confirmation that goblet cells are not contributing to the metaplastic cell population. It is known that there is overproduction of mucus in COPD. Mucus production by metaplastic cells could be contributing to this. Only a single patient (1/50) patient 49 (Airway & parenchymal change), showed any mucin 5AC positive parenchymal cells. This area of positive cells appears to be an area of bronchiolisation. The metaplastic cell population does not show any expression of mucin 5AC.

Mucin 2 is generally thought of a gut mucus, but it has been documented in upper lung. Buisine *et al.* (1999) have shown that mucin 2 may be present in the trachea, bronchi, glands and occasionally bronchioles. This work was carried out on upper airway samples from transplant tissue and lower airway samples from non-tumour bearing tissue from cancer resections. This may mean that the tissue although appears normal, has an altered mucin profile. Mucin 2 was observed within the lower airways of some COPD samples. It was also observed on 17/28 COPD patients had positive metaplastic cells, however these were usually only occasional cells. Patients 33 (airway changes & fibrosis) and 49 (Airway & parenchymal change) were the only patients where numerous cells were positive. This is by no means a phenotype-defining characteristic. The normal lung periphery has no mucin 2 production, however this positive staining is occurring within a disease setting. Previous work has shown that mucin 2 is upregulated in cystic fibrosis patients. Also TNFα, IL-1β and IL-9 can all upregulate mucin 2

production within the lung, as can bacteria and viruses. Mucin 2 may be expressed as a result of viral or bacterial infection or as a disease stimulated inflammation (Dohrman *et al.* 1998). An alternative explanation may be that the metaplastic epithelium is showing the characteristics of gut epithelium. Winterford *et al.* (1999) examined mucins within the colon and found that mucin 2 was restricted to the goblet cells. Staining was cytoplasmic, concentrated in a mainly perinuclear distribution. Less mature cells lower in the gut crypts were the strongest staining cells. Reparative epithelium showed a larger number of positive cells (Mitsuuchi *et al.* 1999). In Barrett's oesophagus intestinal epithelium is found at the base of the oesophagus (Chaves *et al.* 1999). Maturing goblet cells also express mucin 2. It may be expected that immature metaplastic cells would demonstrate mucin 2 before maturing into goblet cells. The very sporadic expression of mucin 2 within the metaplastic population would suggest there is minimal cell turnover unless there is another stimuli for the production of mucin 2. The evidence of bacterial and viral stimulation appears more likely as COPD patients are susceptible to repeat infection.

Mucin 4 staining within the COPD cohort was seen within many patient airways and within the metaplastic cells of 41/50 patients. Mucin 4 is not normally seen in airway epithelium of the lower respiratory tract although it is seen in large airways and bronchioles (Audie *et al.* 1993). It may be upregulated by inflammation through IL-6 and neutrophil elastase. Neutrophil elastase exposure can lead to squamous metaplasia (Fischer *et al.* 2003). The group suggest that although the specific role of mucin 4 in the lung is unclear, it probably plays a role in lung repair and homeostasis. Mucin 4 is thought to potentiate the activity of the receptor ErbB2. ErbB2 receptor dimerises with EGFR, which might indicate mucin 4 controls growth, repair and maintenance in normal lung tissue.

Mucin 4 staining was seen on vascular endothelium, however capillary endothelium was not stained. There is little documentation of mucin 4 expression by endothelial cells. Jung *et al.* (2002) have demonstrated that human corneal endothelium produces mucin 1. It is thought that the mucin functions as a lubricant and protects from osmotic and pH variation. Lung mucin 4 may have a similar protective function, helping lubricate the lung as it undergoes structural changes and inflammatory influx.

Mucin 4 is seen in foetal airways. Mucin 4 is the earliest mucin detected in the foregut during development, seen at 6.5 weeks of gestation. As development continues it is

found in the trachea, bronchi and some bronchioles, with mucin seen in the more apical epithelial layers and is more widespread in the upper airways (Buisine *et al.* 1999). Mucin 4 is seen at high levels in upper airways during development before there is any real signal from either mucin 5AC or mucin 5B, suggesting that it is expressed on a very primitive preciliated form of epithelium. It was not found within the alveolar bed at any stage of gestation or on any adult samples.

The presence of mucin 4 staining on the metaplastic cell population may be the result of frequent rounds of lung infection and inflammation that are stimulating the metaplastic cells in the same manner as airway epithelium can be stimulated. Mucin 4 could also indicate that ectopic metaplastic cells are a more foetal cell phenotype and are showing primitive developmental characteristics. Lack of ubiquitous staining across samples and the apparent correlation with inflammation in nearby airways gives strength to the argument that the metaplastic cells are behaving in a similar way to airway epithelium.

Mucin 1, mucin 2, mucin 5AC and mucin 6 have been examined previously on atypical adenomatous hyperplasia (AAH), bronchioloalveolar carcinoma (BAC) and adenocarcinoma with mixed subtypes. Mucin 1 was present on AAH with expression diminishing as proliferations progressed to BAC and mixed adenocarcinoma. Expression of mucin 2, mucin 5AC and mucin 6 increased significantly on both tumour types (Awaya *et al.* 2004). The lack of mucin 2 staining on metaplastic cells suggests the cells are not pre-neoplastic.

To conclude, sub populations of metaplastic cells were positive for CK7 or CK18. Dual staining would be necessary to determine whether these populations were exclusive, overlapping or co-localised. The cytokeratin profile of the metaplastic cells appears consistent of simple cytokeratins CK7 and CK18 only. There was no evidence of CK5/6 (basal) or CK14 (squamous) positive metaplastic cells. There may be other simple cytokeratins present such as CK19 but further work would be needed to confirm this. The cytokeratin profile is summarised for the airways and metaplastic cells in Figure 5.17.

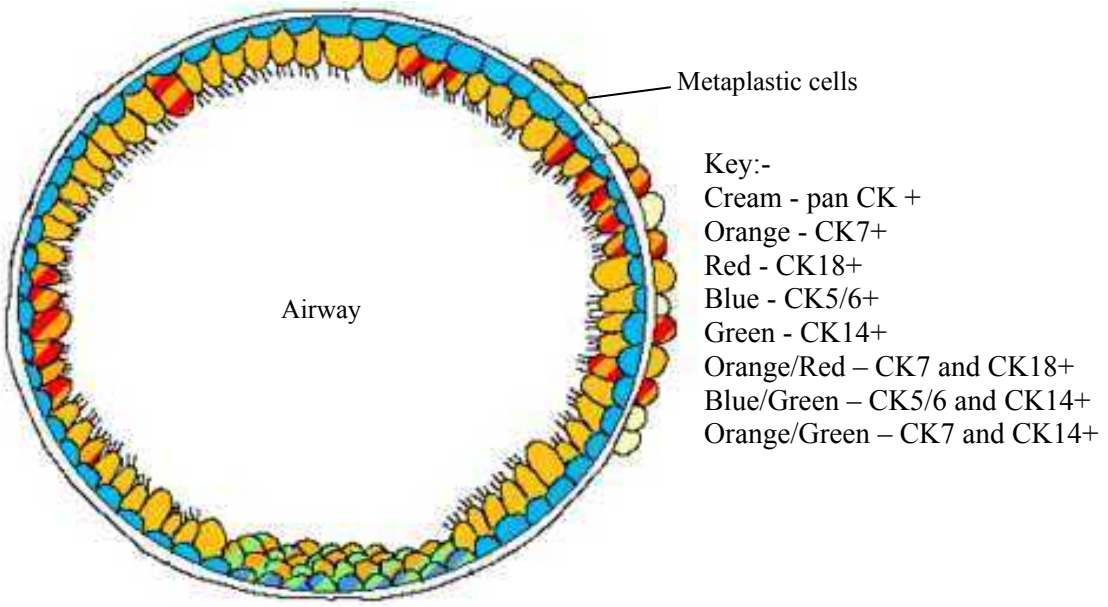


Figure 5.17 Diagram representing cytokeratin airway & metaplastic cell staining
Shows the results found in the COPD cohort of all the cytokeratins used here. The key at the side explains the colour used for each cytokeratin, the cream colour shows that the pan CK antibody highlighted these cells but none of the individual cytokeratin markers highlighted this population.

The metaplastic cells were shown to produce some mucins but mucin 5AC (normal airway mucin produced by goblet cells and mucus glands) was not detected. The metaplastic cells show mucin 4 expression which is not normal on airway epithelium. Upregulation of mucin 4 here may be attributable to inflammatory stimulation. The mucin 2 expression was sporadic in metaplastic cells and did not follow a distribution pattern consistent with maturing gut cells or adenocarcinoma. Occasional mucin 2 positive cells may also be stimulated by inflammation and infection.

The metaplastic cells show evidence of mixed origins with demonstration of both type II pneumocyte (surfactants) and airway epithelial (mucin 4). This is possibly suggestive of a primitive epithelial phenotype.

Chapter 6. Underlying matrix and microenvironment of the metaplastic lesion

The areas discussed in this chapter are the extra cellular matrix associated with metaplastic cells and the microenvironment of the metaplastic cells with regard to hypoxic conditions.

6.1 Extracellular matrix

There are a number of different proteins that form polymers and contribute to the scaffold and structure of organs and tissues. Laminins found in basement membranes are heterotrimeric glycoproteins and are made up of 3 subunits α, β and γ. Currently 5 different α subunits have been identified, along with laminin 3β and 3γ which combine to give at least 15 isoforms. In the adult lung α5 laminin is found in the airway, alveolar, endothelial and pleural basement membranes (Nguyen, Senior 2006).

Collagen forms a long triple stranded helix, where 3 α chains wind round one another. Around 25 α chains have been identified and around 20 different triple helix combinations have been identified. There are fibrous collagens I, II and III which make up the main structural components of collagen. Fibroblasts and epithelial cells produce collagens. Within the lung collagens I, III, IV, V, VI, VII, XII and XVI are found in various locations.

6.1.1 Basement membrane and extracellular matrix

The basement membrane is an acellular structure comprised of matrix layers. The airway basement membrane is made up of three layers; the lamina lucida, lamina densa and lamina reticularis. The lamina lucida is the most luminal layer, in contact with epithelial cells. The lamina lucida comprises collagen XVII, laminins 5, 6 and 10 and contains integrins α6, β4, α and β. The lamina densa is electron dense appearing as a strong dark line using electron microscopy. This membrane comprises collagen IV, laminin 1, entactin and nidogen with proteoglycans, perlecan, bamacan, agrin and collagen XVII and can store FGF-2 growth factor within the matrix. The lamina lucida and the lamina reticularis are both approximately 50nm in depth. The lamina reticularis is made up of fibrils of collagen and is only present in adults. These fibrils are collagens I, III, V, VI and VII with the proteoglycans perlacan, bamacan and collagen XVII.

Airway basement membrane is thicker and more obvious in the central airways. It becomes thinner moving down the respiratory tree almost joining with the capillary basement membrane within the alveolar bed. Entactin and nidogen play an important role in joining matrix proteins to one another, forming complexes with collagen IV, laminin and perlecan. Different membranes have different components which determines the overlying phenotypes depending on organ or location.

Basal cells anchor to the basement membrane via hemidesmosomes, whilst other epithelial cells such as columnar, ciliated and goblet cells adhere to the basal cell (Michelson, Tigue & Jones 2000).

Torikata *et al.* (1985) have shown that fibronectin is normally present in small amounts in capillary and airway basement membrane, however, once fibrosis has occurred fibronectin levels increase significantly. This demonstrates that extracellular matrix and basement membrane are often altered in disease. The lamina reticularis appears thickened in some disease, such as asthma.

Table 6.1 shows the range of structures contributing to vascular and airway networks. Normal adult lung expresses collagen I more strongly than collagen III. Alveolar walls, pleural, peribronchial and perivascular fibrous tissues show low level collagen I and very low levels of collagen III. Similar levels of collagen I and III are seen in the airway submucosa and vascular subintima. Collagens I and III are also observed surrounding granulomas. Type II collagen is only found in the cartilage (Bateman, Turner-Warwick & Adelmann-Grill 1981). Collagen IV lines alveoli, bronchi, vessels and pleura (Kaarteenaho-Wiik *et al.* 2007).

The alveolar basement membrane is composed of collagen IV, (Amenta, Gil & Martinez-Hernandez 1988) collagen V, (Madri, Furthmayr 1980) chondroitin sulfate, a sulfated glycosaminoglycan that forms chains, chondroitin sulfate proteoglycan and heparan sulfate proteoglycan (Sannes *et al.* 1993). Sannes *et al* (1984) have suggested type I and type II pneumocyte distribution is determined by basement membrane components. Fibronectin is differentially distributed on basal and luminal surfaces of type I pneumocytes and infrequently associated with type II pneumocytes.

Table 6.1 Summary of cell types & extracellular matrix contributing to lung membranes

Structure	Composition (cell types)	Extracellular Matrix
Bronchus 5-12mm	Basal cells	Collagens I, III, IV, V, VI and VII, XVII laminins 1, 5, 6 and 10, entactin/nidogen Proteoglycans; perlecan, bamacan, agrin. Integrins α6, β4, α and β
Bronchi 1-5mm	Basal cells	Collagens I, III, IV, V, VI and VII, XVII laminins 1, 5, 6 and 10, entactin/nidogen Proteoglycans; perlecan, bamacan, agrin. Integrins α6, β4, α and β
Bronchioles 0.3-1mm	Basal cells	Collagens I, III, IV, V, VI and VII, XVII laminins 1, 5, 6 and 10, entactin/nidogen Proteoglycans; perlecan, bamacan, agrin. Integrins α6, β4, α and β
Alveolar bed	Type II pneumocytes, type I pneumocytes	Collagen IV isoforms α3, α4, α5, laminin, fibronectin
Elastic artery 5-30mm	Endothelium (tunica intima), sm musc cells (tunica media), fibroblast (adv)	Elastin, fibronectin, fibrillin, fibulin, coll I, II, III, IV, V, VI, proteoglycans
Muscular artery 1-5mm	Endothelial cells, smooth musc cells, fibroblasts	Elastin, fibronectin, fibrillin, collagens I, III, IV, V, VI, proteoglycans
Vein 1-5mm	Endothelial cells, smooth muscle cells	Elastin, fibronectin, collagens I, II, III, IV, VI, XII, XIV proteoglycans
Arteriole ≥50um	Endothelial cells, smooth muscle cells	Elastin, collagens I, III, fibrillin
Venule 20-100um	Endothelial cells, smooth muscle cells, perivascular	Laminin, coll IV, fibronectin
Capillary ≤20um	Endothelial cells, pericytes	Collagen IV, laminin, fibronectin, heparin sulphate proteoglycan (HSPG)
Lymph vessel	Endothelial cells smooth muscle cells	Coll IV, laminin, bigger HSPG, fibronectin

Adapted from (Bou-Gharios *et al.* 2004)

Type II pneumocytes move through the parenchyma to replace type I pneumocytes in times of damage and during normal turnover. Lesur, Arsalane & Lane (1996) demonstrated that growth factors, TGFα, EGF, HGF and aFGF and matrix components such as laminin, fibronectin and collagen I, all promote cell migration. Examination of COPD tissue for these components will indicate type II pneumocyte attraction. Type II pneumocytes can produce fibronectin and laminin, therefore fibroblast produced matrix is not necessary (Dunsmore *et al.* 1996). When cultured on fibronectin alone type II pneumocytes lose mature phenotype and develop a more type I phenotype (Rannels *et al.* 1987). Biological activity of type II pneumocyte matrix may depend on the fibronectin-to-laminin ratio. Crouch & Longmore (1987) have found that type II cells

preferentially bind to fibronectin but can also bind to laminin. This may help stimulate repair of lost type I cells.

In lung disease fibrotic IPF lungs undergo extensive vascular remodelling and matrix deposition. In early fibrosis collagen III deposition increases, as the lesion progresses collagen I increases. Collagen I is rigid so increased amounts make tissue less flexible and breathing more difficult.

The extracellular matrix protein tenascin is seen in foetal lung development and in adult tumours. Tenascin C presence in tumours is believed to be linked to establishment of malignant activity (Chilosi *et al.* 1993). Tenascin C is present in pulmonary fibrosis, particularly usual interstitial pneumonia, a known subgroup of idiopathic pulmonary fibrosis (Paakko *et al.* 2000). Tenascin mRNA expression has been demonstrated in myofibroblasts and type II pneumocytes of active foci in usual interstitial pneumonia. The location of tenascin C throughout development suggests that it is involved with assisting epithelial-mesenchymal interactions (Kaarteenaho-Wiik *et al.* 2001). The presence of tenascin C in adult tissue is indicative of disease.

Pre-neoplastic lesions are often identified, using altered cell morphology, within tissue surrounding lung tumour. Fisseler-Eckhoff *et al.* (1990) have examined the basement membrane components in these areas. Membrane thickness was assessed and found to remain normal underlying metaplasia and micropapillomatosis. Micropapillomatosis are small projections of muscle pushing the basement membrane into convolutions. Membrane was thickened underlying basal cell hyperplasia and even further thickened under goblet cell hyperplasia. The thicker membrane seen with basal and goblet cell hyperplasia appeared to be due to increased collagen III deposition. In severe dysplasia the basement membrane became discontinuous with weak fibronectin and collagen III staining. The only continuous component was laminin. In the early cancer samples, the membrane becomes split into very thin layers. It was broken and discontinuous whereupon laminin was the only matrix underlying the tumourlets and even individual cells. This suggests that tumour cells degrade all other matrix components and are capable of depositing laminin. It should be noted that neo-vascularisation often accompanies these basement changes.

To summarise, basement membranes form around bronchioles, alveoli, vessels and capillaries. Membranes are also located around all muscle blocks, airways or

vasculature as well as surrounding peripheral nerves and the pleura. These membranes determine structure, compartmentalise the lung, control proliferation, migration, differentiation and apoptosis of the many cells that adjoin these membranes.

6.2 Field cancerization

Conceptually, both COPD and lung cancer can be considered as a primary lesion which then phenotypically alters the local cell environment to induce repair dysregulation. A useful concept here is 'field cancerization'. This model was first introduced by Slaughter, Southwick & Smejkal in 1953, based upon studies of oral tumours. Field cancerization has been used to describe cellular genotypic and phenotypic switching that occurs around tumour foci. Neoplastic tissue is surrounded by abnormal tissue, which, in itself is not neoplastic, but exhibits many molecular features of a neoplastic cell. The 'field cancerization' model has also been applied to a theoretical model of cancer development. Epithelial cells are not the primary initiating cell, but respond to phenotypically altered stromal cells or matrix that induce profound epithelial phenotype alterations. It is thought that microenvironment determines overall tumour phenotype. The new signal repertoire from transformed epithelium influences neighbouring cells amplifying the tumour signal. Figure 6.1 shows a diagrammatic representation of field effect. Other lung tumour work has found that the majority of cases demonstrate altered and abnormal surrounding tissue (Solomon, Greenberg & Spjut 1990). Interestingly, Jensen *et al.* (1994) showed in NSCLC and mesothelioma resection margins, that CC10 and CK14 are increased in areas of parenchymal bronchiolisation; these cell populations were both undergoing proliferation. Numerous cell types are influenced by tumour field effects indicating a phenomenon that does not solely affect epithelial cells. All malignant tumours investigated by Slaughter, Southwick & Smejkal (1953) had benign abnormal, hyperplastic and fibrotic epithelium spreading beyond the boundaries of malignant growth. There were also smaller foci in surrounding tissue, supporting the idea of local influence with positive feedback and multiple lesion origins.

Other influences may include chronic inflammation, aberrant repair, particulate or chemical damage. The presence of squamous metaplastic foci near lung tumours has been examined by Klein *et al.* (1993). Foci number was assessed, the presence of *myc* protein or p53 accumulation, both known oncogenes, was examined in foci and tumours. One third of patients showed between 1 and 4 foci squamous metaplastic foci.

Tumour samples had *myc* mutations in 6/16 samples, p53 overexpression in 4/16 and only one sample had an altered *ras*. 3 patients showed *myc* changes in the metaplastic foci and 2 patients had increased p53, where one of the patients with altered p53 in the foci did not demonstrate altered p53 in the tumour. This data highlights that local changes may be a direct result of a field effect or independent changes. Solomon, Greenberg & Spjut (1990) suggested different lung compartments respond to carcinogens at different rates and this may explain why some tumours, for example adenocarcinoma, are found more often in specific lung compartments.

Field effects are very important during development. Signal gradients in foetal development control growth or phenotype change. Freund *et al.* (1987) examined epithelial and mesenchymal cells of salivary rudiments. Single cell type infection with polyoma virus had no effect. If the two cell types recombined a neoplastic response took place. This shows epithelial-mesenchymal interaction was necessary for tumorigenesis to occur. Field effect was occurring in normal adult tissue to maintain tissue order. Culturing embryonic stem cells has shown that different concentrations of the same chemical lead to different cell types forming.

One mouse strain develops spontaneous testicular teratocarcinoma, if these multiple phenotype tumours are excised and put into blastocysts, a normal mouse, with a genetic mosaic is produced. The tumour cells have followed the same local cues as the host tissue and mature into appropriate cell types according to tissue location (Mintz, Illmensee 1975).

Field effects may also be demonstrated by released signals. Released matrix metalloproteinase 9 (MMP9) plays an important role in epithelial migration and lung inflammation, influencing behaviour in COPD lung. Varying levels of MMP9 are expected in the parenchyma of the COPD lung and MMP9 may highlight damaged areas or active repair (Warburton *et al.* 2006).

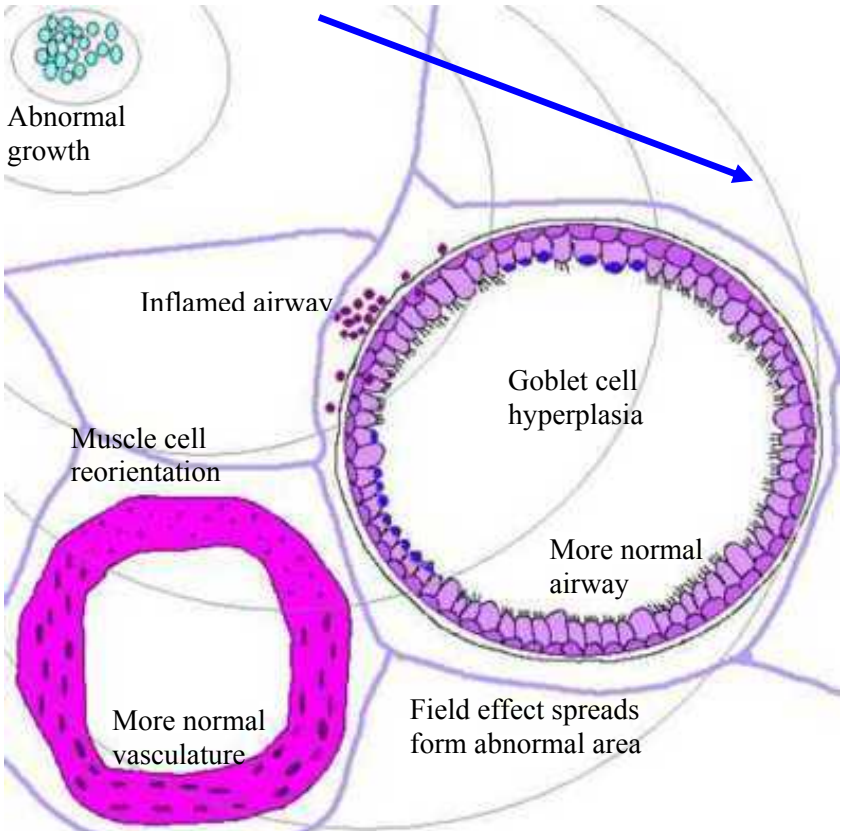


Figure 6.1 Diagrammatic representation of field effect

A diagram to demonstrate the field effect from abnormal growth. The vessel and airway would also be sending out altered signals and the inflammation would alter things further. The airway shows inflammation, goblet cell hyperplasia and relative normality. The vessel shows areas of apoptosis.

6.3 Cell microenvironment

In this context microenvironment refers to any cell or structure's immediate surrounding. This locale may have altered O_2 levels, matrix, growth factor or mediator imbalances that influence the growth, survival or phenotype of cells. Hypoxia is known to cause damage in the lung and COPD lung is known to have gas-trapped areas that are possibly hypoxic (Rice & Nicholson 2009).

Hypoxia inducible factor-1, (HIF-1) accumulates rapidly when hypoxia occurs. HIF is a heterodimer made up of an α subunit, with three alternatives α1, α2 or α3 and a β subunit ARNT. Under hypoxic conditions the α unit is stable, binding to ARNT and is able to recognise HIF-response elements in the promoter regions of hypoxia responsive genes. When conditions are normoxic the α subunit interacts with Von Hippel-Lindau (VHL) is ubiquinated and rapidly degraded.

For VHL to recognise HIF two proline residues, 402 and 564, must be hydroxylated. Hydroxylation is carried out by prolyl hydroxylases. In hypoxic conditions HIF is not

hydroxylated and rapidly accumulates. This leads to heterodimer formation and the production of hypoxia gene products.

HIF has been shown to induce the chemokine, stromal cell-derived factor-1, (SDF-1) which is thought to influence progenitor cell recruitment (Ceradini *et al.* 2004). Cellular recruitment may influence the repair phenotypes occurring and identifying areas of hypoxia may indicate areas to investigate in more detail.

Another potential hypoxia marker is carbonic anhydrase IX (CA IX). This is produced in hypoxic tissue as a result of increased HIF levels. As CA IX is upregulated downstream from HIF, it may not be as specific as HIF for use as a marker of hypoxia (Groenman *et al.* 2007, Kim *et al.* 2005). CA IX has a relatively long half-life so areas that have recently become normoxic may express both CA IX and the normoxia marker VHL.

The presence of CA IX has been associated with poor prognosis in a range of tumours. Kon-no *et al.* (2006) examined CA IX expression in adenocarcinomas. CA IX was observed in around one quarter of adenocarcinoma samples examined. These cases all appeared to be more aggressive and invasive without the presence of lepidic growth. CA IX is thought to function as a pH regulator when tissue or tumours are hypoxic. Normal lung tissue did not show any CA IX expression in either type II pneumocytes or airway epithelium.

As well as the normoxic degradation of HIF 1α,Von Hippel Lindau protein has been linked to VHL disease where genetic alteration leads to an autosomal dominantly inherited predisposition to a variety of tumours. Corless *et al.* (1997) examined the normal distribution of VHL strong epithelial staining was seen in all tissues examined. Normal lung epithelium was not examined however, the presence of epithelial staining in small cell carcinoma, mesothelioma and adenocarcinoma was confirmed. This would suggest that a VHL antibody should highlight airway epithelium and possibly alveolar bed.

Using CA IX to demonstrate areas in which HIF controlled gene upregulation may have occurred it was hoped that hypoxic areas of tissue could be identified. VHL usually accumulates in areas of normoxia, it was hoped that this would give an opposite profile to CA IX allowing assessment of areas of hypoxia and normoxia, neither of these

antibodies allow absolute detection as the protein expression is downstream in a pathway or may be influenced by other factors.

6.4 Aims and Objectives

The aim of chapter 6 was to determine whether there were features of the local environment necessary for the development of a metaplastic lesion.

Are metaplastic cells associated with underlying mesenchymal cells?

Mesenchymal cells were investigated using desmin (muscle), vimentin (mesenchymal) and S100A4 (fibroblast).

Is there a particular matrix associated with metaplastic cells?

Antibodies for collagens I, III and IV, laminins containing α1 or α2 subunits and elastin (EVG) were investigated.

Is there evidence for an association between local hypoxic conditions and metaplastic cell localization?

COPD damage including, airway obliteration, fibrosis and angiogenesis all indicate local hypoxia within the COPD lung. Areas of possible hypoxia were investigated using immunohistochemistry for carbonic anhydrase IX to indicate hypoxia and von Hippel Lindau accumulation to outline normoxia.

6.5 Results for Surface Phenotype

The following section summarises the immunohistochemistry findings for mesenchymal cells, matrix markers and oxygenation indicators.

6.5.1 Results for Mesenchymal cells: Vimentin IHC

The structural protein vimentin is found in a range of cells including fibroblasts, smooth muscle cells, macrophages and lymphocytes. This marker was used to identify mesenchymal cells. The question addressed here was firstly whether mesenchymal cells contributed to the metaplastic cell population and secondly, if not directly contributing, were the cells consistently found to be underlying the colonising cells.

Vimentin antibody was applied to COPD patients 1-10. Vimentin was seen extensively within cell cytoplasm and on some matrix. Macrophages and lymphocytes were positive, with a speckling of positive stain through the vascular muscle and many fibres

appeared stained. The airways in patients 2 (Goblet cell airway changes), 3 and 4 (Airway changes & fibrosis) showed some positive cells within epithelium. All samples had occasional positive cells or fibres underlying the metaplastic cells regardless of location (Figure 6.2A, C, E, 6.3A). Table 6.2 summarises vimentin underlying metaplastic cells. Vimentin presence was not consistent for all areas of metaplastic cells or patients. Vimentin positive cells do not determine the presence of metaplastic cells.

Table 6.2 Vimentin staining around metaplastic cells

Pat	Pathology Code	Vimentin
1	Airway changes & fibrosis	+ Underlying SP, hilar & adventitial cells
2	Goblet cell airways change	Airway epithelium positive, + adventitial & SP & Fibrotic cells
3	Airway changes & fibrosis	Airway epithelium positive, + Underlying SP, hilar & adventitial cells
4	Airway changes & fibrosis	Airway epithelium positive, + underlying SP & adventitial cells
5	Airway changes & fibrosis	+ Underlying adventitial cells
6	Goblet cell airways change	+ Underlying adventitial & alveolar cells
7	Normal	+ Underling SP & adventitial cells
8	Airway & pleural change	+ Underlying SP & adventitial cells
9	Goblet cell airways change	+ Underlying SP & fibrotic cells
10	Fibrosis & inflammation	+ Underlying adventitial, fibrosis & hilar cells

6.5.2 Results for Smooth muscle cells: Desmin IHC

Mesenchymal cell presence in close association with the metaplastic cell population needs further investigation to determine whether these cells are smooth muscle cells or fibroblasts. Muscle does not produce any significant matrix or maturation cues whereas fibroblasts may be able to fulfil this role. Desmin is a structural protein that should be seen in muscle cells. This marker was run on COPD patients 1-10. All patients demonstrated positive muscle cell staining. This muscle staining was apparent in the vessel walls, in muscle blocks surrounding airways and in any fibrotic blocks. There were instances of positive cells adjacent to or underlying the metaplastic cell population. No metaplastic cells stained positive for desmin. It appears that muscle cells may be able to provide part of the structure that metaplastic cells are colonising these cells are not the sole contributor (Figure 6.2B, D, F, 6.3B). This is not a determining feature as there were areas of uncolonised muscle.

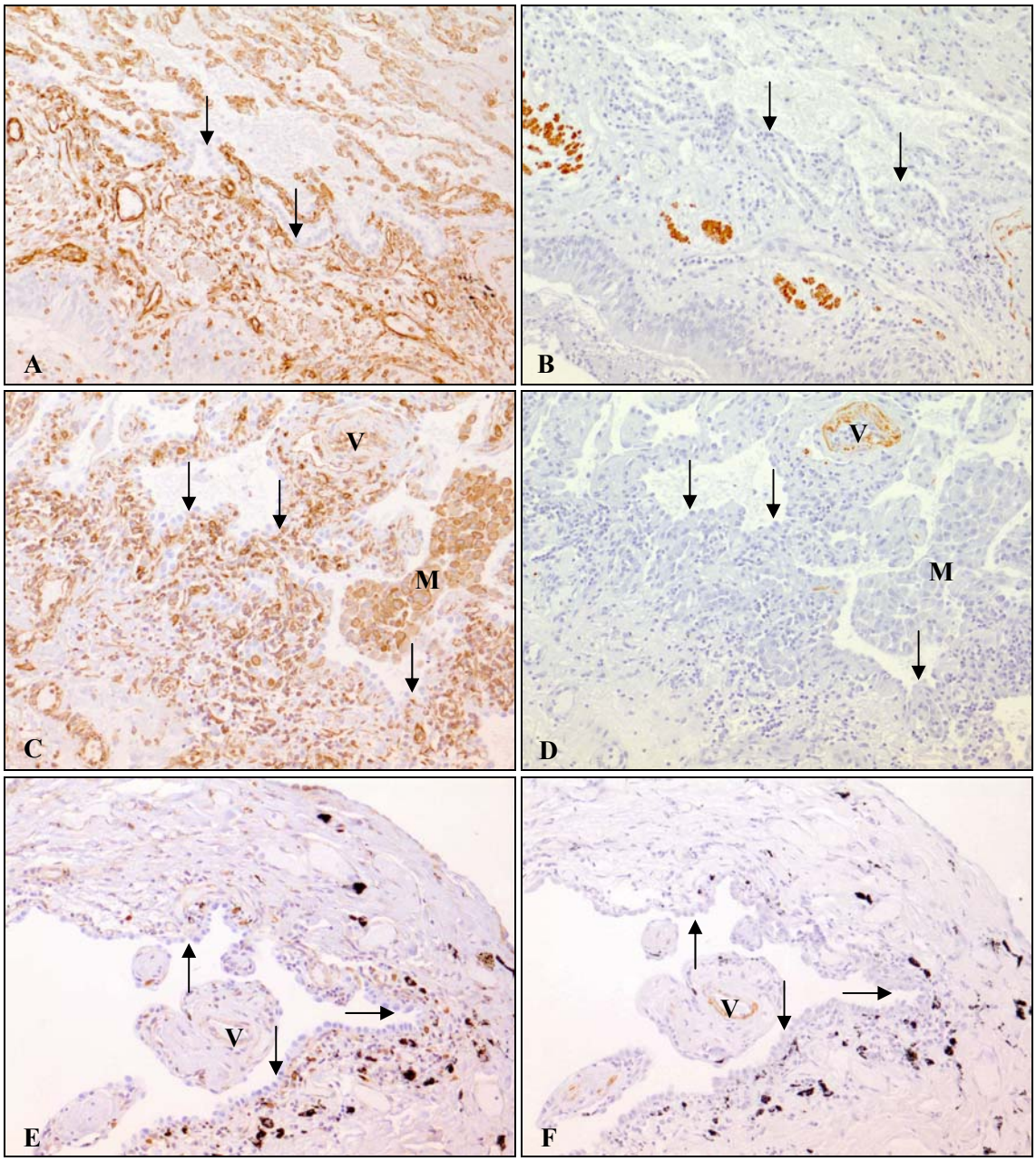


Figure 6.2 Mesenchymal cells, desmin & vimentin IHC

Figure 6.2A)Vimentin positive staining (Patient 8: Airway & pleural change), the bottom left of the picture has part of an airway with vimentin positive cells within the epithelium. There is also vimentin positive staining in the muscle surrounding the airway and any smooth muscle or fibroblast cells within the parenchyma.

Figure 6.2B) Desmin positive staining (Patient 8: Airway and pleural change) can be seen within the muscle blocks surrounding the large airway which can be seen in the bottom left of the picture.

Figure 6.2C)Vimentin positive staining (Patient 6: Goblet cell airway changes) underlying uniform metaplastic cells (arrows), there is also positive vimentin staining within the macrophages (M)

Figure 6.2D) Desmin positive staining (Patient 6: Goblet cell airway changes) underlying uniform metaplastic cells and positive macrophage population (M), the only staining seen was in a small vessel (V)

Figure 6.2E) Minimal vimentin positive staining (Patient 4: Airway change & fibrosis) underlying macrophage-like metaplastic cells (arrows) on an area of sub pleura.

Figure 6.2F) Desmin staining on a corresponding area to Figure 6.2E (Patient: Airway changes & fibrosis), tissue underlying mac-like metaplastic cells (arrows) is negative, only real staining is of small vessel in centre of picture (V).

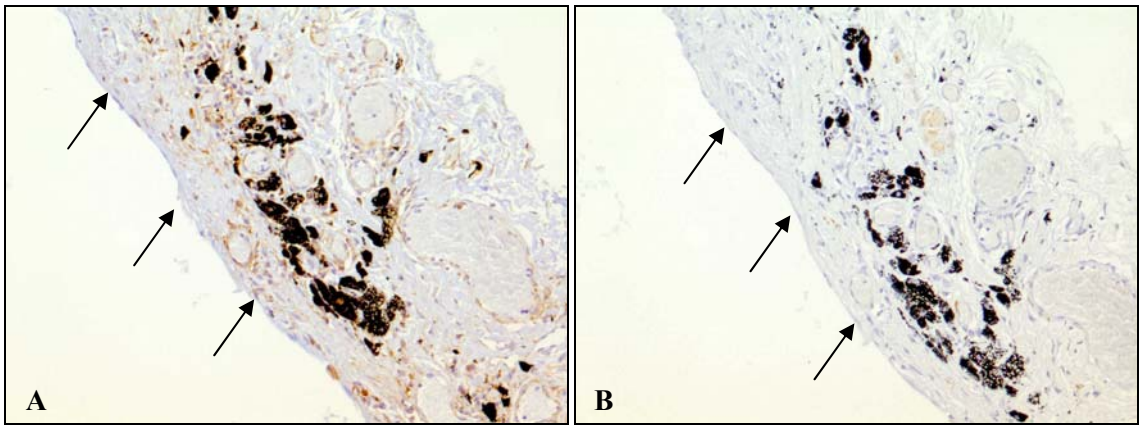


Figure 6.3 Mesenchymal cells continued, desmin & vimentin IHC

Figure 6.3A) Vimentin positive staining (Patient 10: Fibrosis & inflammation) underlying squamous metaplastic cells, arrows indicate surface lined with squamous cells

Figure 6.3B) Desmin staining (Patient 10: Fibrosis & inflammation) on a corresponding area to Figure 6.3A, tissue underlying squamous metaplastic cells (arrows) is negative for desmin, the only positive signal comes from a muscle block at the centre of the picture

6.5.3 Results for Fibroblasts: S100A4 IHC

S100A4 was used as a fibroblast marker on samples from patients 1-10, examples of staining may be seen in Figure 6.4. This marker highlighted a subset of lymphocytes which have an obvious rounded morphology and were distinguishable from S100A4 positive fibroblasts. Patients 1-10 all showed a positive wash within macrophages. All patients had lymphocytes sitting underneath the metaplastic cell layer in a range of sites (Figure 6.4A). Lymphocytes were not consistently present but were visible in patches or clusters in all 5 locations of colonisation. There were also some positive fibroblasts underneath some metaplastic cells, these were sporadic. Patient 9 (Goblet cell airway changes) had some positive cells sitting within sub pleural, adventitial and fibrotic tissue metaplastic cells (Figure 6.4B, C, D). These could be underlying cells in areas of discontinuous metaplastic cells or lymphocytes infiltrating the metaplastic cell layer but the morphology was not consistent with fibroblasts (Figure 6.4E, F). Overall fibroblasts were not directly contributing to the metaplastic cell population and although fibroblasts were underlying metaplastic cells, fibroblast presence was not the determining feature.

This concludes the mesenchymal cell staining. These stains collectively demonstrate that mesenchymal cells are occasionally present underlying metaplastic cells however; it was not a requirement for the development of metaplastic cells.

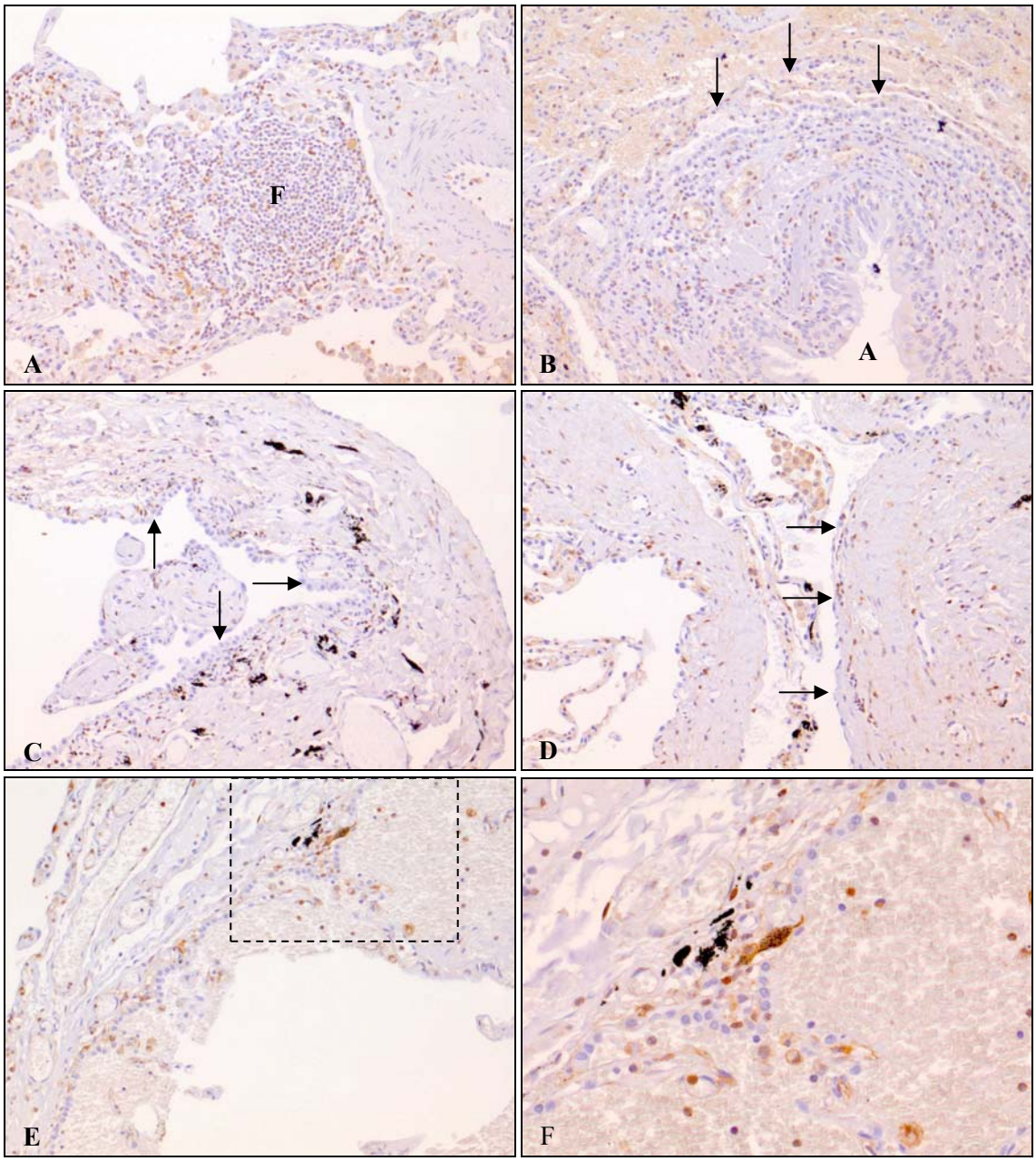


Figure 6.4 Fibroblasts S100A4 IHC

Figure 6.4A) S100A4 positive staining Patient 3: Airway changes & fibrosis) within a sub population T cells within an inflammatory foci (F).

Figure 6.4B) S100A4 staining (Patient 6: Goblet cell airway changes), airway (A) and uniform metaplastic cells (arrows), diffuse staining in tissue, metaplastic cells negative.

Figure 6.4C) S100A4 minimal positive staining (Patient 4: Airway changes & fibrosis) underneath an area of sub pleural metaplastic cells .

Figure 6.4D) S100A4 minimal positive staining (Patient 1: Airway changes & fibrosis) underneath an area of squamous metaplastic cells on an area of fibrosis, there are a number of positive cells.

Figure 6.4E) S100A4 was seen within the metaplastic population in patient 9 (Goblet cell airway changes).

Figure 6.4F) x20 objective of the S100A4 positive cells suggests that the cells are lymphocytes within the metaplastic population.

6.6 Results for matrix markers

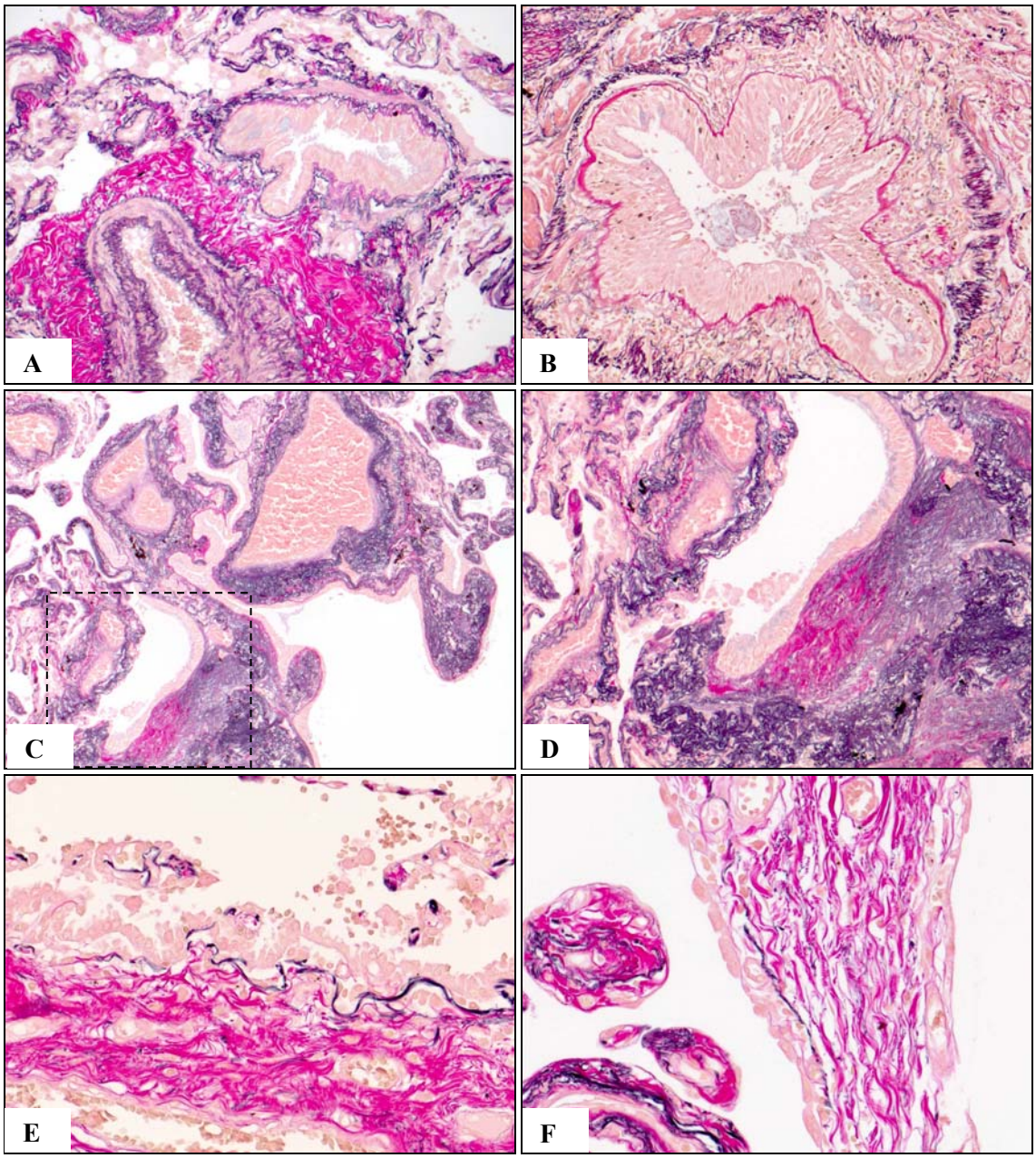


Figure 6.5 Elastic van Gieson matrix staining

Figure 6.5A) EVG (Patient 4: Airway changes & fibrosis) shows a small airway with elastin, collagen layers directly underlying epithelial cells, epithelial cells are yellow, elastin is black and collagen is pink.
Figure 6.5B) EVG (Patient 20: Airway changes & fibrosis) on a small airway, here there is a clear pink collagen layer but no elastin directly under the epithelium.
Figure 6.5C) EVG x5 objective (Patient 10: Fibrosis & inflammation) shows an area of bronchiolisation the spreading epithelial cells are pale yellow. The underlying tissue shows extensive black elastic and an area of elastin and pink collagen.
Figure 6.5D) EVG (Patient 10: Fibrosis & inflammation) shows a x10 objective of the boxed area in Figure 6.5C. This shows the predominant elastin signal with some collagen underlying spreading cells.
Figure 6.5E) EVG (Patient 28: Airway changes & fibrosis) shows an area of metaplastic cells with a partial thin layer of elastin and extensive collagen within the layers under the metaplastic cells.
Figure 6.5F) EVG x20 objective (Patient 10 Airway changes & fibrosis) shows an area of metaplastic cells with very oedematous underlying tissue. The metaplastic cells are adhering to elastin or a pale layer lacking both elastin and collagen, this with Figure 6.5E demonstrates variable underlying matrix.

6.6.1 Results for Elastic van Gieson staining

Tinctorial stain elastic van Gieson was employed on patients 1-10 and a range of blocks from other patients to give a comparison of the surface underlying the metaplastic cell population compared with that of the airways. EVG shows elastin fibres in dark grey or black with collagen fibres staining vivid pink and the remaining tissue having a yellow appearance. The airways showed either a layer of black elastin directly underlying the epithelial cells or a vivid pink layer representative of collagen fibres (Figure 6.5A, B). In cases where there was collagen directly underlying epithelium there was a black elastin layer further into the sub mucosa. Areas of bronchiolisation showed extensive collagen and elastin deposition (Figure 6.5C, D). Areas underlying metaplastic cells showed a mixed response with areas of collagen, or elastin and areas where both were absent (Figure 6.5E, F). Based on elastin and collagen staining provided by EVG, neither matrix component is consistently found underlying the metaplastic cell population.

6.6.2 Results for collagen I IHC

A collagen I antibody was used to highlight matrix deposition throughout the lung. Collagen I is found largely associated with vasculature, with little contribution to the airway basement membrane. The objective was to assess the presence and influence of collagen I underlying the metaplastic cells. Collagen I staining was carried out on patients 1-10. Positive staining was observed in all samples, collagen I was present within media and sometimes adventitia of smaller vessels (Figure 6.6E, 6.7A). Vascular staining was variable with not all vessels being highlighted. Collagen I was also seen within the pleura, some fibrosis, the adventitia of some larger vessels and some airway smooth muscle (Figure 6.7A). Tissue was examined for the presence of staining directly underneath the metaplastic cells. Although some areas showed a wash in underlying tissue there was no clear stain directly under colonising cells (Figure 6.7C). Patient 2 (Goblet cell airway changes) showed staining within the epithelial and metaplastic cell populations. This staining was small granules within the cytoplasm suggestive of collagen I production by these cells.

6.6.3 Results for collagen III IHC

A collagen III antibody was used to highlight the deposition of this collagen. The objective was to examine the influence of collagen III underlying the metaplastic cells

and to contrast it with airway basement membrane. Collagen III staining was examined on samples from patients 1-10. Positive staining was observed in the adventitia and media of all vessels as expected, however the intensity of staining was variable on some patients (Figure 6.7F). Smooth muscle in airway sub mucosa was consistently positive (Figure 6.7B). The pleura where present, showed positive staining, along with hilar projections into the tissue. Any fibrotic clubs within the lung parenchyma were also collagen III positive. On many occasions the adventitial surface underlying metaplastic cells was positive for collagen III (Figure 6.7E). Collagen III presence was not consistent under all metaplastic cells.

6.6.4 Results for collagen IV IHC

A collagen IV antibody was used to examine the presence of this membrane collagen. Collagen IV is widespread in the lung, being found underlying airways and within the alveolar bed. This antibody was examined on COPD patients 1-10. Collagen IV presence underlying metaplastic cells is summarised in Table 6.3. This has a similar distribution to collagens I and III but is also seen around some airways. Small and large vessels often showed medial and intimal staining (Figure 6.9C). The airways had staining through much of the sub mucosal layers (Figure 6.8D). The metaplastic cell population often had positive underlying collagen IV in a range of locations (Figure 6.9A). Positive staining under the metaplastic cells was not seen ubiquitously, it does not appear to be crucial for metaplastic cell colonisation.

Table 6.3 Summary of collagen IV IHC under metaplastic cells

Pat	Pathology Code	Underlying metaplastic cells
1	Airway changes & fibrosis	- Underneath
2	Goblet cell airways change	+ Under SP, airway remnants - underneath
3	Airway changes & fibrosis	+ Under adv
4	Airway changes & fibrosis	+ Under SP
5	Airway changes & fibrosis	+ Under few adv
6	Goblet cell airways change	+ Under SP, + under remnants
7	Normal	-Ve underneath mets
8	Airway & pleural change	+ Under SP
9	Goblet cell airways change	-Ve under few adv
10	Fibrosis & inflammation	+Ve under few adv

6.6.5 Results for laminin IHC

An antibody against laminin 1 and laminin 2 was applied to the whole sample panel of 50 COPD patients and 6 control samples. Laminin staining was seen extensively throughout the lung as expected. Laminin stained strongly around all airways (Figure

6.8E). Laminin also highlighted vascular membranes within large vessels and small capillaries. Large vessels often had extensive laminin 1/2 staining through the intima and occasionally media (Figure 6.9D). There were also laminin deposition in areas of fibrosis, parenchymal fibrotic clubs scarring where the alveolar connectivity were often positive. The surface underlying the metaplastic cells was always positive. Laminin intensity was measured in areas underlying metaplastic cells and compared with airway basement membrane. There was no minimum signal associated with metaplastic cell colonisation, some areas were very weak and others were very strong (Figure 6.9B, E). There did not seem to be any association with laminin intensity and the presence or absence of inflammation. The white area values, darkest DAB staining and laminin staining were measured according to the red, green and blue colour levels using Leica QWin analysis software.

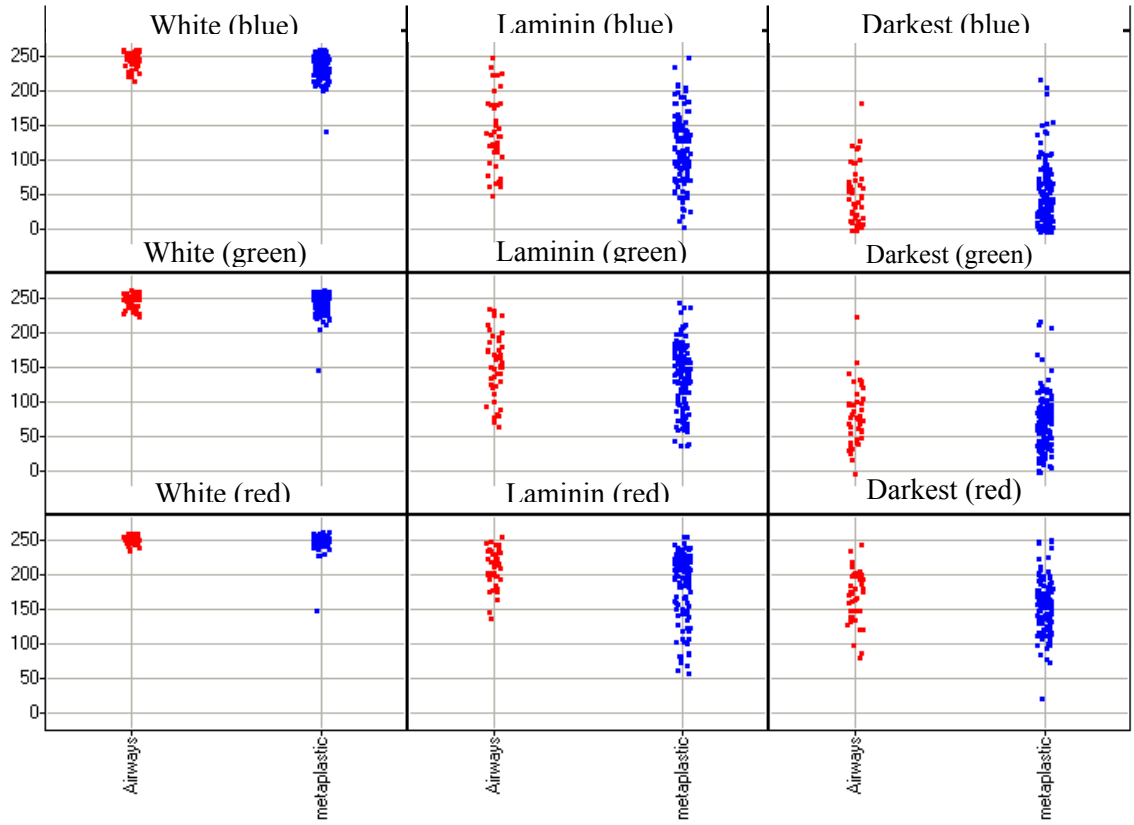


Figure 6.6 Laminin staining intensity underlying airways & metaplastic cells
Intensity values for the airways (red) and the intensities for the metaplastic cells (blue) were plotted to examine if there was a minimum threshold or range for laminin underlying metaplastic cells. The white light values show that the photographs were all taken at approximately the same light readings. The laminin values for blue and green show complete overlap between airways and metaplastic cells. The values for red laminin have a greater range than the airways.

Strongest laminin staining underneath airways and metaplastic cells were measured. There does not appear to be any threshold determining metaplastic cell presence and these findings are demonstrated in Figure 6.6. The spread over the darkest graphs shows

that the strongest staining intensity varied quite a lot between slides. This would be in part due to biology and in part, sample and experiment variability.

Collagen I˙Collagen III

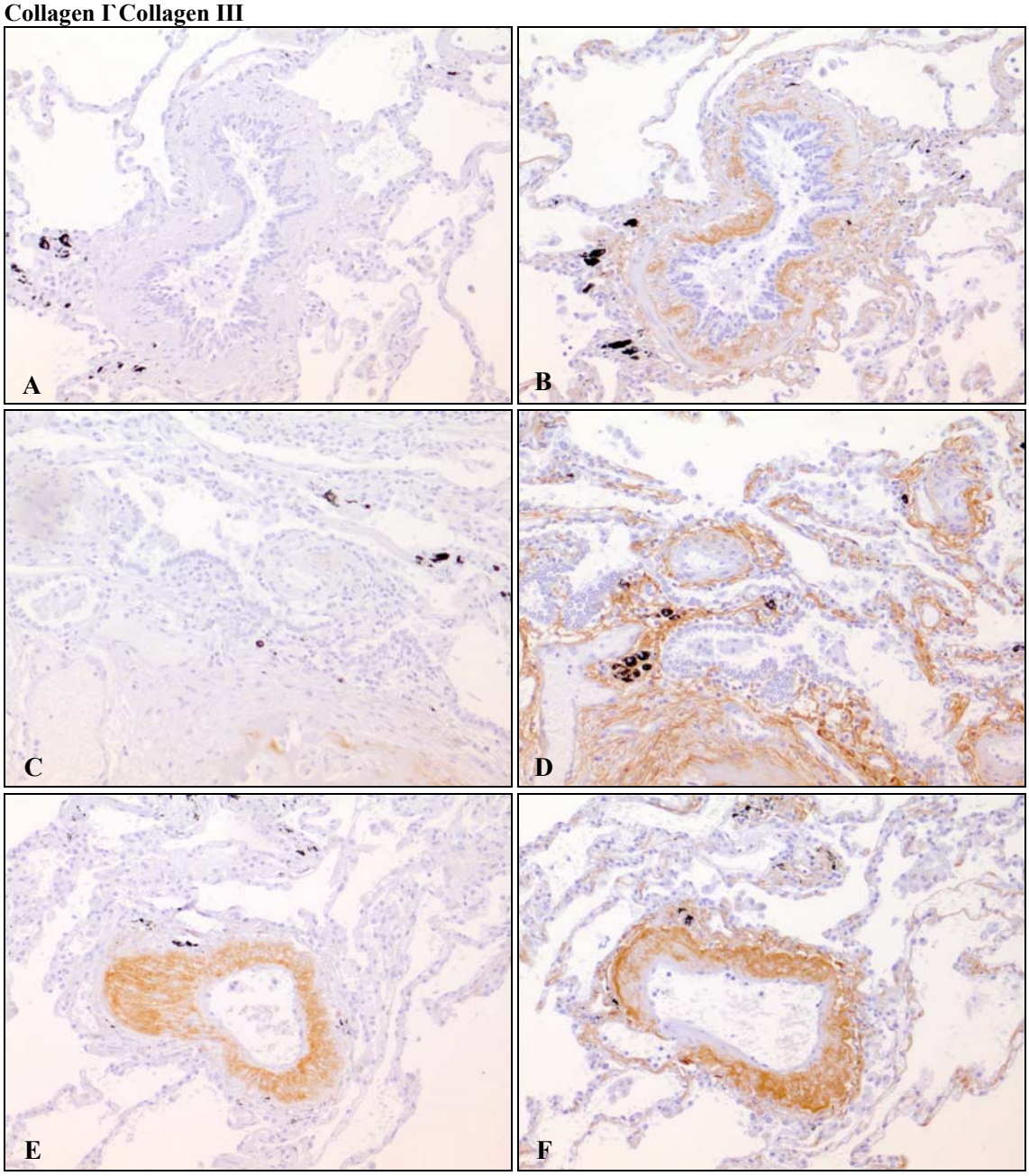
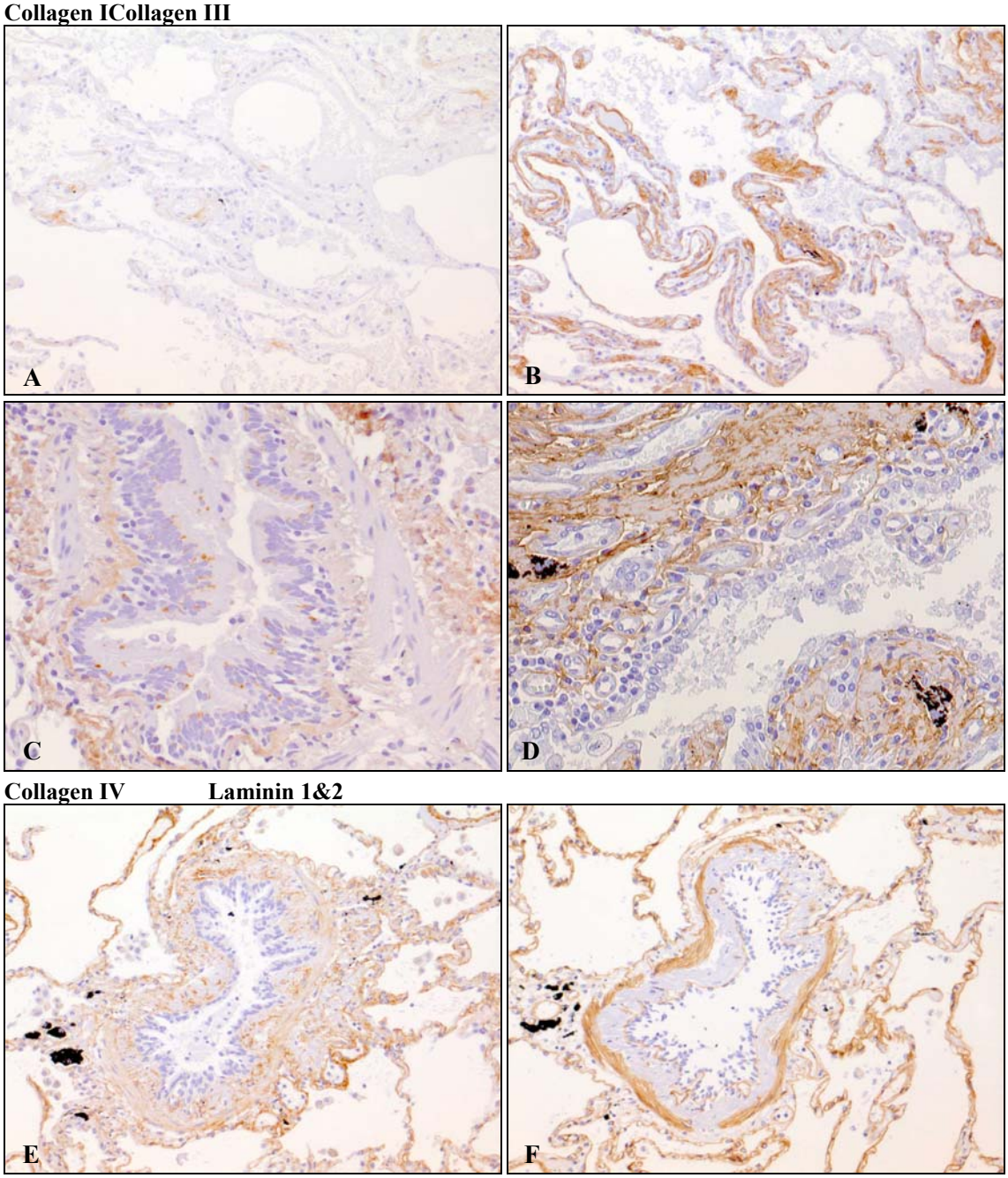


Figure 6.7 Matrix IHC, Collagen I & Collagen III

Figure 6.7A) Collagen I staining (Patient 9: Goblet cell airway changes) on the airways.
Figure 6.7B) Collagen III staining (Patient 9: Goblet cell airway changes) on the airways.
Figure 6.7C) Collagen I staining (Patient 5: Airway changes & fibrosis) negative underlying the metaplastic cells
Figure 6.7D) Collagen III staining (Patient 5: Airway changes & fibrosis) negative underlying the metaplastic cells
Figure 6.7E) Collagen I staining (Patient 9: Goblet cell airway changes) in the small vessels
Figure 6.7F) Collagen III staining (Patient 9: Goblet cell airway changes) in the small vessels

Collagen ICollagen III



Collagen IV Laminin 1&2

Figure 6.8 Matrix IHC, Collagen I, Collagen III, Collagen IV & Laminin

Figure 6.8A) Collagen I staining (Patient 5: Airway changes & fibrosis) in the parenchyma, only seen in small vessels.

Figure 6.8B) Collagen III staining (Patient 5: Airway changes & fibrosis) in the parenchyma, seen in all smaller vessels.

Figure 6.8C) x20 objective of Collagen I staining (Patient 2 Goblet cell airway changes) shows some granular collagen I within the airway epithelium suggestive of collagen production.

Figure 6.8D) x20 objective Collagen III staining is minimal directly underlying metaplastic cells.

Figure 6.8E) Collagen IV staining (Patient 9: Goblet cell airway changes) positive in the airways.

Figure 6.8F) Laminin 1&2 staining (Patient 9: Goblet cell airway changes) positive around the airways.

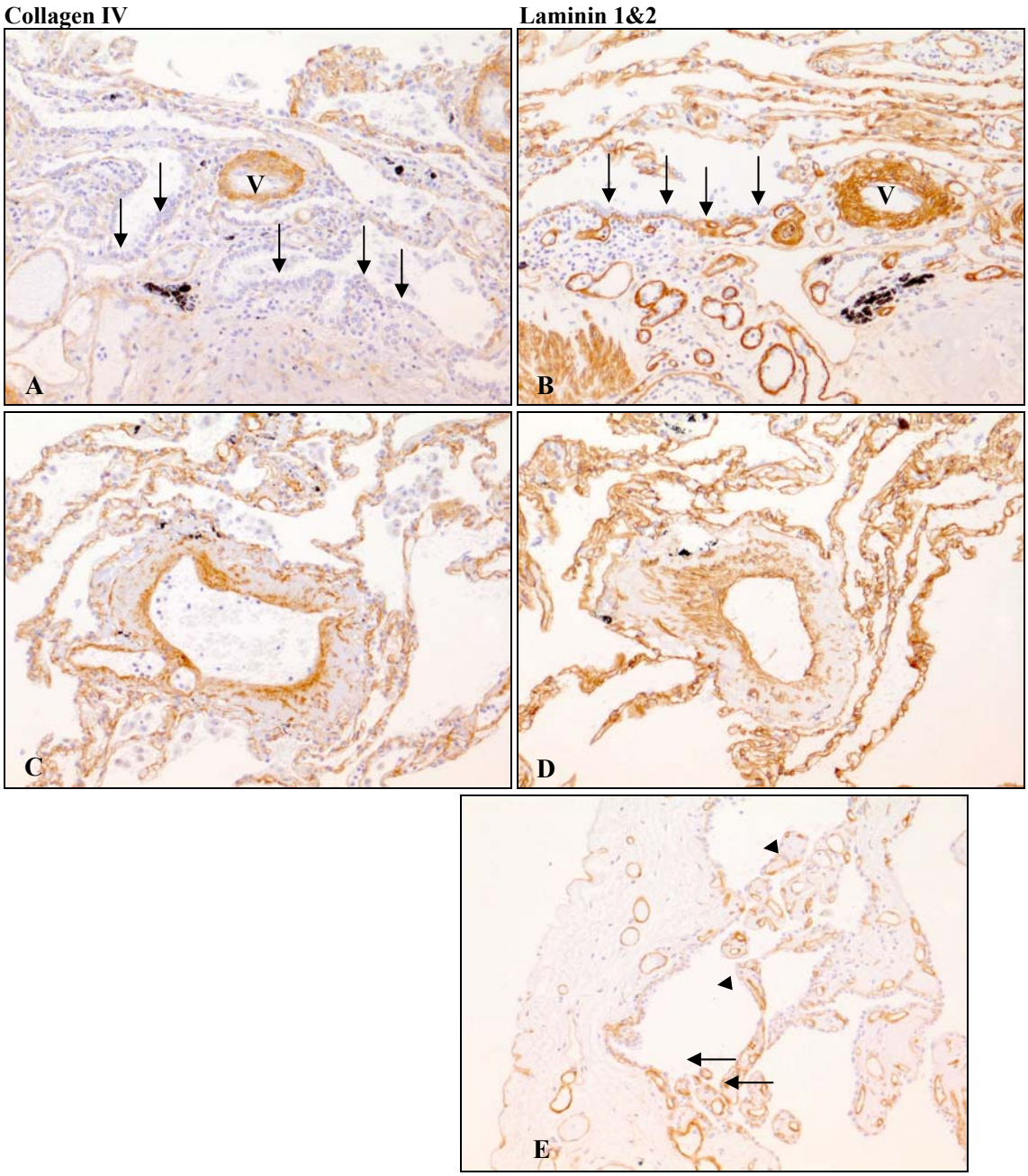


Figure 6.9 Matrix IHC, Collagen IV & Laminin

Figure 6.9A) Collagen IV positive staining (Patient 5: Airway changes & fibrosis) underlying the metaplastic cells.

Figure 6.9B) Laminin 1&2 positive staining (Patient 5: Airway changes & fibrosis) underlying the metaplastic cells, the laminin stain pre-treatment damages the epithelial cells, the cells are still visible at higher powers, this is an area of strong staining underneath the metaplastic cells.

Figure 6.9C) Collagen IV staining (Patient 9: Goblet cell airway changes) strong positive within the media of many vessels.

Figure 6.9D) Laminin 1&2 staining (Patient 9: Goblet cell airway changes) positive within the muscle wall of vessels.

Figure 6.9E) Laminin 1/2 variable staining (Patient 38: Fibrosis) underlying the metaplastic cells, this was an area of sub pleural metaplastic cells that show strong underlying stain (arrows) and weak underlying stain (arrow heads).

6.7 Results for microenvironment investigation: CA IX and VHL IHC

An antibody for Von Hippel Lindau protein was used to highlight areas of normoxia, examples of this staining are in Figure 6.10. A second antibody, carbonic anhydrase IX (CA IX) was used to highlight areas of hypoxic cells or tissue. Both VHL and CA IX antibodies were examined on the sub panel that consisted of 28 COPD patients and 6 control samples. VHL protein was found on epithelial cells as expected, positive cells were seen in airway epithelium, type II pneumocytes and metaplastic cells (Figure 6.10A, C, E). Staining was not observed on 100% of cells in all locations or samples. Table 8.3 in the appendix summaries the approximate percentage of positive cells in various lung locations. On some occasions where staining was really strong, there appeared to be positive vascular muscle cells. This occurred in patient 11 (Goblet cell airway changes), patient 19 (Airway changes & fibrosis), patient 21 (Goblet cell airway changes) and patient 37 (Goblet cell airway changes).

CA IX staining was observed predominantly in vascular muscle cells and pleural connective tissue. There were areas of parenchyma with staining, along with a subset of inflammatory cells, both macrophages and lymphocytes. On a few samples there was CA IX staining seen within airways and metaplastic cells, which was usually minimal and weak (Figure 6.10B, D).

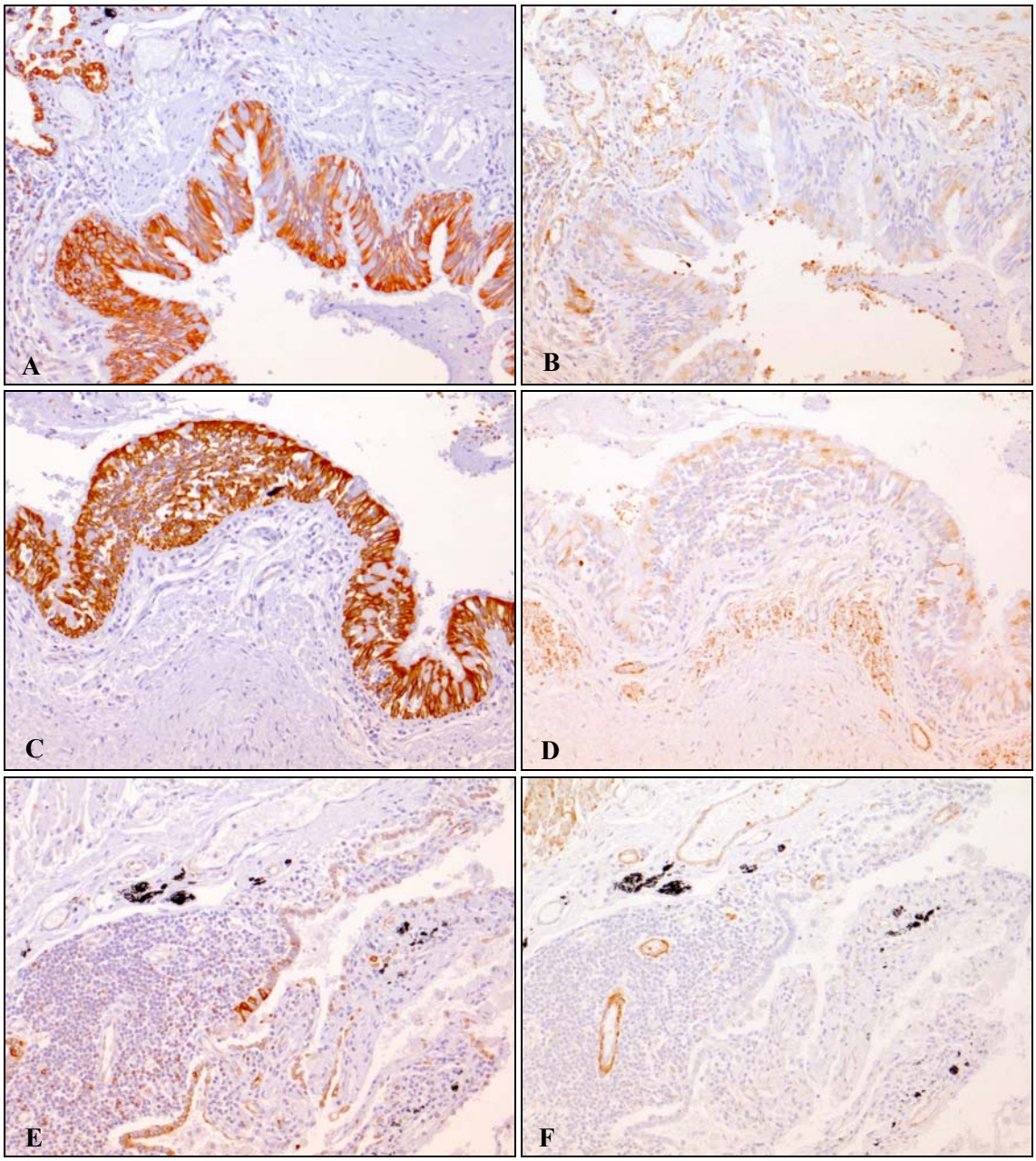


Figure 6.10 Carbonic anhydrase IX & Von Hippel Lindau IHC

Figure 6.10A) VHL staining (Patient 5: Airway changes & fibrosis) positive within the small airways.
Figure 6.10B) CA IX staining (Patient 5: Airway changes & fibrosis) strong positive within the small airways.
Figure 6.10C) VHL staining (Patient 5: Airway changes & fibrosis) within the large airways.
Figure 6.10D) CA IX staining (Patient 5: Airway changes & fibrosis) within the large airways and muscle blocks.
Figure 6.10E) VHL staining (Patient 5: Airway changes & fibrosis) variable along metaplastic cell string.
Figure 6.10F) CA IX staining (Patient 5: Airway changes & fibrosis) comparable area with no staining in metaplastic cells, positive staining in the vasculature.

6.8 Discussion of Surface Phenotype Differences

6.8.1 Mesenchymal cells

There is growing literature demonstrating epithelial to mesenchymal transition alongside evidence that underlying mesenchymal cells can influence epithelial phenotype. These events make it important to consider whether mesenchymal cells are contributing to or underlying the metaplastic cell population. Mesenchymal cells could determine the locations colonised and influence epithelial phenotypes. The phenomenon of epithelial to mesenchymal phenotype change when put under extreme stress has been demonstrated in the lung. An example of this is BOS (Bronchiolitis Obliterans Syndrome), which is seen in chronic rejection of lung transplants (Borthwick *et al.* 2009). The marker used to demonstrate the mesenchymal cell population was vimentin. This marker was seen extensively throughout lung tissue, present in airway and vascular muscle, in fibrotic tissue and in an acellular manner on basement membrane and submucosal fibres. Three patients showed evidence of vimentin staining within the airway epithelium but there was no influence of vimentin stain within the metaplastic cell population. The airway positive signal may have been epithelial to mesenchymal transition or more likely these cells are lymphocytes within the epithelium.

One member of the mesenchymal cell family is the fibroblast. Fibroblasts are a complex cell phenotype, which are very difficult to label with one antibody. Positive vimentin and negative desmin staining identifies fibroblasts and myofibroblasts. Fibroblasts may also be tissue specific. Evidence shows epithelial cells cultured overlying fibroblasts from another organ, can change epithelial phenotype (Myerburg *et al.* 2007). With this in mind, it was of interest whether fibroblasts could be detected underneath metaplastic cells. Fibroblast staining with S100A4 was variable underlying metaplastic cells in all 10 patients examined. This indicates fibroblast presence is not necessary for metaplastic cells to populate a surface. It also implies that fibroblasts are not needed for differentiation or maturation signals.

Previous work has shown that fibrocytes migrating into the lungs of IPF patients may be highlighted initially by CD34 but that as the cell matures this epitope is lost. The fibrocyte must be considered when interpreting results here as there is an element of fibrosis in COPD (Andersson-Sjoland *et al.* 2008). Although CD34 within the metaplastic cell population was shown in chapter 4, there is no S100A4 positive stain

suggesting that the CD34 positive cells are not maturing into fibrocytes. Further examination of these cells is necessary to make this final conclusion, by dual staining of CD34 with S100A4 and smooth muscle actin.

Desmin is an intermediate filament found in smooth muscle cells and myofibroblasts. The production of desmin was not seen within metaplastic cells. There is no indication from metaplastic cell morphology that muscle is contributing to the cell population. The underlying tissue was also examined for the presence of desmin, although there are areas of airway, vascular and *de novo* muscle under the metaplastic cells, it was not consistently seen under all cells. Metaplastic cells can colonise desmin positive muscle but are also able to adhere to other types of mesenchyme.

6.8.2 Underlying Matrix

The use of elastic van Gieson staining showed that there was variable matrix underlying the metaplastic cell population. Black *et al.* (2008) have used EVG staining and fluorescence to evaluate elastic fibre loss within small airways disease. This work suggested that there were matrix changes occurring surrounding airways and within parenchyma. Matrix loss and remodelling is constantly occurring in COPD tissue, these changes may stimulate new matrix deposition. Shifren *et al.* (2007) have shown that although elastin is not usually produced in the adult lung, fibroblasts can produce elastin in disease. The surface on which metaplastic cells adhere was examined for the presence of collagen I, III and IV and laminin 1/2. Collagens I, III and IV were found only sporadically underlying the metaplastic cells. The presence or absence of these collagens did not appear to affect the spread or adhesion of metaplastic cells. The only consistently present component was laminin 1/2. The antibody used for this study detected any laminin that contained α1 or α2 chains. There was also a clear basement membrane seen using electron microscopy (Figure 4.23), airway basal cell of the airways and type II pneumocytes are the only cells that adhere directly to basement membrane (Michelson, Tigue & Jones 2000, Sannes 1984). Other airway epithelium is thought to join to basal cell desmosomes. This gives airways an additional defence mechanism allowing rapid shedding of all luminal layers. Previous staining with CK5/6 and ΔN-p63 has discounted basal cells from contributing to the metaplastic cell population, which leaves the type II cell. Vaccaro & Brody (1981) used EM to examine the structure of the alveolar bed which showed that capillary membrane and alveolar membrane have different characteristics. Ruthenium red staining was indicative of

different anionic charges. Although the overall matrix composition may appear similar, proteogylcan content, or anionic charge can greatly alter membranes.

Coraux *et al.* (2005) have shown that embryonic cells cultured on collagen I become Clara cells. These Clara cells when put in air liquid interphase (ALI) culture demonstrated the capability of producing, bulbous non-ciliated Clara cells, basal, intermediate and ciliated cells. Differentiation to a Clara cell phenotype occurs on other matrix proteins but takes almost twice as long. This may suggest a synergistic effect between collagen I and other growth factors. The ability of embryonic cells to develop into a range of cell types suggesting that de-differentiated epithelial cells exist in the foetal lung. Other recent studies by Ali *et al.* (2002) have shown embryonic epithelial cells being capable of developing to become type II cells.

COPD airways have been shown to have increased amounts of collagens I, III and IV, fibronectin and laminin (Kranenburg *et al.* 2006). This may have led one to expect metaplastic cell basement to exhibit these matrix proteins. The metaplastic cells did not consistently have collagen I, III or IV underlying the lesion. The lack of collagen staining further highlights differences between metaplastic cells and nearby airways showing these cells are not Lambertosis. Kranenburg suggested that widespread increases in airway basement matrix deposition was due to remodelling. It has been shown that underlying matrix increases in goblet cell hyperplasia (Fisseler-Eckhoff *et al.* 1990), the absence of matrix constituents underlying COPD metaplastic cells further supported by the lack of PAS staining, showed that the metaplastic cells were not goblet cell hyperplasia.

Alveolar spaces in IPF are lined with increased collagen III in early disease, as remodelling developed collagen I increases. Collagens XII and XIV also increased but this appears to be more associated with vascular remodelling (Tzortzaki *et al.* 2006). Chilosi showed laminin and tenascin underlying areas of colonised alveolar bed in IPF. This emphasized that a range of matrix proteins underlie the lining cells of IPF (Chilosi *et al.* 2006). This seemingly collagen led matrix profile does not correspond to the laminin only COPD metaplastic cell matrix profile. This divergent matrix profile suggested that the fibrosis of underlying tissue was not the main or sole pre-requisite for deposition of colonising cells.

Bronchopulmonary dysplasia lungs show an increase in fibronectin. Fibroblasts produce this but production by reparative and cuboidal epithelium also occurs (Sinkin *et al.* 1998). Overall collagen levels surrounding the saccules seemed to be increased in BPD. This might have been due to the increased size of the saccule pushing the collagen scaffold outwards and compressing it (Thibeault *et al.* 2003).

Work on pre-neoplastic lung lesions demonstrated that as epithelial alterations progressed towards dysplasia, the underlying matrix was divided and degraded leaving only laminin (Fisseler-Eckhoff *et al.* 1990). Bronchioloalveolar carcinomas (BAC) are unusual; tumour growth does not alter lung structure instead utilising existing alveolar surfaces. It has been shown in non-mucinous BAC that there are irregular patches of laminin and collagen IV present underlying tumour cells. Alveolar bed connective tissue showed collagen III and fibronectin indicative of fibrosis. The alveolar bed showed degradation of normal continuous collagen IV and laminin (Guedj *et al.* 2004). Other work has suggested that matrix loss only occurs in more severe sclerosing cases of BAC (Goto *et al.* 2001). Overall these lining tumours showed little or no matrix profile alteration and laminin was usually present.

Epithelial cells are capable of producing certain matrix proteins including tenascin, β2 laminin (Altraja *et al.* 2008), fibronectin and collagen (Bodo *et al.* 2001). This would suggest that the colonising cells are able to produce the underlying laminin matrix.

Haddad & Massaro (1968) examined the abnormal epithelial proliferations in IPF and the link to cancer formation. This work suggested there may be links between fibrosis, scarring, surface colonisation with epithelial-like cells and a progression to cancer. Walter & Pryce (1955) examined over 200 tumours and found that 55% of those in the peripheral lung were associated with lung scarring. This work suggested that fibrosis and scarring provide an appropriate surface for lining cells to colonise and that the altered cells may be a pre-neoplastic lesion. Although COPD has an element of fibrosis, it is not usually as marked as IPF. The lack of underlying collagen and colonisation of areas other than fibrosis do not support the theory of a pre-neoplastic lesion in COPD.

There are a number of matrix components not examined in this work, these include membrane constituents collagen 7 and laminin 5, as well and tenascin seen in foetal development and tumours and fibronectin (Weinacker *et al.* 1995). Laminin 5 would not have been highlighted by the antibody used here as it contains α3 chains, it is found in

lung basement membrane throughout development and in adulthood (Coraux *et al.* 2002).

6.8.3 Microenvironment

The two markers used to interrogate the microenvironment were carbonic anhydrase IX and von Hippel lindau protein. CA IX is a downstream enzyme from HIF 1α, which should demonstrate areas where hypoxia has occurred or there is some stress signal upregulation and VHL may be used to demonstrate normoxia. The majority of airway epithelial and type II cells were highlighted with VHL as was expected. There were areas of both airway and metaplastic cells that appeared to have a staining gradient within the epithelial cells; some were very strongly stained whereas others were quite weak. This change in intensity may not be a true reflection of the oxygen available as staining changed from one cell to the next. Normal distribution of VHL was strong signal within epithelial cells (Corless *et al.* 1997).

Local oxygenated blood is supplied to the metaplastic population lining the external adventitia of vessels through the vasa vasorum. The structure of the vasa vasorum has been studied in the coronary artery and within other organs of the body, demonstrating a branching tree-like structure. This should mean that throughout the area supplied by the vasa vasorum oxygen levels are similar. This makes it impossible for oxygen levels to vary in the distance of a single cell, the whole area should have the same oxygen levels (Galili *et al.* 2004, Gossl *et al.* 2003). One explanation for differing staining intensity may be different cellular oxygen so cells do not exhibit any lowering of normoxic proteins such as VHL and do not show any expression of stress markers such as HIF 1α or CA IX. Although some cells showed lower staining VHL suggestive of lower VHL expression these cells were not showing any positive stain for CA IX. This indicates low oxygen level but not hypoxia. The majority of samples showed low level CA IX within vascular muscle and pleura. This may result from the high level of remodelling occurring in these areas.

Little is known about normal physical oxygen levels for specific cells, the variability of VHL staining may highlight different tolerances of epithelial cells. This tolerance may be influenced by prior exposure to altered oxygen levels, cell maturation or other factors. Although some patients show more CA IX than others there are only a few patients with expression of this stress-related pH mediator within the metaplastic cell population. This suggests that cells are not hypoxic and under stress so perhaps are not

present as a result of hypoxic damage. This is difficult to confirm the severe emphysema patients of this cohort will be receiving oxygen therapy and so may have been able to re-oxygenate previously hypoxic areas.

Asikainen *et al.* (2006) have used a baboon model of bronchopulmonary dysplasia to examine the effects of hypoxia in the lung. This work showed that HIF activation induces prolyl hydrolases that stimulate angiogenesis and help the lung recover from BPD. This may suggest that low-level hypoxia that is sufficient to stimulate HIF may have protective effects within the lung and that hypoxia may not be always detrimental.

The use of mesenchymal markers has shown that there are regions of metaplastic cells associated with underlying fibroblasts. These cells are not always present perhaps indicating that the cells are not vital for lesion formation. Further investigation using dual staining of desmin, vimentin, S100A4 and other markers such as smooth muscle actin would be needed to further characterise the fibroblast population.

The only matrix component that was consistently found directly underneath the metaplastic cell population was laminin. Collagen IV is found extensively throughout the lung therefore it was often in close proximity to metaplastic cells. Collagen I and III were present within the tissue but not directly adjacent to the metaplastic lesion, suggesting these are not fundamental matrix components for metaplastic cells. There are a number of other matrix proteins such as tenascin, fibronectin and collagen VII that may underlie metaplastic cells and further work would be necessary to complete this study. The metaplastic cells may be capable of laying down the underlying matrix. The matrix profile of metaplastic cells is very similar to pre-neoplastic lesions and tumours where there is no collagen and only laminin present.

The markers used to demonstrate areas of normal or low oxygen tension did not seem to suggest that low or normal oxygen tension was predictive of metaplastic cell presence. More responsive markers such as HIF would be beneficial to confidently determine areas of hypoxia. COPD may have had ventilation before surgery, which may have altered the oxygen tension within the lung.

Chapter 7. Genetic alterations and indications of pre-neoplasia

7.1 Why cancer in COPD?

COPD and lung cancer both affect the lungs, usually of older individuals and predominantly in those who smoke. The incidence of COPD is around 20% in smokers. The incidence of lung cancer in smokers is around 11% (Decker 2002). Recent publications suggest that those with chronic lung diseases such as emphysema and COPD are at increased risk of developing cancer (Gao *et al.* 2009).

Smoking is the main risk factor for both COPD and lung cancer, however, cigarette type, frequency, and inhalation habits are thought to influence the tumour type that develops. The predominant tumour seen in smokers has altered as cigarette-smoking habits have changed. Initially central lung tumours, adenocarcinoma and oat cell carcinoma, were seen with smoking. With filtered and low nicotine cigarettes individuals may inhale more deeply, leading to changes in peripheral lung. A meta-analysis carried out in 2000 showed that squamous cell carcinoma and small cell carcinoma are more strongly associated with smoking than adenocarcinoma and large cell carcinoma (Khuder 2001). Squamous and large cell carcinomas are both peripheral tumours (Corrin 2006). Papi *et al.* (2004) carried out a study looking at the risk of developing certain types of lung cancers if the individual has COPD already. It was found that the presence of COPD greatly increases the risk of developing squamous cell carcinoma. A study examining the occurrence of COPD in lung cancer found that 45% of adenocarcinoma patients, 51% of squamous carcinoma patients and 47% of NSCLC patients also had COPD (Young *et al.* 2009). Papi *et al.* (2004) have examined NSCLC tumour subtypes associated with COPD and determined a three-fold increased risk of developing squamous cell carcinoma in the presence of COPD. There was less association with chronic bronchitis and adenocarcinoma. Histologically the metaplastic lesion in COPD shows similarity to some adenocarcinoma subtypes, such as lepidic bronchioloalveolar carcinoma. The peripheral nature of the lesion may be more suggestive of squamous cell carcinoma. Examination of the presence of tumour characteristics or indications of altered phenotypes may be indicative of a pre-neoplastic lesion.

To allow further understanding of the possible overlapping pathways to the development of COPD and cancer the genetic susceptibility of both diseases have been extensively studied, a few recent findings for COPD are outlined in section 1.4.4.

A genetic predisposition for COPD development shows that disease is greatly influenced by small gene changes. The molecular and genetic changes necessary for cancer development have been extensively studied, so it is perhaps worth considering what types of changes these are and whether similar changes may occur in COPD some of these similarities are outlined in section 1.6.

Breuer *et al.* (2005) have previously examined lung pre-neoplastic lesions that may develop into squamous cell carcinoma. The work examined changes observed within airway epithelium; sixty percent of patients investigated had COPD and thirty-five percent of these individuals progressed in a non-stepwise fashion to develop squamous cell carcinoma. This work demonstrates that COPD patients often harbour pre-neoplastic lesions and that these lungs are capable of developing tumours.

7.2 Cell characteristics in cancer development

One theory of how cancerous cells may develop is through normal cells acquiring abnormal changes. The key cellular changes are:

Autocrine - Autocrine growth signals, down regulation or inhibition of growth lost.

Cell death - The evasion of apoptosis and normal cell death regulation.

Replication - The ability to replicate indefinitely, the avoidance of cell senescence.

Angiogenesis - Angiogenic ability, new vascular development supplying new tissue.

Invasion - Invasive ability, basement membrane breach for migration and metastasis.

Each of these changes can take place via numerous mechanisms. Important growth factors include epidermal growth factor receptor (EGFR, ERBB1) and HER2/neu (ERBB2). These may both be independently expressed in NSCLC. Small cell lung carcinoma (SCLC) is often associated with stem cell factor and tyrosine kinase receptor CD117 (c-kit). de Boer *et al.* (2006) have looked at the influence of EGFR in COPD and smokers. This showed that damaged epithelium expressed EGFR regardless of smoking or COPD status, however, intact epithelium showed an overall higher level of

expression in COPD compared to non-COPD patients. This suggests COPD epithelium may exhibit similar changes to pre-neoplastic change.

Markers linked with cell death avoidance are p53 gene and BCL2 proto-oncogene. BCL2 is found in 75-95% of SCLC. BAX is a BCL2 related protein promoting apoptosis and is downstream transcription target of p53. BAX expression is usually low in SCLC when the tumour is p53 deficient. The ability to override normal growth inhibition signals can be identified by looking for methylation of various promoters. COPD seems to have increased levels of apoptosis. Hodge *et al.* (2005) have shown increases in airway epithelial apoptosis and T cells apoptosis in COPD.

Telomerase is an enzyme capable of adding telomeres. Telomeres are shortened after each round of replication therefore there is a finite number of divisions any cell can undergo. With telomerase, telomere length never becomes short enough to stop replication, therefore there is limitless replication capability. All lung cancers express some level of telomerase activity.

Sustained angiogenesis is necessary for the tumour to support itself as it increases in size. Implicated in this role VEGF is important in foetal lung vascular development. VEGF levels are altered in COPD, whereas levels appear decreased in emphysema, but increased in chronic bronchitis (Fong *et al.* 2003).

K-ras mutation occurs quite late in tumour development so even if the metaplastic cells are precancerous K-ras may not be altered. However, K-ras is also smoking related so very relevant to COPD tissue (Keohavong *et al.* 2004).

Another gene of interest is p53. Investigation of changes in lung cancer gave the following results: small cell lung carcinomas 75-100% altered, 47% of non-small cell lung carcinomas, 51% of squamous-cell carcinomas, 54% of large-cell carcinomas and 39% of adenocarcinomas. These proportions were calculated using meta-analysis of data available (Sekido, Fong & Minna 2003). p53 gene alteration is seen in all tumour types it is also an early event in tumour development that has been detected in pre-neoplastic lung lesions and so perhaps is more likely in COPD (Sozzi *et al.* 1992).

7.3 Markers of altered epithelium

Proliferation rates are used as an indication of a pre-neoplastic lesion. Tumours are known to have high proliferation and apoptosis rates (Cavarga *et al.* 2009). If the

diffuse metaplasia in COPD represents a pre-neoplastic lesion it may be expected to have a high proliferative compartment.

Said *et al.* (1983) have found that involucrin is absent in normal bronchial epithelium. Involucrin is expressed on cultured tracheal epithelial cells and in bronchial mucosa with squamous metaplasia. Squamous cell carcinoma has also been found to strongly express involucrin. The COPD metaplastic cell population has squamous metaplastic morphology with many flattened cells adjacent to fibrosis it may therefore be expected to express involucrin.

p63 highlights more cells than basal cells, seen on squamous epithelial cells it is used in tumour diagnosis. It is involved in cell turnover and is on progenitor cells or a p53 type pathway where cells will be sent into apoptosis. Chilosi *et al.* (2002) also suggest that p63 may be linked with dysregulated epithelial growth and repair. Previous work looked at acute interstitial pneumonia examining the presence of p63 and p21 both of which are closely related to p53. The acute interstitial pneumonia samples had hyperplastic type II pneumocytes that stained positive for p53. The type II pneumocytes of COPD tissue are continually repairing and so may be expected to show p53 staining. p53 overexpression was examined in a large number of pre-neoplastic and tumours. Metaplastic samples showed little p53 overexpression and the group concluded that alterations of p53 in lung cancer is a very late event (Chyczewski *et al.* 2001). p63 has a number of isoforms that can be categorised as transactivating TA-p63 which functions in the same way as p53 and dominant negative ΔN-p63 that has a negative effect on p53. The two isoforms appear to have opposite functional effects. Truncated, ΔN-p63, is also referred to as p40, is seen on basal epithelial cells and can be thought of as being found on progenitor or proliferative cells.

Epidermal growth factor receptor (EGFR) is normally only found in airway basal epithelium (Rusch *et al.* 1995). It is involved in normal repair processes. Injured cells rapidly release IL-13, which stimulates the phosphorylation and activation of EGFR. Allahverdian *et al.* (2008) have shown that blocking IL-13 signalling attenuates repair. Damaged bronchial cells also release several EGFR ligands. This pathway is also involved with goblet cell metaplasia generation (Tyner *et al.* 2006). This is a feature of COPD further suggesting increased EGFR in COPD.

EGFR has been examined in the peripheral airways of cystic fibrosis (CF) patients due to the influence of EGFR on mucous production. EGFR appears to be increased in CF epithelium when compared to normal patients. Burgel *et al.* (2007) showed a marked increase in mucin 5AC and mucin 5B in CF epithelium. It was surmised that increased EGFR mediated mucin over-expression. EGFR over-expression has been shown in 44% of NSCLC. This over-expression was also observed on early bronchial neoplasia suggesting it is an early event in tumour development (Rusch *et al.* 1995, Rusch *et al.* 1993). Meert *et al.* (2003) have also examined EGFR over-expression in pre-invasive and early invasive bronchial lesions. Normal and hyperplastic epithelium showed basal staining only. The majority of airway metaplasia also showed normal basal staining. In addition several cases showed some or all of the layers above having positive staining. This may be reflective of normal repair mechanisms as mild and moderate dysplasia all demonstrated normal basal cell staining only. More than half of severe dysplasia samples examined showed staining throughout epithelial layers and most carcinoma *in situ* and microinvasive tumours showed positive staining through the full thickness of epithelium. Kurie *et al.* (1996) have shown that in metaplastic cells EGFR expression loss is an indication of reversal to a normal phenotype. This further supports the suggestion that expression may be part of normal repair and a reversible event.

Over-expression of EGFR may be advantageous to tumour cells as it seems to disrupt integrin α6β4 at hemidesmosomes, which allows tumour cells to migrate and invade more easily (Mariotti *et al.* 2001).

EGFR mutations in cancer have been reported previously. Sasaki *et al.* (2006) have examined a large Japanese cohort of lung cancer patients for a number of EGFR mutations. The arginine for leucine substitution at amino acid 858 and a deletion in exon 19 are thought to be the most common alterations. This work showed that 63 patients out of 575 examined had the L858R mutation. These patients had a mix of lung cancer types: those with an L858R mutation all had adenocarcinoma and represented 17% of adenocarcinoma patients having the L858R mutation.

7.4 Cancer morphology and phenotype

Jensen-Taubman, Steinberg & Linnoila (1998) have examined epithelial cell spread in tumour bearing lungs, lesions referred to as bronchiolisation of the alveoli (BOA). A grading criteria was assigned to these lesions as follows:

Grade 0: simple metaplasia with uniform cell size and shape in a single layer, ciliated and non-ciliated lining both sides of alveolar septae, having a round or oval nucleus and granular chromatin.

Grade 1: mild atypia, occasional multilayer patches, slightly variable cell shapes, mildly enlarged round or oval nucleus, some with darker granular chromatin.

Grade 2: moderate atypia, frequent multilayer cells, shape ranges from columnar to polygonal, non-ciliated, increase in nuclear/cytoplasm ratio, variable nuclei.

Grade 3: marked atypia, multilayer with no organisation, non-ciliated, variable cell size and shape, high nuclear/cytoplasm ratio, condensed chromatin with variation between cells.

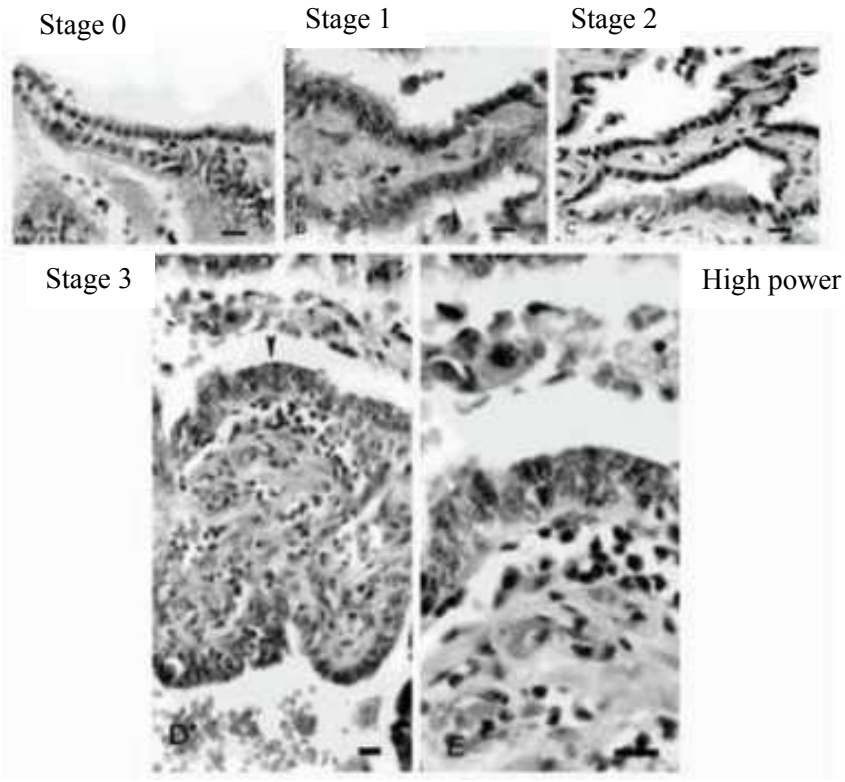


Figure 7.1 Grades assigned to BOC during lung cancer diagnosis.
Lesions within the lung periphery that fulfil the cancer grading for stages 0-3 of the WHO criteria. These are not tumours but the epithelial characteristics are similar to cancer. Progressive alveolar bronchiolisation in non-small cell carcinoma has been reproduced with kind permission from Dr Linnoila.

Many cellular changes were seen within parenchymal proliferations in COPD, suggesting altered epithelium in tumour bearing patients that may indicate the influence of field effect change. In the COPD cohort, lesions with a similar appearance may be identified. This raises the question of whether these lesions constitute a pre-neoplastic lesion. This may have implications on treatment and management of COPD patients.

Another example of tumour bearing lungs that have cells which may resemble the diffuse response on COPD are the papillary subtype of adenocarcinoma and the lepidic, surface lining growth seen in the adenocarcinoma sub type and non-mucinous bronchioloalveolar subtype (Flieder & Koss 2004). These are further examples of epithelial cells lining the periphery of the lung. This further supports the need to investigate the lesion occurring in COPD. Based on H&E examination alone there are similarities between the peripheral lesions of COPD and the epithelial cells of lepidic bronchioloalveolar carcinoma.

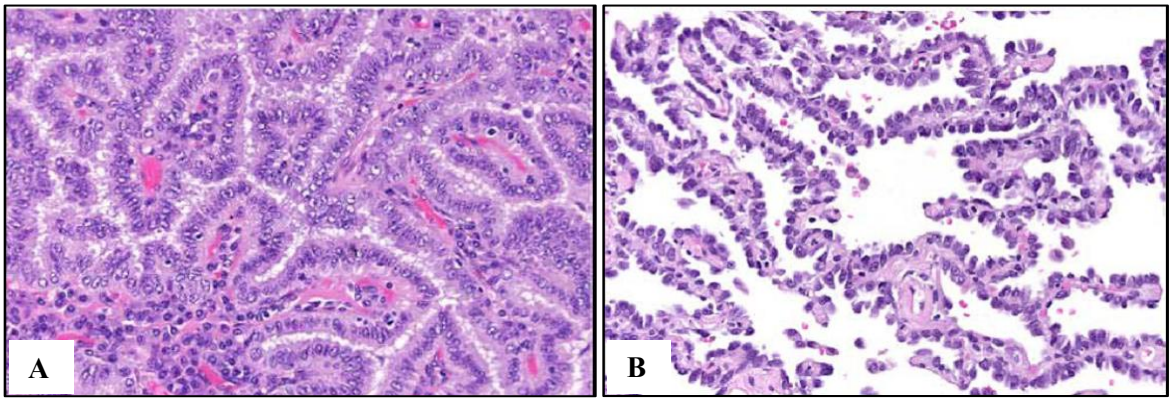


Figure 7.2 Examples of cells seen in adenocarcinoma
Figure 7.2A shows the papillary subtype of adenocarcinoma, note the epithelial cells covering all surfaces and Figure 7.2B shows non-mucinous bronchi alveolar subtype of adenocarcinoma, this shows unciliated epithelial cells lining all surfaces. These pictures are reproduced with kind permission from D Flieder.

7.5 Aims and Objectives

Chapter 7 aimed to address the question of whether the metaplastic cells were likely to constitute or could progress to a pre-neoplastic lesion.

> What is the distribution of proliferating metaplastic cells and is it similar to a pre-neoplastic lesion?

This was examined using Ki67 staining on both airways and metaplastic cells to identify the distribution of proliferating cells.

Are there epithelial alterations in the metaplastic cells in accordance with those seen in tumour margins or neoplasia present?

Markers of altered phenotype were assessed using antibodies for p63, ΔN-p63, involucrin and EGFR.

Is there any evidence of the L858R mutation in EGFR in the metaplastic cells?

This was carried out by laser capture microdissection DNA extraction and PCR analysis for the presence of L858R EGFR mutation. This is an early change in adenocarcinoma and may therefore suggest the metaplastic cells are a pre-neoplastic lesion.

Do the metaplastic cells have a morphology or localization similar to the metaplasia observed surrounding tumours?

The position and morphology based on H&E staining were compared with those found in areas of bronchiolisation with alveolar bed associated with tumours.

7.6 Results: Altered cell phenotype

7.6.1 Results for proliferation: Ki67 IHC

A Ki67 antibody was used to examine the number of cells proliferating within the tissue. This marker allowed comparison of metaplastic cell and airway epithelial

turnover. COPD patients 1-10 were stained and examined, staining is shown in Figure 7.1. Positive staining produced a nuclear signal. The metaplastic cells showed variable positivity, ranging from negative to 5% positive in isolated cases. The approximate percentage of cells and cell morphology of metaplastic cells are in Table 7.1 along with the approximate airway percentages.

Table 7.1 Ki67 summary percentage of positive cells

Adv = adventitia, SP = sub pleural, Fib = fibrosis, H=hilar The number in brackets refers to the positive cell morphology, (1)=uniform, (2)=macrophage-like, (3)=squamous. The percentage values in this table are approximate.

Pat	Pathology Code	Adv (%)	SP (%)	H (%)	Fib (%)	Airway (%)
1	Airway changes & fibrosis		0 (2)		1 (1,3)	0-5
2	Goblet cell airway changes	0			2 (3)	0-10
3	Airway changes & fibrosis	2 (1)		1 (1)	2 (2)	5-40
4	Airway changes & fibrosis	1 (1)	1 (1,2), 2 (3)			1-5, sq 50
5	Airway changes & fibrosis	0.5-1 (1)			5 (3)	5 by mets 10
6	Goblet cell airway changes	3 (1)				2-80
7	Normal	5 (1)	0 (1)			3 by mets 10
8	Airway & pleural change		2 (1), 0 (3)		1 (1or2)	2-50 inflam
9	Goblet cell airway changes	1 (1)	3 (2or3)	1 (3)		2-10
10	Fibrosis & inflammation	1 (1)	1 (2)		1 (1)	2

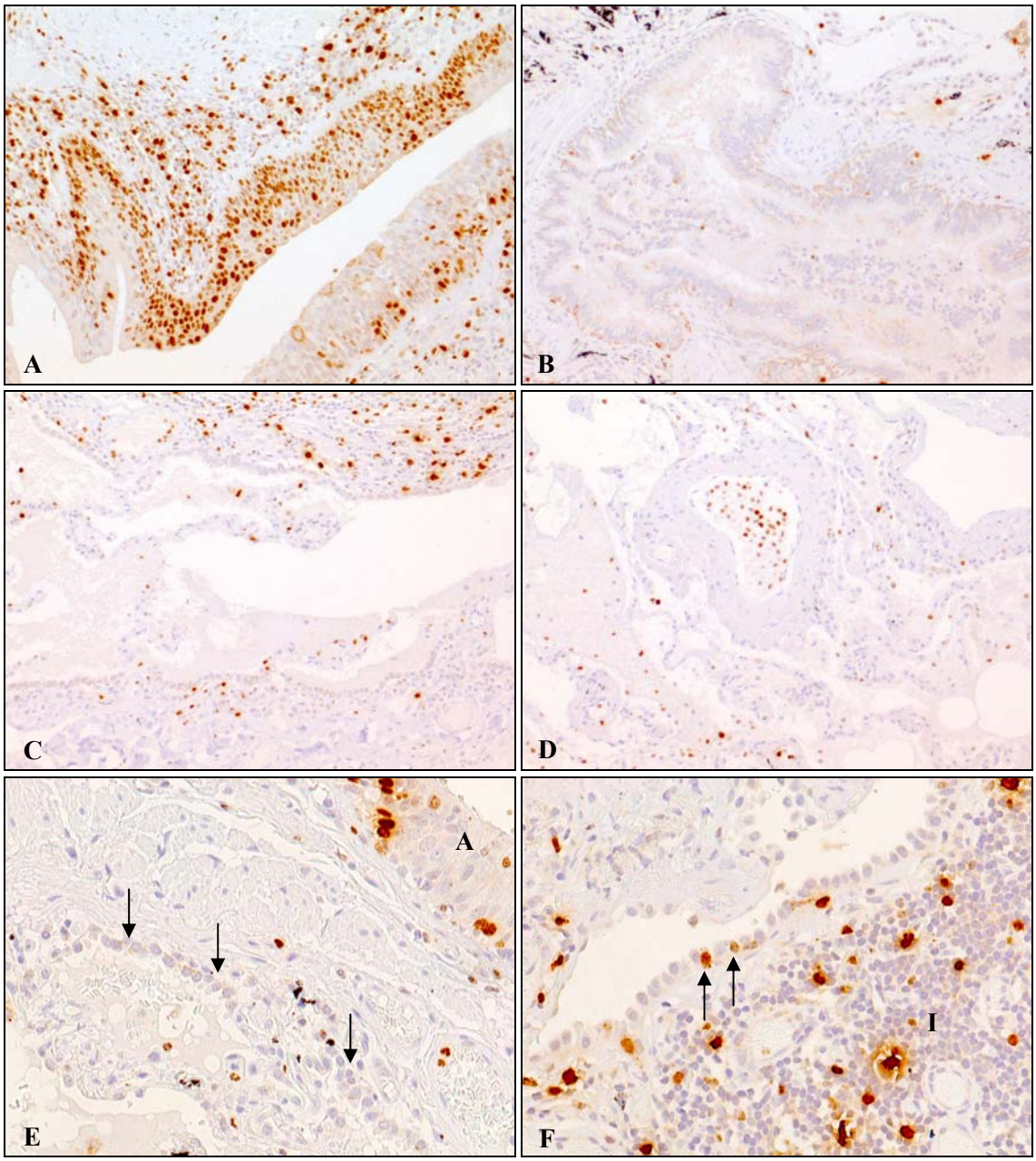


Figure 7.3 Proliferation, Ki67 IHC

Figure 7.3A) Ki67 staining (Patient 6: Goblet cell airway changes) numerous positive cells in the airways and underlying submucosa, mainly epithelial cells and inflammation.

Figure 7.3B) Ki67 staining (Patient 4: Airway changes & fibrosis) low numbers of positive cells in the airways.

Figure 7.3C) Ki67 staining (Patient 6: Goblet cell airway changes) negative in the metaplastic cell population, positive cells on the slide are inflammatory cells.

Figure 7.3D) Ki67 staining (Patient 6: Goblet cell airway changes) positive within inflammatory foci within a small vessel and surrounding parenchyma.

Figure 7.3E) x20 objective Ki67 staining (Patient 4: Airway changes & fibrosis) negative metaplastic cells (arrows), positive epithelial cells within the airways (A).

Figure 7.3F) x20 objective Ki67 staining (Patient 4: Airway changes & fibrosis) sporadic positive metaplastic cells and positive cells within inflammatory foci (I).

7.6.2 Results for p63 IHC

A p63 antibody was used to examine progenitor cells and cells that may be about to undergo apoptosis via the p53 pathway. The p63 marker was examined on the full COPD panel of 50 patients and 6 control samples, examples of staining in Figure 7.4. The predominant signal was from airway basal cells (Figure 7.4A). This included some suprabasal cells, not in direct contact with basement membrane. 16 patients showed some strongly stained metaplastic cells within the lung periphery. These positive cells were found in all locations, Table 7.2 summarises the p63 staining on metaplastic cells. There were also 25 patients that showed weaker positive staining on metaplastic cells in all locations. Patient 19 had the only occurrence of a large number of positive cells seen together within the metaplastic cell population (Figure 7.4E). This corresponds to the area that was positive for CK5/6 and ΔN-p63 and was thought to be bronchiolisation (Figure 7.4C).

Table 7.2 p63 staining on metaplastic cells

Few=mild, some=moderate, lots=severe

Pat	Pathology Code	Airways	Metaplastic cells	Parenchymal p40 staining
1	Airway changes & fibrosis	Some +	Few + fib	
2	Goblet cell airway changes	Lots ++	Few ++ fib	
3	Airway changes & fibrosis	Lots ++	Few ++ fib, SP	
4	Airway changes & fibrosis	Lots ++	Some ++ SP	
5	Airway changes & fibrosis	Lots ++	Few + adv	
6	Goblet cell airway changes	Some ++	Few ++ adv	
8	Airway & pleural change	Some ++	Some +SP	2 ++ areas bron
9	Goblet cell airway changes	Some +	Few + sp, fib	
10	Fibrosis & inflammation	Some ++	Few + hilar, some ++ SP/fib	1 ++ area bron
11	Goblet cell airway changes	Lots +++	Few + adv, SP	
12	Airway changes & fibrosis	Some +++	Few + adv	
13	Airway changes & fibrosis	Some +++	Some + adv	
14	Fibrosis & inflammation	Some ++	Few ++ sp	++ on cystic areas
15	Airway changes & fibrosis	Some ++	Few + adv, SP	
16	Widespread ongoing damage	Some ++	Few + adv, SP, fib	
17	Airway changes & fibrosis	Some ++	Few ++ adv, fib	
18	Airway changes & fibrosis	Some ++	Few + hilar, fib, SP	++ on cystic area
19	Airway changes & fibrosis	Some ++	Lots ++ SP,	++ on cystic areas
20	Airway changes & fibrosis	Lots ++	Few + SP, adv	
21	Goblet cell airway changes	Some ++	Few + adv, SP, fib,	
22	Goblet cell airway changes	Lots ++	Few + SP, adv	
24	Airway changes & fibrosis	Some ++	Few + SP, hilar	
27	Fibrosis	Some ++	Few ++ SP	

28	Airway changes & fibrosis	Lots ++	Few fib, SP, alv, ++ in P	
29	Goblet cell airway changes	Range ++	Some + adv, Few + in P	
31	Fibrosis	Few +++	Few ++ SP, fib,	
32	Airway & pleural change	Some ++	Few ++ SP, fib	++ on cystic area
33	Airway changes & fibrosis	Few ++	Few + adv	
34	Fibrosis	Lots ++	Few + adv	
35	Fibrosis & inflammation	Few ++	Few + SP	
36	Goblet cell airway changes	Few ++	Few + adv	
37	Goblet cell airway changes	Some ++	Few + adv, SP, few ++ in P	
38	Fibrosis	Some ++	Few + SP, adv	
39	Airway & parenchyma	Some ++	Some ++ SP, adv, hilar, fib	++ on cystic areas
40	Goblet cell airway changes	Few ++	Few + adv	
41	Airway changes & fibrosis	Some ++	Few ++ adv	
43	Airway & parenchyma	Some ++	Few ++ adv	
44	Airway & parenchyma	Some ++	Few ++ adv	
46	Airway & parenchyma	Lots +++	Few + adv	
48	Widespread ongoing damage	Lots +++	Few + SP	
49	Airway & parenchyma	Lots +++	Few ++ adv, SP	
N5	Normal	Few ++	Few ++ fib	

7.6.3 Results for ΔN-p63 IHC

A ΔN-p63 antibody was used to identify the true progenitor cells highlighted with p63, staining also found in Figure 7.4. Any additional staining seen in p63 over what is seen in ΔN-p63 is the TA-p63 isoform that acts like p53. ΔN-p63 was applied to the whole panel of 50 COPD patients and 6 controls.

As with p63 the predominant positive stain was airway basal and suprabasal cells (Figure 7.4B). The instances of ΔN-p63 in the parenchyma listed below, were much more limited and determined to represent bronchiolisation (Figure 7.4D). The metaplastic cells were negative for ΔN-p63 (Figure 7.4F).

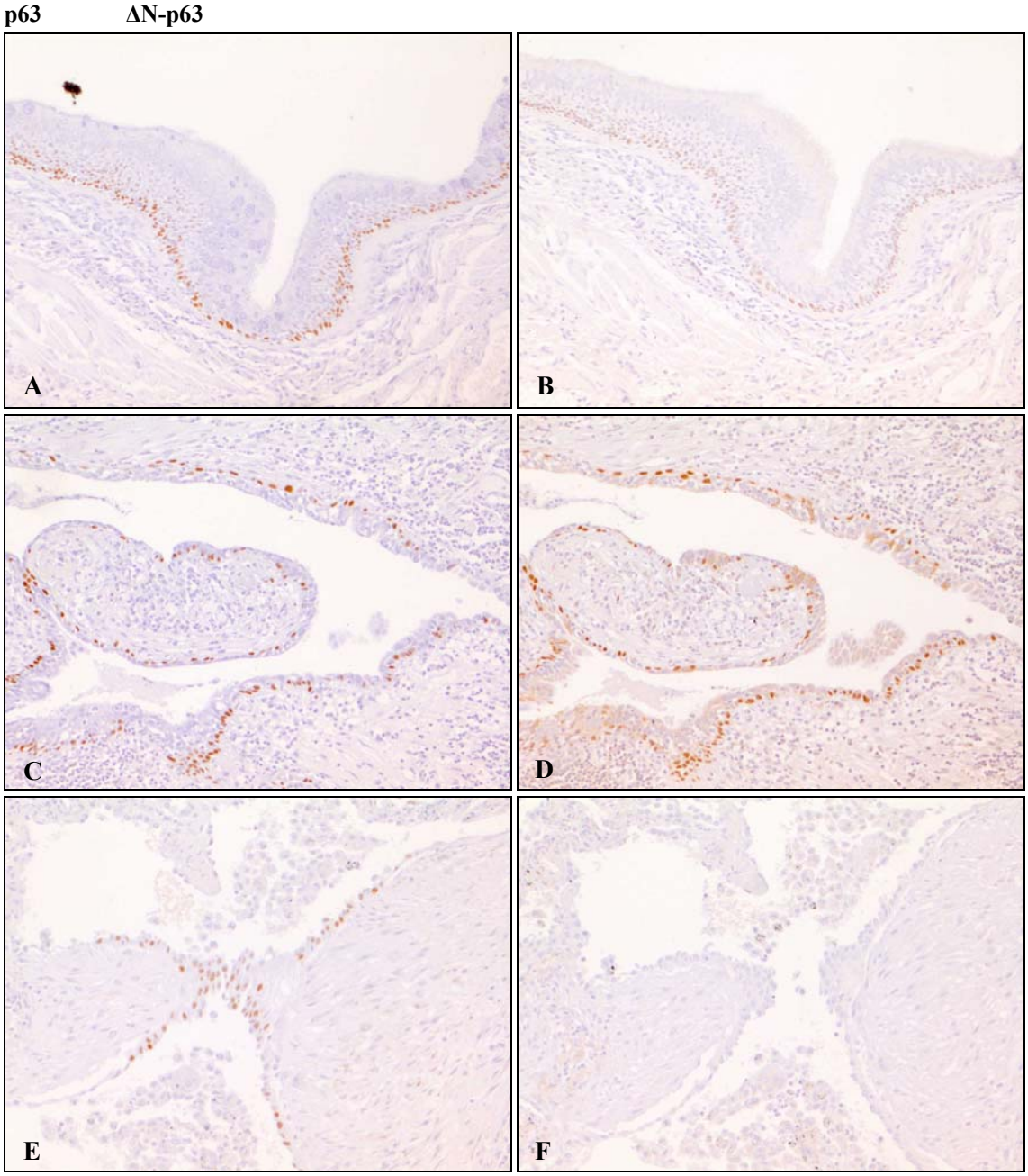


Figure 7.4 p63 & ΔN-p63 IHC

Figure 7.4A) p63 positive staining (Patient 7: Normal), in the basal layer of a large airway, p63 demonstrating basal cells.

Figure 7.4B) ΔN-p63 positive staining (Patient 7: Normal) shows the same cell population as Figure 7.4A being highlighted with ΔN-p63.

Figure 7.4C) p63 positive staining (Patient 19: Airway changes & fibrosis) in an areas of bronchiolisation within the parenchyma.

Figure 7.4D) ΔN-p63 positive staining (Patient 19: Airway changes & fibrosis) shows the same population of epithelial bronchiolisation also being highlighted with ΔN-p63.

Figure 7.4E) p63 positive staining (Patient 17: Airway changes & fibrosis) on an area of metaplastic cells sitting on fibrosis.

Figure 7.4F) ΔN-p63 (Patient 17: Airway changes & fibrosis) no staining on a corresponding area to Figure 7.4E showing that the metaplastic cells have the p63 isoform that is similar to p53 and is involved with cell death when damage occurs. These metaplastic cells are not demonstrating ΔN-p63 the progenitor cell isoform.

7.6.4 Results for involucrin IHC

An involucrin antibody was used to determine altered epithelial cell presence, as involucrin is found in squamous cells. Involucrin staining was carried out on the sub panel of 28 COPD patients and 6 control samples, examples are found in Figure 7.5. Involucrin was only seen on airway epithelial cells and occasional metaplastic cells. All samples, except patient 4 (Airway changes & fibrosis) with no airway, showed some airway epithelial staining (Figure 7.5A). In some cases positive involucrin was only observed within airway remnants as opposed to intact airway (Figure 7.5B). There was also some granular macrophage staining suggestive of ingestion of positive material rather than de novo production. Positive metaplastic cells were observed in 20/28 patients examined. The 2 control samples with metaplastic cells had no positive involucrin staining. The metaplastic cells showed positive stain on sub pleura, adventitia and fibrosis, not hilar or alveolar areas. These are much less frequent lesions. Metaplastic cell morphology of involucrin positive cells was also noted where there was only one incidence of macrophage shaped metaplastic cells. There was lots of uniform cells that showed some squamous staining and any positive staining on metaplastic cells is summarised in Table 7.3 below. There was one incidence of granular metaplastic cells. This may be packaged involucrin or non-specific staining (Figure 7.5D, E).

Table 7.3 Involucrin staining on metaplastic cells

1=uniform, 2=macrophage-like, 3=squamous. Patients without positive metaplastic cells were omitted.

Pat	Pathology Code	Metaplastic cells
4	Airway changes & fibrosis	SP2 granular +
5	Airway changes & fibrosis	SP1 granular +
6	Airway changes & fibrosis	Adv 1 granular +
13	Airway changes & fibrosis	Adv 1 granular +, fib 3 +
18	Airway changes & fibrosis	Adv 1 granular +
19	Airway changes & fibrosis	Fib 3 +
20	Airway changes & fibrosis	SP1 & adv1 granular +
21	Goblet cell & airway changes	Adv 1 & fib 1 granular +, some adv strong
22	Goblet cell & airway changes	Adv -ve
23	Airway changes & fibrosis	SP1 -ve
24	Airway changes & fibrosis	SP3 +
27	Fibrosis	SP1 granular +
32	Airway & pleural change	Adv1 & SP 1&3 granular +
33	Airway changes & fibrosis	Fib1 & SP + granular +
37	Goblet cell & airway changes	SP1 granular +
41	Airway changes & fibrosis	Fib1 granular +
46	Airway & parenchymal change	Adv1 granular +
48	Widespread ongoing damage	Hilar1 granular +, fib3 +, bronchiolis^{ation} occ +
49	Airway & parenchymal damage	Fib3 +, sp/fib1 granular + occasional 3 +

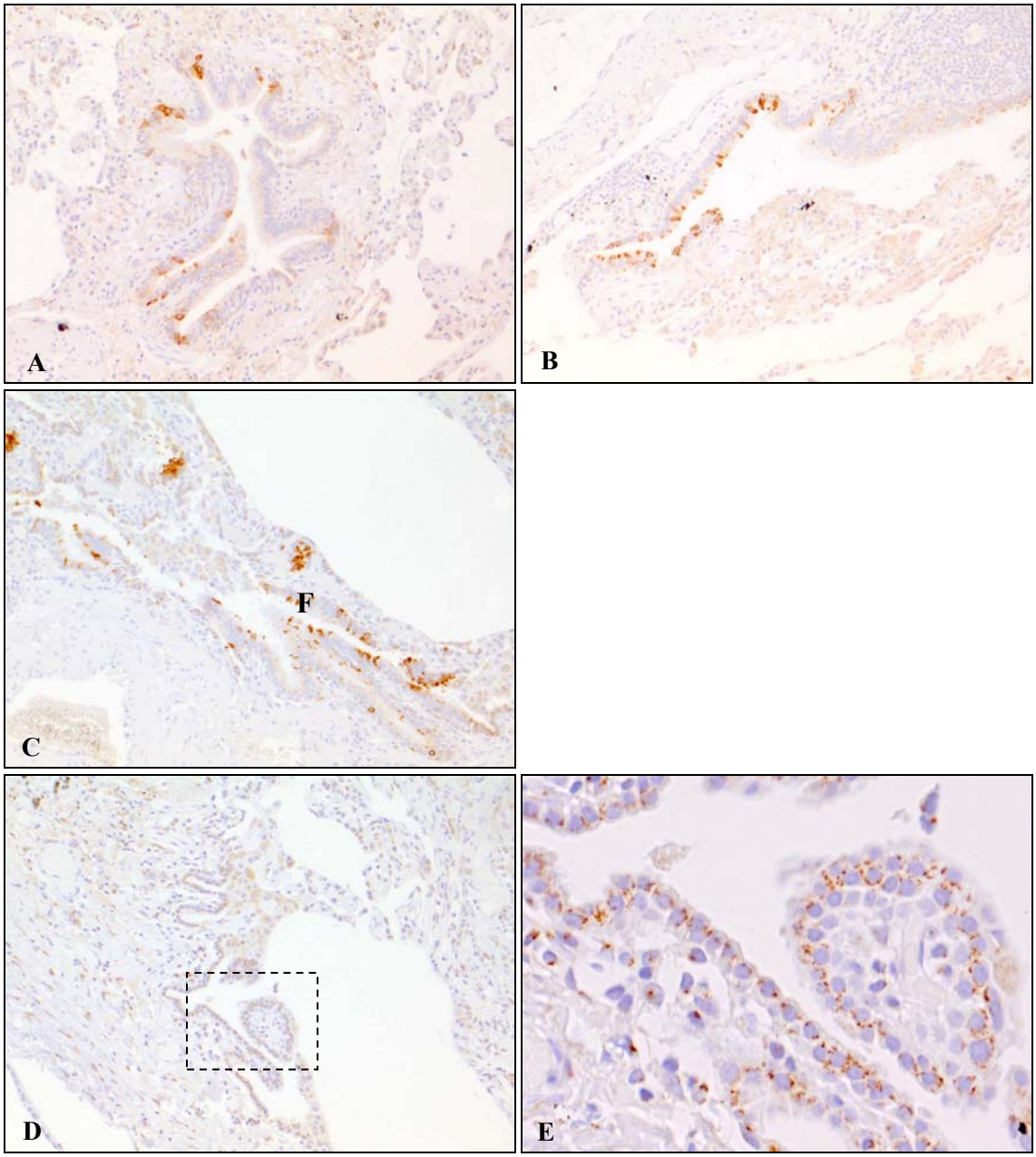


Figure 7.5 Involucrin IHC Staining

Figure 7.5A) Involucrin positive staining (Patient 49: Airway & parenchymal change) within the epithelial cells of a small airway. This was quite uncommon in intact airways.

Figure 7.5B) Involucrin staining (Patient 41: Airway changes & fibrosis) on an area of airway remnants.

Figure 7.5C) Involucrin positive staining (Patient 21: Goblet cell airway changes) on an area of colonising epithelial cells, the airway remnants are spreading into the tissue, this may be considered to be bronchiolisation.

Figure 7.5D) Involucrin staining (Patient 21: Goblet cell airway changes) possible weak positive stain on an area of metaplastic cells.

Figure 7.5E) x40 objective of involucrin staining shows the boxed area on Figure 7.5D, staining is vesicular and not throughout the cytoplasm as in all other positive cells.

7.6.5 Results for epidermal growth factor receptor (EGFR) IHC

An antibody for epidermal growth factor receptor (EGFR) was used to examine epithelial cell and metaplastic cell expression, staining can be seen in Figure 7.6. IHC was carried out on 50 slides with good examples of adventitial metaplasia. General findings on metaplastic cells are summarised in Table 7.4. Patients 1, 4, 27 and 34 showed no metaplastic cell staining. Airway basal cells were positive for EGFR (Figure 7.6B). There were occasions where all the epithelial layers showed positive staining (Figure 7.6A). This may have demonstrated reparative epithelium. There was also ganglion nerve staining within airway sub mucosa (Figure 7.6D). Metaplastic cells had cytoplasmic or membrane orientated staining (Figure 7.6E, F).

Table 7.4 EGFR within the airways & metaplastic cells

Pat	Pathology Code	Metaplastic cells
2	Goblet cell airway changes	SP + adv + bron -
3	Airway changes & fibrosis	+
5	Airway changes & fibrosis	Adv +
6	Goblet cell airway changes	+
7	Normal	Membrane ++ mets
8	Airway & pleural change	Fib, adv +
9	Goblet cell airway changes	+
10	Fibrosis & inflammation	Variable
11	Goblet cell airway changes	Bron few +
12	Airway changes & fibrosis	-
13	Airway changes & fibrosis	+ wash only
14	Fibrosis & inflammation	Bron few ++
15	Airway changes & fibrosis	Adv +, SP -
16	Widespread ongoing damage	Few +, most -
18	Airway changes & fibrosis	Fib, adv +
19	Airway changes & fibrosis	Membrane +
20	Airway changes & fibrosis	-
21	Goblet cell airway changes	+ and membrane +
24	Airway changes & fibrosis	+ membrane
28	Airway changes & fibrosis	+
29	Goblet cell airway changes	Some +
31	Fibrosis	Occ +, most -
32	Airway & pleural change	Occ +, most -
33	Airway changes & fibrosis	Most -
36	Goblet cell airway changes	Most -
37	Goblet cell airway changes	Occ +, most -
39	Airway & parenchymal change	Most -
40	Goblet cell airway changes	Some +
41	Airway changes & fibrosis	Most -
42	Normal	Occ +, most -

44	Airway & parenchymal change	Some +, some membrane ++
45	Goblet cell airway changes	Lots +
46	Airway & parenchymal change	Most +
47	Airway changes & fibrosis	Most -
48	Widespread ongoing damage	+
49	Airway & parenchymal change	Most -
50	Widespread ongoing damage	Some +++

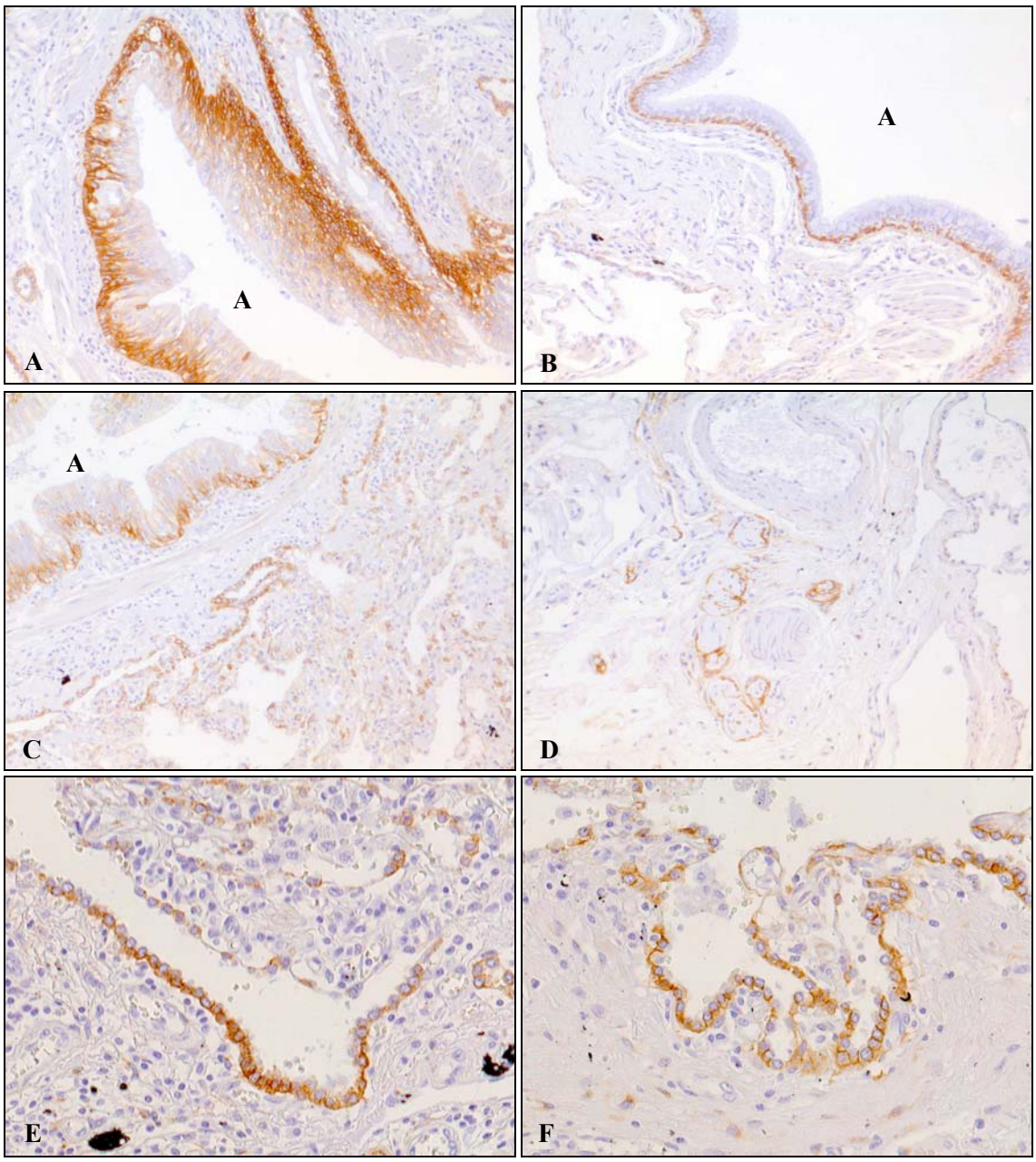


Figure 7.6 EGFR IHC staining

Figure 7.6A) EGFR (Patient 5: Airway changes & fibrosis) on multiple basal and suprabasal layers.
Figure 7.6B) EGFR (Patient 7: Normal) on a single basal layer of epithelial cells of the airways.
Figure 7.6C) EGFR (Patient 6: Goblet cell airway changes) positive adventitial metaplasia. Positive airway (A).
Figure 7.6D) EGFR (Patient 7: Normal) on a number of nerve bundles adjacent to an airway.
Figure 7.6E) EGFR x20 objective (Patient 5: Airway changes & fibrosis) adventitial metaplastic cells with cytoplasmic staining.

Figure 7.6F) EGFR x20 objective (Patient 5: Airway changes & fibrosis) adventitial metaplastic cells with membrane staining.

7.7 EGFR mutation detection

The process for DNA extraction is outlined in the materials and methods section 2.7.1. The first step was to laser capture microdissect the metaplastic cells of interest. Figure 7.7 shows microscope slides before and after laser capture has been carried out.

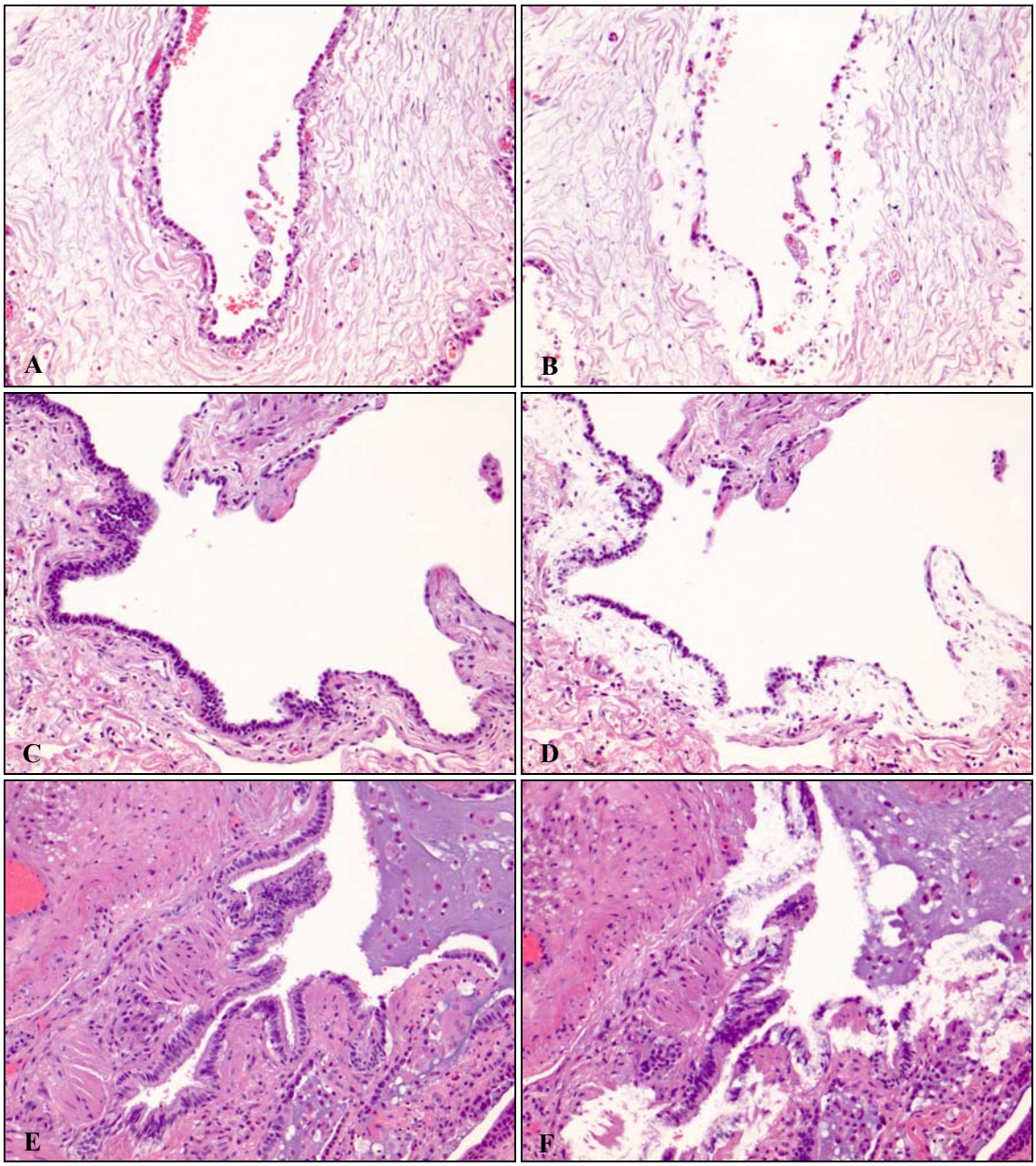


Figure 7.7 Laser capture before & after photographs

Figure 7.7A) Patient 20 (Airway changes & fibrosis) H&E prior to laser capture, metaplastic cells.
Figure 7.7B) Patient 20 (Airway changes & fibrosis) H&E after laser capture, cells are missing.
Figure 7.7C) Tumour resection corresponding to sample 15 tumour resection run 2 prior to laser capture.
Figure 7.7D) Tumour resection corresponding to sample 15, cells are missing.
Figure 7.7E) Tumour resection corresponding to sample 10 tumour resection run 2 prior to laser capture.
Figure 7.7F) Tumour resection corresponding to sample, cells are missing.

Table 7.5 EGFR PCR results I

Sample	Patient number	Metaplastic cell location	Result
1	39	Fibrosis	Wild type
2	12	Adventitia	Wild type
3	31	Alveolar	Wild type
4	17	Alveolar	Wild type
5	6	Adventitia	Wild type
6	32	Sub pleural	Wild type
7	30	Hilar	Wild type

First extraction run contained the following samples as shown in Table 7.5. Table 7.6 shows the results from the second extraction run containing the following samples.

Table 7.6 EGFR PCR results II

Sample	Patient number	Metaplastic cell location	Result
1	32	Fibrosis	No amplification
2	30	Fibrosis	Wild type
3	45	Adventitia	Wild type
4	28	Hilar	Wild type
5	20	Hilar	No amplification
6	45	Alveolar	Wild type
7	27	Fibrosis	Wild type
8	49	Sub pleural	Wild type
9	33	Hilar	Wild type
10	Tumour resection	Tumour resection	No amplification
11	128/0020pa	Adenocarcinoma sample	Wild type
12	Tumour resection	Tumour resection	Wild type
13	Tumour resection	Tumour resection	Wild type
14	46/38	Sub pleural	Wild type
15	Tumour resection	Tumour resection	Wild type
16	21	Sub pleural	Wild type
17	5	Adventitia	Wild type
18	Tumour resection	Tumour resection	Wild type
19	Tumour resection	Tumour resection	Wild type
20	N6	Tumour resection	Wild type
21	Tumour resection	Tumour resection	Wild type
22	Tumour resection	Tumour resection	Wild type

Control cell line DNA was run as part of each experiment and showed consistent demonstration of the mutant probe showing that the experiments all worked. There were no instances of mutated DNA from any of the COPD cohort or the tumour surround samples examined here. The probe levels are plotted for, heterogeneous positive control DNA for cell line NCI-H1975 in Figure 7.8A and negative control tonsil DNA in Figure

7.8B. Figure 7.9 shows one replicate for each of the seven COPD samples examined in the first run.

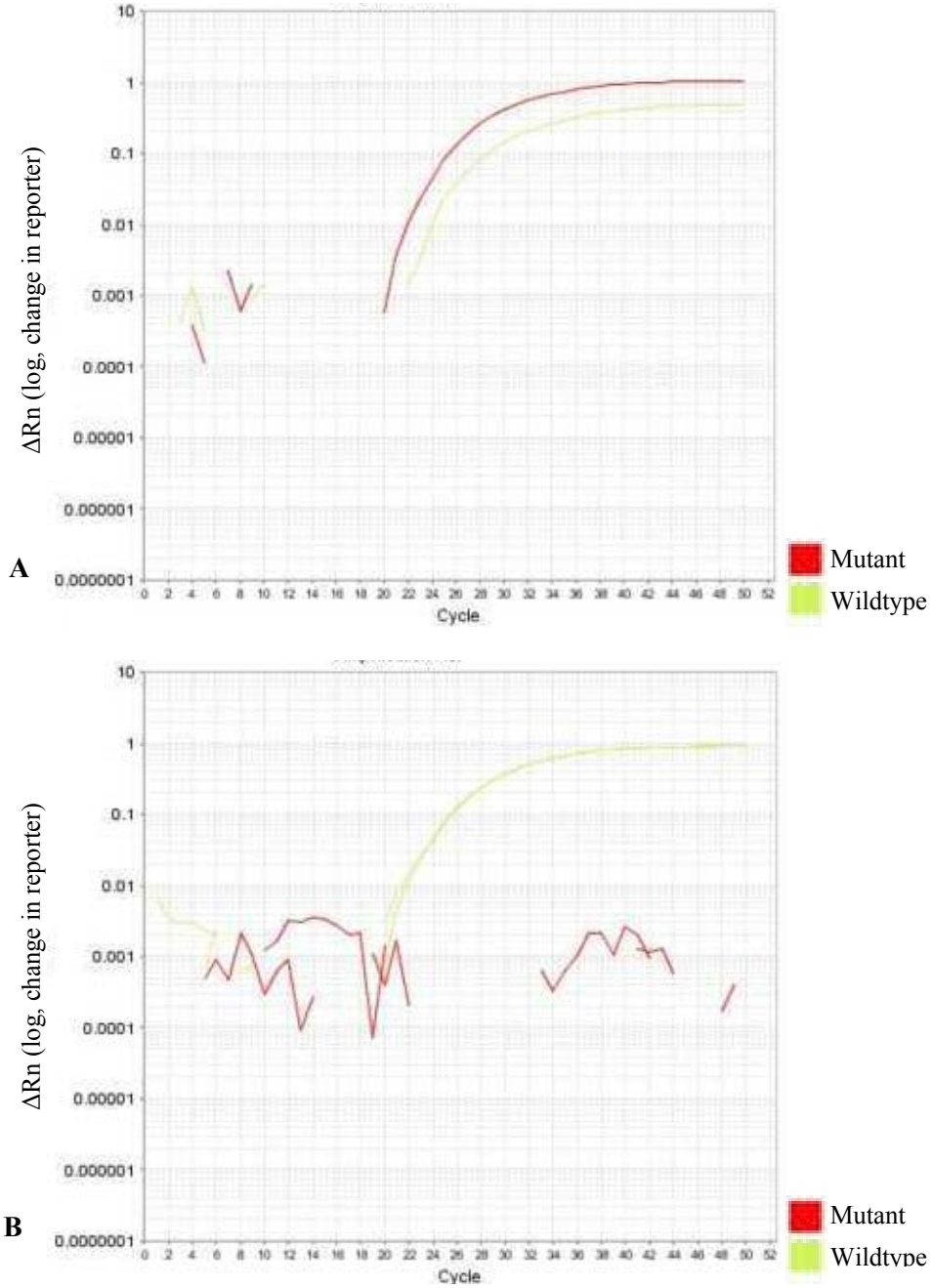


Figure 7.8 Positive and negative control PCR results

Figure 7.8A) shows cell line NCI-H1975, that is heterogeneous for the EGFR mutation L858R. The red line represents the probe detecting the mutated DNA, and the green line represents the probe detecting the wildtype DNA.

Figure 7.8B) shows wildtype tonsil DNA that does not contain any mutated DNA. Wildtype DNA is shown in green.

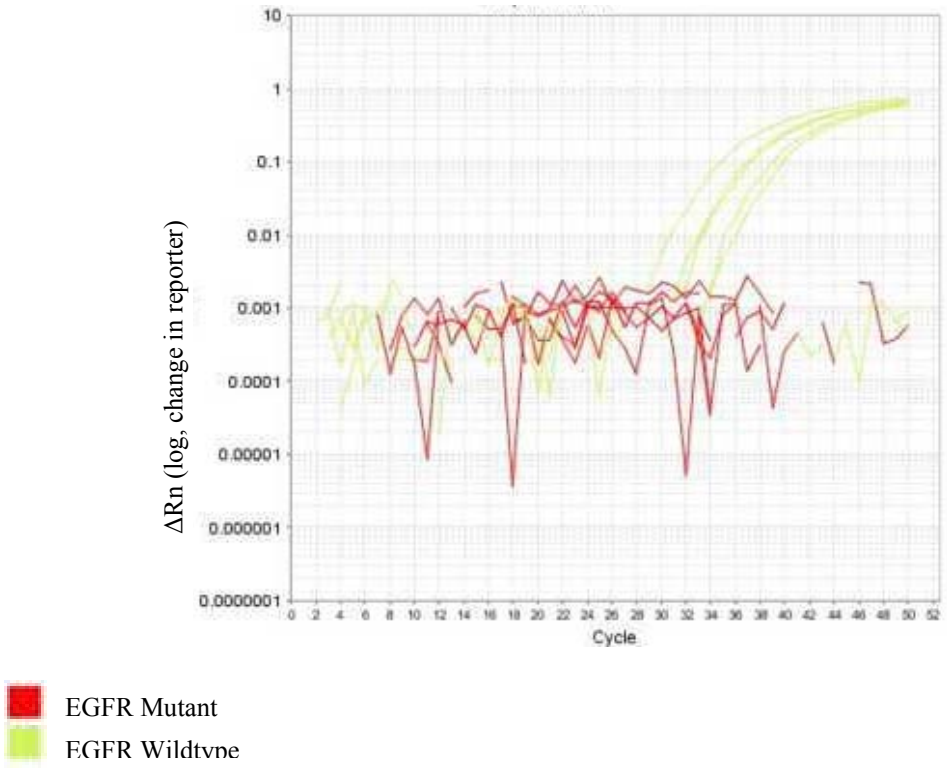


Figure 7.9 Run 1 results for PCR mutation detection of EGFR L858R
The results of the seven samples in run 1 (Table 7.7). None of the mutant DNA has shown amplification. This shows that there are no mutated samples present.

7.8 Morphology, position & characteristics in COPD metaplastic lesion and tumours

Examples of grades similar to those observed in the tissue surrounding tumours, originally described and documented by Jensen *et al.* (1994) were found within the metaplastic cell lesions of the COPD cohort.

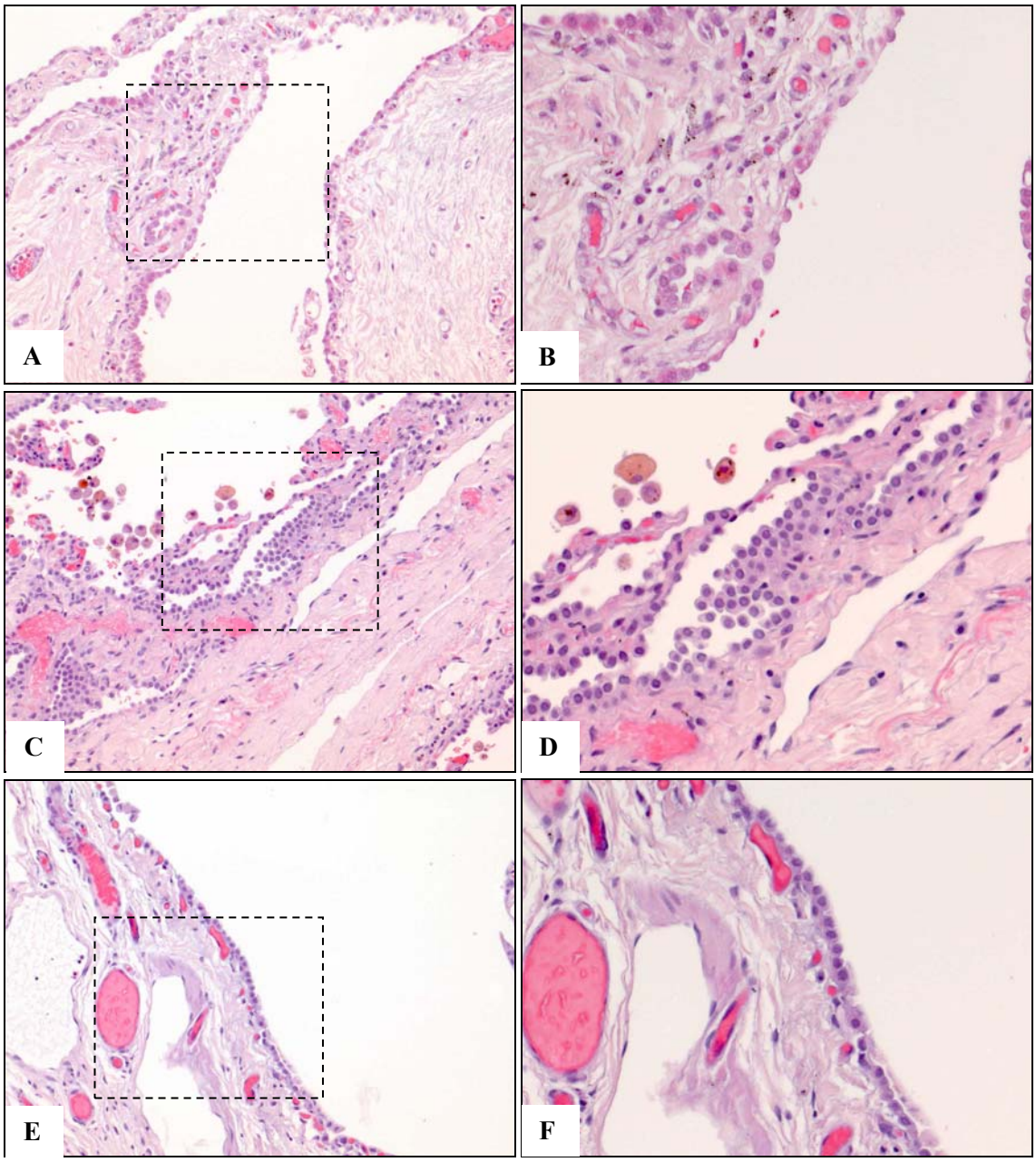


Figure 7.10 Metaplastic cells with BOA tumour diagnosis grades

Figure 7.10A) x10 objective, has the appearance of grade 1

Figure 7.10B) x20 objective shows a grade 1 area on more detail. This string of cells is spaced out there is no cell-cell contact between cells

Figure 7.10C) x10 objective grade 2

Figure 7.10D) x20 objective grade2/3

Figure 7.10E) x10 objective should show grade 3 these cells are in contact with one another and are more flattened in appearance

Figure 7.10F) x20 objective Zoom in of E

7.9 Discussion

7.9.1 Proliferation

Proliferation levels identified in COPD airways here varied greatly from a baseline of around 2% to approximately half the cells staining positive for Ki67. The airways with high levels of staining were inflamed. The metaplastic cell populations varied in number of proliferating cells from 1-10%. Yokohori, Aoshiba & Nagai (2004) have shown increased proliferation and apoptosis within the COPD parenchyma. There was significantly higher staining for both proliferation markers PCNA and topoisomerase Iiα. Cell types were not discussed and there was no mention of colonising cells. Although it showed that there was increased proliferation and apoptosis in the lung periphery it did not appear to include consideration of any metaplastic epithelial lesions. Miller *et al.* (2007) examined proliferation marker Ki67 in COPD patients, contrasting it with healthy smokers and non-smokers. The findings suggested that proliferation was not increased as a result of COPD. This suggested that proliferation was determined by gender with men showing increased levels, and smoking, non-smokers and quit smokers had much lower levels of Ki67 staining. Samples examined were obtained via bronchoscopy and so are not from the appropriate anatomical location to consider peripheral colonisation. Miller *et al's* work suggested proliferation in our study should not be high as surgical criteria include smoking cessation. Another bronchoscopy study showed that smoking status greatly influences epithelial proliferation supporting Miller's findings (Lapperre *et al.* 2007). It has been shown in pre-neoplastic epithelium that Ki67 labelling was increased in metaplasia and further increased in dysplasia compared to normal epithelium. This did not reach significance with the Ki67 marker. Normal epithelium showed proliferation of approximately 3.5% of cells (Munoz-Antonia *et al.* 2007). There does not appear to have been an assessment of the proliferating cells of the lining cells in IPF or COPD. This makes it difficult to predict what proliferation would be expected in this COPD cohort. If the cells were to be considered a pre-neoplastic lesion then perhaps numbers of around 40% would be expected (Cavarga *et al.* 2009). High proliferation within a pre-neoplastic lesion appears to correlate with a loss of p16 expression (Lantuejoul *et al.* 2005). Accumulation of p16 would lead to a more senescent pre-neoplastic lesion. In this instance the cells seem to be a more long term scarring type lesion. Type II hyperplasia has usually been

considered to be an acute more transient resolving lesion that may therefore be expected to undergo high levels of proliferation before maturation and resolution.

7.9.2 Epithelial characteristics

ΔN-p63 and p63 were used to examine the possibility that metaplastic cells represented a progenitor cell pre-neoplastic lesion. The presence of p63 but not ΔN-p63 was observed on a number of metaplastic cells. The only incidences of ΔN-p63 positive cells within the parenchyma were determined to be areas of bronchiolisation. These areas had transitional cells and other markers such as Clara cell CC10. The presence of p63 on a large number of squamous cell carcinoma has been demonstrated previously (Jorda *et al.* 2009). It is thought the ΔN-p63 isoform of p63 may be observed on tumour cells. Tumour cells are immortal and can act as progenitor cells proliferating indefinitely. The presence of p63 but not ΔN-p63 on the metaplastic cell population suggested that the cells are not capable of proliferating indefinitely, but are more likely to be about to undergo apoptosis and clearance as p63 helps to recognise and kill damaged cells. This is another characteristic that differs from most tumours. The lack of progenitor cell capability also explains the relative lack of proliferation compared to many tumours. This perhaps further supports that these cells are unlikely to be a pre-neoplastic lesion.

Involucrin was used to examine squamous nature of the metaplastic cell population. Positive staining was very occasional within airways and more frequent on airway remnants. This staining profile was much less extensive than the squamous and basal marker CK14. This suggests that although the airway epithelium may be altered and undergoing repair processes it is not sufficiently altered to express involucrin. Metaplastic cells had occasional positive cells in 20 of the 28 samples examined, rather than a consistent phenotype. Metaplastic cells are exposed to inflammation in the same way as airway epithelium and so could be responding to the same damage or stress signals. Dakir, Feigenbaum & Linnoila (2008) have used a mouse model to show that as epithelium becomes more altered and progresses towards tumour involucrin expression increases. Like p63, involucrin is found in a large proportion of squamous cell carcinomas (Suo, Holm & Nesland 1993). If the metaplastic cell population was a pre-neoplastic lesion it would perhaps express high levels of involucrin. This was not the case providing further evidence that the cells are not pre-neoplastic. There was some staining granular on metaplastic cells, this altered pattern was not found within previously the literature and may be non-specific.

7.9.3 Over expression and somatic mutation of EGFR

EGFR expression was assessed using IHC and detection of single nucleotide mutation L858R within exon 21 was assessed using PCR. EGFR positive staining was observed on airway basal cells. There was a range of cytoplasmic and cell membrane staining on sub populations of the metaplastic cells. The other positive cells were membranes on nerve ganglion. The presence of EGFR staining on nerve ganglion has not been documented previously but was a consistent finding throughout all patients examined. There are several incidences of positive EGFR peripheral nerve sheath tumours staining so a normal nerve sheath also expressing EGFR is perhaps unsurprising (Holtkamp *et al.* 2008, Tawbi *et al.* 2008).

Takeyama *et al.* (2008) employed an allergen sensitized rat model to examine the role of EGFR in goblet cell hyperplasia maintenance. EGFR pathway inhibitors confirmed the relationship. EGFR staining was seen in the apical side of the airway goblet cells. The metaplastic cells lack basal or goblet cell phenotype markers so is not representative of a normal basal cell EGFR population or a reparative goblet cell phenotype.

The mix of cytoplasmic and membrane orientated staining may reflect cell maturation, activation or phosphorylation state of EGF receptor. The majority of staining within airway basal cells was membrane staining, showing the established nature of most airways. The metaplastic cells often showed cytoplasmic EGFR staining and a number of metaplastic cells also exhibited strong E-cadherin cytoplasmic staining. The cytoplasmic expression of both these proteins may be indicative of recent protein production for example the production of new cells. Deeb *et al.* (2004) examined EGFR and E-cadherin distribution within NSCLC samples. EGFR was seen in normal airway basal cells and not alveolar bed whereas E-cadherin was observed in most epithelial layers and the alveolar bed. Within adenocarcinoma tissue cytoplasmic E-cadherin was associated with negative EGFR staining. This contrasted with cytoplasmic E-cadherin that was associated with membrane positive EGFR staining. There does not seem to be a clear relationship between the two markers within the metaplastic cell population in our study. As this tissue is not tumour there may be reparative expression of EGFR that does not follow the same pattern as permanent tumour over-expression. This may be true also for E-cadherin.

A lack of EGFR L858R mutation in the experiments here may reflect mutation frequency in lung cancers. Positive control DNA worked consistently well in all experiments carried out. EGFR mutation incidence in squamous cell tumours is zero in some studies and in adenocarcinoma is around 20% (Sasaki *et al.* 2006). Tissue used as potential positive control was obtained from tumour resection margins. Mutation incidence within tumour surround has not been documented within lung but there is evidence for pancreatic cancer and k-ras mutation. 81% of patients showed k-ras mutation in the primary tumour and 53% showed mutation within resection margins (Kim *et al.* 2006). This suggests that although incidence is lower it is possible to detect some mutations within tumour resection margins. Also there was no information on the tumour type from of the resection margins available. There may be only a few adenocarcinoma samples and of those only 20% would be expected to exhibit this mutation and this percentage applies only to tumour tissue.

EGFR is involved in a number of key cell functions and pathways; it has been shown to influence mucus production, cell motility and cell migration. These functions make EGFR important during cell repair but are also beneficial to tumour cells. Previous documentation of metaplastic lesions in diseases such as IPF and BO have been thought of as a pre-neoplastic lesion (Haddad, Massaro 1968, Souza, Krishnan & Spechler 2008). EGFR over-expression may indicate pre-neoplasia however the failure to detect any mutation evidence within the metaplastic cells may suggest a 'normal repair type' transient over-expression rather than tumorigenic change. The metaplastic response in COPD may be a repair or damage response that is common to both COPD and cancer. It may not be an automatic precursor to cancer development. The up-regulation of EGFR is associated with normal repair processes. One repair function of EGFR is to allow cell motility. Cells migrate to areas of damage and the lack of mucin involvement in these cells suggests that motility is the property conveyed by EGFR expression.

7.9.4 Cell morphology and characteristics

The H&E photographs in Figure 7.9 were taken of metaplastic cells in the COPD cohort. These cells are very comparable to bronchiolisation of alveolar bed shown to surround tumours by Jensen-Taubman, Steinberg & Linnoila (1998), with rounded cell morphology and similar cellular spacing, from individual cells to neat single rows and occasionally multiple layers of cells. Based on general H&E morphology alone these altered cells in the tumour surrounding tissue bear the closest resemblance to the

metaplastic cell population in COPD. Based on this observation alone the cells could be thought of as a pre-neoplastic lesion.

In conclusion, the metaplastic cells showed low numbers of proliferating cells throughout the samples and in some cases, these numbers were lower than nearby damaged airways. This low proliferation would not be consistent with a pre-neoplastic lesion.

There was some evidence of TA-p63 but not ΔN-p63 within the metaplastic cell population. This suggests that these cells may undergo cell cycle arrest and apoptosis via a similar pathway as p53. This cell death is triggered by detection of abnormality within the cell. This is more indicative of normal epithelium than a pre-neoplastic lesion. Involucrin was sporadically seen on metaplastic cells suggesting that the majority of cells were not squamous. Many tumours express a high level of involucrin the lack of staining here is not suggestive of pre-neoplasia. The presence of EGFR within the metaplastic cell population suggests that the cells are motile. EGFR expression is found in reparative cells and tumour cells. The mix of cytoplasmic and membrane staining may be indicative of metaplastic cell maturation. Further work would be needed to gain more information about this staining profile.

The absence of L858R mutation within the metaplastic cells is perhaps unsurprising as this mutation is found in only 20% of adenocarcinoma and absent from other lung tumours. Therefore even if this mutation is an early event of pre-neoplasia it would not be expected in many samples.

The evidence for a pre-neoplastic lesion is low. There are low levels of TA-p63, minimal involucrin, low proliferating cell numbers and no L858R EGFR mutation detected. However EGFR was found to be expressed, which may be indicative of a pre-neoplastic lesion.

Chapter 8 Conclusions

This COPD cohort exhibited many of the characteristic changes noted for COPD, for example, goblet cell hyperplasia, alveolar tissue loss, inflammation. The heterogeneity of the samples reflects the multifactorial nature COPD (Snider 1986). Goblet cell hypertrophy and hyperplasia, medial hypertrophy and vascular remodelling, chronic and acute inflammation and loss of alveolar tissue have all been reported previously (Saetta *et al.* 2000, Santos *et al.* 2002). The lung function available for most of the patients shows that all individuals were GOLD 3 or 4 COPD sufferers, this means severe COPD. It would therefore be expected that there would be extensive tissue damage and change.

Corrin describes an obstructive small airways disease that features bronchiolar goblet cell hyperplasia and surrounding inflammation. He suggests that chronic inflammatory changes lead to airway wall thickening, peribronchiolar fibrosis and inflammation, all of which may be observed in the LVRS cohort (Corrin 2006).

The morphology of surface lining cells in COPD were categorised into three cell types, uniform; similar size cells, where cell and nucleus were both rounded. A macrophage-like morphology; cells differed in size, variable sized nuclei. The third morphology more often associated with fibrosis, was a squamous flattened phenotype. These cell morphologies are similar to other epithelial cells. The pluripotent epithelium in the pseudoglandular phase of lung development shows round single layer morphology. Pulmonary sequestration and BPD, both a developmental retardation, have similar rounded cells. Simple round cells are also found in IPF, alongside lining cells that are perhaps more squamous. In lepidic bronchioloalveolar carcinoma cells that line the alveolar bed are uniform rounded cells. Round cells are also present bronchiolisation of the alveoli, the altered lesions surrounding some tumours. Type II hyperplasia can occur as part of disease or normal repair processes also display the same morphology. In summary these cell morphologies can be seen in development, repair and disease, including cancer. The morphology alone is not really indicative of any characteristics other than an epithelial phenotype, perhaps a default basic phenotype.

The distribution and spreading pattern of the metaplastic cells does not clearly indicate any maturation from one morphology to another. The position of the cells is similar to lepidic BAC that seems to spread into the alveolar bed. Many of the lung diseases such as IPF, BPD, pulmonary sequestration and sclerosing hemangioma have got

underdeveloped or altered lung structure, due to fibrosis and airway loss that it is difficult to closely compare the colonising cell locations. All cells seem to sit on muscularised or fibrotic blocks of tissue. The use of matrix staining demonstrated that colonising cells required only laminin, of those matrix components examined, as a matrix surface. This is very similar to the findings by Fisseler-Eckhoff *et al.* (1990) who examined the matrix underlying a pre-neoplastic lesion. In IPF there has been demonstration of increased amounts of collagen I and collagen III production in the tissue, however precise locations of this deposition in relation to IPF lining cells was not discussed (Hetzel *et al.* 2005). Further work would include the examination of matrix constituents such as fibronectin, tenascin, collagen 7 and laminin 5.

The lung progenitor markers examined in the metaplastic population showed only evidence of type II cell contribution to the population. It was thought that if the cells represented a reversion to a foetal phenotype the metaplastic cells may have also expressed CC10 Clara cell marker as previous work shows pluripotent epithelium showing markers of several cell types (Wuenschell *et al.* 1996). Cells contributing from the local airways may be thought of as Lambertosis, this is defined as a spread of the airway epithelium through the crypts of Lambert into the alveolar tissue (Lambert 1955). The lack of any airway progenitors along with the lack of ciliated cells and general airway structure allowed this possibility to be discounted. Tumours also often exhibit ΔN-p63; this demonstrates a similarity to basal cells but also the potential for continued proliferation. This was absent from the metaplastic cell population but there was evidence of progenitor cell markers such as CD133 that have been identified in tumours (Monzani *et al.* 2007). Although cells expressing CD133 can contribute to tumour development, progenitor cells can also contribute to normal repair processes (Mattsson *et al.* 2004). The benign lung lesion alveolar adenoma is usually a single lesion, lined with simple epithelial cells. Lining cells are cytokeratin positive, non-ciliated and do not stain for CC10. The cells were positive for type II cell markers, surfactant and TTF-1. This is a very similar profile to the metaplastic cells seen within the COPD lung. Alveolar adenoma appears to be quite proliferative being classed as PCNA giving strong staining on all the samples examined. Burke *et al.* (1999) conclude that these lining cells are type II pneumocytes. So although first impressions suggest that these cells share many characteristics with a neoplastic lesion, this benign lesion perhaps suggests that the metaplastic lesion in COPD will not progress to malignancy.

These findings show that the presence of progenitor cell influence could indicate either a pre-neoplastic lesion or a normal attempt at repair albeit in an inappropriate location. The other progenitor cell identified was the type II cell.

Although initial staining suggests that the metaplastic cells are type II cells with a contribution from cells with multipotent possibly bone marrow markers, further staining with additional markers put this into question. Pan cytokeratin and CK7 and CK18 staining are all type II cell characteristics. The production of surfactants A and C are also type II cell features. The production of occasional mucin 2 and more frequent mucin 4 are not type II cell behaviours. Mucin 2 is thought of as upper airway or gut cell produced. Mucin 4 is seen in early development or is upregulated by inflammation. In the gut mucin 2 is seen in maturing goblet cells, the distribution of positive cells in the metaplastic population is not suggestive of maturation and the morphology is not that of goblet cells. This does not appear to gut epithelial phenotype. The mucin 4 production may be explained by the extensive inflammation within the COPD lung. This leaves some cells showing type II surfactants and some cells showing epithelial mucin production. Mucin 1 production has been demonstrated in type II cells but no other mucins are documented as being produced by pneumocytes (Jarrard *et al.* 1998). Unfortunately there has been no co-localisation work carried out with surfactant C and mucin 4 to determine whether the same cells are producing both secretions. Not all metaplastic cells produce surfactant, it could be argued that two populations contribute to the lesion: type II cells expressing surfactant and bone marrow cells becoming simple epithelium producing mucin. I think this is unlikely as quite large areas of adjacent cells all show mucin 4, also the cell morphologies are uniform, cells of different origins may well look different.

The cytokeratin profile examination allowed consideration of whether the epithelium was behaving like repairing airway, Lambertosis, normal airway or epithelium from another organ. Some of the metaplastic diseases considered here are difficult to interrogate using the cytokeratin profile, as there is little published information. The lesion in BPD is pan CK positive showing epithelial origin (Davis *et al.* 2008), it has been shown to produce mucin 1, this could suggest either airway or type II cells (Ogihara *et al.* 2000).

Epithelial research in pulmonary sequestration has only been carried out on one case that resembles a neuroendocrine tumourlet. This case was pan CK positive and a few

exhibited basal cell markers. There was extensive positive staining for neuroendocrine markers (Pelosi *et al.* 1992). Perhaps most other pulmonary sequestration examples are pan cytokeratin positive with a few basal cells. This would suggest that these proliferations are part of an attempt at repair although unlike the COPD metaplasia, originate from airway epithelium.

Lambertosis has not had cytokeratin profiling carried out but by definition it should be similar to the airways and so the lack of CK5/6 in the metaplastic population suggests that it is not the same profile as Lambertosis. There has not been much published on the cytokeratin profile on the airways of the COPD lung. There is some evidence that there is an increase in goblet cells within the smaller airways (Saetta *et al.* 2000). The metaplastic cells do not resemble the airways of COPD or normal airway epithelium and further confirms the exclusion of Lambertosis as a diagnosis.

Reparative epithelium often shows a simple cytokeratin profile and may be expected to exhibit involucrin and EGFR (Burgel & Nadel 2004). These characteristics are seen in the COPD metaplastic cells so may suggest repair behaviour, although cells have no maturation cues so cannot complete repair and mature.

Previous work examining IPF ling cells generally conclude that the cells are type II cells, as a direct result of the production of surfactant. Shilo *et al.* (2007) noted cells had the appearance of adenocarcinoma, cells showed mitotic activity and were described as cuboidal to columnar with prominent nuclei and vesicular chromatin. More inflated areas showed altered cells lining existing surfaces of the alveolar bed, Shilo *et al* likened these to the lepidic growth of bronchioloalveolar carcinoma. The samples were stained with TTF-1, a type II marker and all cuboidal and columnar lining cells were positive. The description and appearance of this type II hyperplasia was similar to that of the metaplastic cell population in the COPD cohort. TTF-1 is seen within the airway epithelium during development but not in adult airway epithelium (Chapin *et al.* 2005). Kawanami *et al.* (1978) demonstrated anchoring fibrils occasionally present under lining cells, showing some membrane adhesion. The work here confirmed the presence of areas of multilayer cells, multilayer cells are very occasionally observed within the metaplastic cells of COPD and it was difficult to be confident that this was not just a consequence of tissue orientation and plane of cut. The lower power electron microcopy pictures of the IPF cells look very similar to the COPD metaplastic cells, the IPF cells

also demonstrate grooved and irregular nuclei, variable nuclear colour intensity, microvilli present on the surface and dense inclusions within the cytoplasm.

COPD metaplastic cells resemble goblet cell hyperplasia based on cytokeratin profile (Stacher *et al.* 2004). This phenotype was quickly discounted based on PAS tinctorial staining that highlighted mucinous goblet cell contents. The PAS stain was not seen on any of the metaplastic cell population. Although clearly epithelial and productive, metaplastic cells were not producing goblet cell mucins.

Lepidic bronchioloalveolar carcinoma is the closet epithelial phenotype to the COPD metaplastic cell population, cytokeratin and mucin profiles are similar (Sato *et al.* 2006).

Barrett's oesophagus and Crohn's disease both seem to have an element of simple cytokeratin expression that was similar to the COPD metaplastic cell profile, however, this must be put into context (Buning *et al.* 2004). The normal cytokeratin profile of the oesophagus was CK19, with some CK18 and CK13 staining, and minimal expression for CK7, 8 or 10 (Lam *et al.* 1995). Normal oesophagus is positive for CK10/13 and negative with regard to CK7, 8, 18 and 20 (van Baal *et al.* 2008). This suggested that the profile seen in both Barrett's and Crohn's was a simple epithelial phenotype that was not similar to that seen in normal surrounding lung tissue.

Hypoxia and normoxia markers were not strongly associated with the metaplastic cell lesions. There may be a broad influence exerted by lung hypoxia, stimulating the mobilisation of progenitor cells or triggering angiogenesis but the immediate microenvironment does not suggest hypoxic control.

The lack of collagen and matrix requirements underlying the metaplastic cells indicates a lack of maturation or phenotypic cues from the underlying tissue. This may explain why surfactant C levels appeared higher in the presence of inflammation and leak, as these were the only local cues. Muscle underneath the metaplastic cells almost suggests a lack of cues leading to a default epithelium.

Barrett's oesophagus shows a complete epithelial shift from oesophagus epithelial phenotype to intestinal epithelial phenotype. Although the metaplastic cells do not resemble airway epithelium, there is no clear alternative cell phenotype emerging, as the cells seem to remain primitive or immature. Barrett's is in part caused by repeat cycles of acid reflux bathing the epithelium. This universal damage may be what leads

epithelial maturation arrest. COPD lungs are exposed to numerous damage cycles of cigarette smoke, infection, and environmental pollution with chronic and acute inflammation. COPD is heterogeneous with different phenotypes of airway epithelium. This heterogeneity may lead to a plethora of growth and maturation signals in the parenchymal tissue, preventing the colonising cells from maturing.

Local crypt progenitor cells appear to contribute to Crohn's disease. There are areas of normal appearing mucosa and inflamed metaplasia within the small intestine. This mixed reaction is similar to the heterogeneity of COPD. The local nature of these progenitor cells suggests that the local progenitor cells within the lung may be capable of producing a metaplasia.

The lack of mutation findings for EGFR is perhaps unsurprising as the general findings suggest that the lesion is not pre-neoplastic but a default reparative epithelium.

Potential roles of this default epithelium include;

Surfactant and mucin production to maintain compliance in damaged or gas-trapped areas of the lung. Hyperinflation in COPD makes inspiration and expiration difficult. In addition, emphysematous change and alveolar loss alters the tortional strain on the remaining tissue. Contribution to the innate immune defence via mucin 4 production. This and the physical barrier of the cells may help prevent infection spread, across the lung or into the bloodstream from within the parenchyma. It may be concluded from this work that the metaplastic response is epithelial, possibly a de-differentiated primitive form of epithelium.

The metaplastic cells from COPD patients have been characterised here as either rounded and uniform, large irregular resembling macrophages or squamous and flattened. These cells were found to be localised to the external adventitia of airways and vessels, the sub pleural zone, hilar regions, ectopic fibrotic deposits and the external facet of alveolar epithelium. The presence of metaplastic lesions was associated with but not dictated by the presence of airway hyperplasia, goblet cell hypertrophy, goblet cell hyperplasia and airway inflammation.

Immune phenotyping showed that basal, Clara, goblet, mucus gland and mesothelial cells do not contribute to the metaplastic cell population. A sub population of metaplastic cells showed a type II pneumocyte phenotype and very occasional cells demonstrated markers of multipotent progenitor cells. Multipotent progenitor cells are

known to be upregulated by damage, so the presence of these markers suggest a reparative behaviour. The cytokeratin profile identified was consistent with simple epithelium, further confirming the divergence from an airway epithelial phenotype. This apparent lack of airway progenitor cells and simple cytokeratin profile refutes the theory of Lambertosis.

The mixed cytokeratin profile confirms a divergent population suggested by surfactant staining. Sporadic mucin 4 expression and very occasional mucin 2 expression on the metaplastic cells may be a potential consequence of inflammation and further support the mixed, immature reparative phenotype.

There was some evidence of mesenchymal cells, possibly fibroblasts, underlying some metaplastic cells. Metaplastic cells are known to adhere to expanded mesenchyme so further work would need to be carried out to examine the relationship with fibroblast populations. Of the matrix markers examined, laminin was the only consistent matrix protein underlying the metaplastic cells. Other basement membrane components such as collagen 7, laminin 5 or fibronectin may be influential and require additional investigation. The presence of laminin and the absence of collagens I, III and IV directly underneath the cells is similar to that of a pre-neoplastic lesion. This makes further matrix examination important.

There was no evidence of a relationship between the presence of metaplastic cells and either CA IX positive hypoxic areas or von Hippel Lindau normoxic areas, although tissue fibrosis and the obliterative nature of COPD tissue suggest hypoxic damage. CA IX is a downstream protein from hypoxia inducible factors so may not reflect current oxygen status. Alternative hypoxia markers such as hypoxia inducible factor demonstration would provide further confirmation of hypoxic areas of tissue. These severe COPD patients may have been ventilated before surgery altering the oxygen within the lungs.

There was little evidence of TA-p63 or involucrin within the metaplastic cells. The presence of EGFR may reflect a repair phenotype as well as possible pre-neoplastic change so conclusions cannot be made about this receptor expression and possible links to neoplasia. The lack of detection of the L858R EGFR mutation seen in some adenocarcinoma may however suggest that these cells do not constitute a pre-neoplastic lesion.

Potential roles of this default epithelium include;

Surfactant and mucin production to maintain compliance in damaged or gas-trapped areas of the lung. Hyperinflation in COPD makes inspiration and expiration difficult. In addition, emphysematous change and alveolar loss alters the tortional strain on the remaining tissue. Contribution to the innate immune defence via mucin 4 production. This and the physical barrier of the cells may help prevent infection spread, across the lung or into the bloodstream from within the parenchyma. It may be concluded from this work that the metaplastic response is epithelial, possibly a de-differentiated primitive form of epithelium.

Future work could include an extension of the investigation carried out here in milder COPD to try and identify the beginnings of the lesion. Further investigation of the anatomy of the lesion through the lung lobe would be helpful. This would need whole lobe samples rather than rind resections gained from LVRS. This would allow a natural history of the evolution of metaplastic cells to be examined.

Table 8.1 Key Findings

Key findings	**Implications/Inferences**
Metaplastic cells were pan CK positive	Epithelial phenotype
Metaplastic cells produced prosurfactant C	Showed type II cell characteristics
Metaplastic cells showed come CD34 positive cells	Contribution to metaplastic cell population by bone marrow progenitor cells
Metaplastic cells showed mixed cytokeratin profile	Suggestive of mixed epithelial phenotype
Metaplastic cells produced Mucin 4	Shows epithelial phenotype other than type II cell present, possibly similar to airway epithelium
Only consistent underlying matrix is laminin 1/2	Shows this is not an extension of the airways and shows some similarity to preneoplastic lesions
0-5% of metaplastic cells showed Ki67	Proliferation rate lower than expected for preneoplastic lesion

From this, we can conclude that diffuse metaplasia in COPD peripheral lung is not consistent with Lambertosis. Overall, characteristics including surfactant and mucin presence, suggest a primitive epithelial phenotype, potentially a result of damage and aberrant repair.

Publications

I would like to publish two papers based on the work carried out in this thesis. The first paper would describe the occurrence of metaplastic cells in the lung periphery of COPD, this has not previously been documented and would allow comparison to other lung diseases. The paper would then discuss the phenotype of the metaplastic cells with regard to the common assumption that these cells are type II cell hyperplasia and also with consideration of the phenomenon of the canals of Lambert. This would include immunohistochemistry work examining cell phenotype markers and cytokeratins.

The second publication would aim to discuss the underlying surfaces and matrix of the cells, this paper would look at connective fibre markers such as collagens and laminin and perhaps also discuss adhesion molecules such as E cadherin. It may also be useful to re- examine possible markers for tenascin and fibronectin as these stains were unsuccessful previously.

9.0 Appendix

9.1.1 LVRS Glenfield Consent form – applicable to samples 1-40

13.6.2007:Version 1

Subject Information Leaflet and Consent Form

Study Title: Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

Principle Investigator: Professor Andrew Wardlaw

Study Funded by: Astra-Zeneca

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

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.

What is the purpose of the study?

In order to find the causes of lung disease such as COPD and lung cancer and to find new ways of treating these diseases we have to do more research. It is ideal to do the research on tissue from human lungs because we are investigating a human disease. The lung research teams at Glenfield Hospital, Leicester Birmingham Heartlands Hospital, Walsgrave Hospital, Coventry and AstraZeneca (a pharmaceutical company) have joined together in order to collaborate on studies into lung disease using human lung tissue. A numbers of diseases will be studied and the lung tissue will be used in a number of different laboratory studies. AstraZeneca will use the lung tissue in the understanding of lung disease and development of new treatments for lung disease. These experiments will be done on lung tissue that has been removed from patients as part of their medical treatment, which would otherwise be destroyed. In some cases we may also wish to take a blood sample to compare the findings in the lung tissue and blood.

Why have I been chosen?

You have been chosen because your doctor has said that you may need to have some of your lung removed to treat your disease.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

You are about to undergo an operation for your current condition. We would like to retain some of the spare lung tissue that will be removed as part of your operation, which would otherwise be destroyed. If you are willing to take part in this research, we will pass the surplus lung tissue to the collaborating hospitals and AstraZeneca. The surgeon will <u>not</u> remove any extra lung tissue for this research. We will also record some information about your recent medical history, medicines taken and reason for the operation from your medical records. In terms of your operation, stay in hospital and subsequent follow up there will be no difference to what will happen to you whether you take part in the study or not except that in a few cases we may wish to take an extra blood sample of approximately 15mL (about three tablespoons full) before your operation.

What happens if I don't want to take part?

Nothing, you simply don't sign this form. This will not affect your medical care or your legal rights in any way.

What rights do I have to the results of the research?

You are being asked to donate your tissue as a gift to the researchers in the hospitals involved and AstraZeneca. Any information derived directly or indirectly from this research by the collaborating hospitals or by AstraZeneca, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result, are the sole property of the company (or their successors, licensees, and assigns) and may be used for commercial purposes. You have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this research. However, in signing this form and donating a blood sample, you do not give up any rights that you would otherwise have as a participant in research.

What do I have to do?

There is nothing extra to do as a result of being part of this study

What are the possible disadvantages and risks of taking part?

There are no disadvantages or risks to taking part in the study over and above the normal risks associated with this surgery, which you require as part of your care. If you are asked to donate an additional blood sample there may be some discomfort of the needle being inserted into a vein in your arm and the possibility of bruising developing afterwards around the area that the needle was inserted. This should disappear in a few days

What are the possible benefits of taking part in the study?

There are no direct benefits. 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.

What if something goes wrong?

We do not think there is any significant risk of any harm occurring as a result of participating in this study. However if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.'

Will my taking part in this study be kept confidential?

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. In all instances, your confidentiality will be maintained, in accordance with the Data Protection Act or as local laws permit.

AstraZeneca and Regulatory authorities may wish to check that this research has been done properly, they may have access to your files and know your identity, but they are under a duty of confidentiality not to disclose details to others.

What will happen to the samples that I have donated?

The samples will be processed by the research team and used in a range of experiments into the causes of lung disease. Samples may be transported to AstraZeneca or other hospitals in the collaborating group to do further experiments including tests to develop new drugs. Those samples that are not fully used up in experiments may be stored by the research team or by AstraZeneca, for use in future experiments, for up to 20 years.

Who is organising and funding the research?

The research is a collaboration between the lung research teams at the hospitals in Leicester, Coventry, Birmingham and AstraZeneca. The study is organised and operated by the individual hospitals involved and the overall collaboration has been funded by AstraZeneca. The income obtained from AstraZeneca will only be used to support the work carried out as pat of this project.

Can I Withdraw my consent?

You may withdraw your consent to the use of your data and samples at any time. If you withdraw your permission consent before your donated tissue and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent after your tissue sample has been sent for analysis we will ensure that your sample(s) are destroyed. However, if analysis has already been performed neither AstraZeneca nor ourselves are obliged to destroy results of this research.

Who has reviewed the study?

The study has been reviewed by the research teams within the consortium and by the members of the Department of Respiratory Medicine, Allergy and Thoracic Surgery. Individual research projects where we use the lung tissue have been reviewed by a variety of charities and funding organisations

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

Professor Wardlaw or Mr Waller (0116 2563841)

This document must be kept in the investigator's study file and retained for a minimum period of 20 years after completion of the study.

Study Title: Use of Tissue from Lung Resections to Investigate the Molecular and Functional Mechanisms of Human Lung Disease

	Initials
1. I confirm that I have read and understood the patient information form on the above project 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 donate as a gift 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.	☐
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 further information.	☐

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**: ……..

9.1.2 Corris Collaboration Consent form – applicable to samples 41-50

ADULT STUDY SUBJECT INFORMATION & CONSENT FORM

Study Code: HTASP/D/006 Subject Initials:

Enrolment Code:

Full Study Title: A Single-Centre, Open, Exploratory Research Study into the

Cellular and Molecular Mechanisms in COPD Using Tissue Obtained from Diseased Lungs Removed at The Time of Lung Transplantation

Short title: The use of diseased lungs removed during lung transplant to understand why chronic lung disease occurs

Study Doctors: Professor Paul Corris, Professor of Thoracic Medicine

Dr Andrew Fisher, Senior Lecturer in Respiratory Medicine

Dr James Lordan, Consultant Respiratory Physician

Lung Transplantation Team Doctors, Freeman Hospital

Sponsoring Company: AstraZeneca R&D Charnwood in collaboration with the Freeman Hospital, Newcastle Upon Tyne NHS Trust and the University of Newcastle Upon Tyne. You are being invited to take part in a research study as you have a severe lung disease that makes you a candidate for a lung transplant. Before you decide if you want to take part it is important for you to understand why the research is being done, how your information will be used, what the study will involve and the possible benefits, risks and discomforts. Please take time to read the following information carefully. Talk to others about the study if you wish. Please ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the background and purpose of the study?

Your Consultant will have already informed you of the need to have lung transplantation performed for the treatment of your condition. During your lung transplant operation it will be necessary to remove the diseased lung from your body and replace it with the new donor lung. If you agree, some of the lung tissue, blood or material extracted from your diseased lung will be stored in a Tissue Bank for use by the Freeman Hospital, Newcastle Upon Tyne NHS Trust, the University of Newcastle Upon Tyne and AstraZeneca for the purposes of this study.

It is hoped that by looking at the tissue samples donated by you and other patients taking part in this study, a greater understanding of the disease process will be gained. This research could then be used in the development of new treatments for the potential benefit of future patient care.

We are asking patients who require lung transplants for lung diseases, which may include

Chronic Obstructive Pulmonary Disease (COPD), Emphysema, Idiopathic Pulmonary Fibrosis (IPF), Cystic Fibrosis (CF) or Pulmonary Arterial Hypertension (PAH) to take part in this study. You are one of a number of patients who will be approached to take part.

Who is organising the study?

This study is being done in collaboration between the Freeman Hospital, Newcastle Upon

Tyne NHS Trust, the University of Newcastle Upon Tyne and AstraZeneca ("Sponsoring Company").

What do I have to do?

The transplant team has recommended that you have a lung transplant to treat your severe lung disease. The diseased lung(s) is removed as a normal part of your operation. If you are willing to take part in our research, the surgeon performing your transplant will give us some samples of the diseased lung tissue that would normally be disposed of by incineration. The donation of this tissue will be seen as a gift from you for research purposes. We will also record from your medical records information that could be relevant to your disease.

Do I have to take part?

No. It is up to you to decide whether or not to take part in this study and give some of your diseased lung tissue. If you do decide to take part in the study, you will be given this information sheet and consent form(s) to sign and keep. You will have an opportunity to ask questions and have them answered to your satisfaction. Your own GP will be notified about your participation in the study and by signing the consent form you will be agreeing that we can contact them. If you choose not to take part in the study, your decision not to take part will not affect the standard of care you receive.

What are the possible side effects, risks and discomforts of taking part?

There are no drugs, devices or procedures being tested in this study – we will simply collect samples and data. There are no additional risks or side effects to your procedure if you decide to donate tissue.

What are the possible benefits of taking part?

Taking part in this study means that you may possibly help improve the treatment for patients in the future, as information about the changes that occur in lung disease may be used to develop new treatments. You will not directly benefit from taking part in this study. You will not be paid for taking part, but it will not cost you anything either.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure.

Complaints can be sent to:

The Complaints Officer, Freeman Hospital, Newcastle, NE7 7DN.

How will my personal data be used and how will my confidentiality be protected?

Special precautions are taken to ensure the research study is carried out with a high degree of confidentiality. By signing this form you consent to the Study Doctor and his or her staff collecting and using your personal data for the study ("Study Data"). This includes: your date of birth, your sex, your ethnic origin and personal data on your physical or mental health or condition. Samples and data shared with sponsors and collaborators outside the Freeman Hospital, Newcastle Upon Tyne NHS Trust will not include your name, address or National Health Service number. The Study Data shared with the Sponsoring Company is protected by the use of a code (the "Code"), which is a number specific to you. If you agree to participate in the study, a code that is specific to you will be used to label your samples and data and identify all results that are recorded at the Freeman Hospital, Newcastle Upon Tyne NHS Trust. The coding of all information resulting from your taking part in the study is to ensure that the results are kept confidential by keeping your identity and the results separate. The Study Doctor is in control of the Code key, which is needed to connect your Study Data to you. A person appointed by the Sponsoring Company, regulatory authorities or other supervisory bodies may review any of your Study Data held by the Study Doctor. This is to ensure the quality of the work being carried out. Your research records may also be looked at by representatives of regulatory authorities and by authorised people from the Trust to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the Freeman Hospital. Once all data entry and sample processing has been completed, the link between your personal data and the unique codes will be broken. Your samples can then not be traced back to you as an individual.

The Study Doctor will use your Study Data to conduct the Study. The Sponsoring Company may use your Study Data to conduct the Study and for research related to the development of pharmaceutical products, diagnostics or medical aids. The Study Doctor and the Sponsoring Company are each responsible for their handling of your Study Data in accordance with applicable Data Protection law(s). The Sponsoring Company may share your Study Data with other companies within its group, with its service providers and its contractors who will use your Study Data only for the purposes described above. The Sponsoring Company may transfer your Study Data to countries outside of the United Kingdom for the purposes described in this document. Please be aware that the laws in such countries may not provide the same level of data protection as in the United Kingdom and may not stop your Study Data from being shared with others. However, in this study, as all data and samples transferred will be coded and not directly identifiable to you as an individual, there should be no issues related to data protection. Please note, the results of the study may be published in medical literature, but you will not be identified. You have the

right to request information about your Study Data held by the Study Doctor and Sponsoring Company. You also have the right to request that any inaccuracies in such data be corrected. If you wish to make a request, then please contact the Study Doctor, who can help you contact the Sponsoring Company if necessary. If you withdraw your consent the Study Doctor will no longer use your Study Data or share it with others. The Sponsoring Company may still use Study Data that was shared with it before you withdrew your consent. By signing this form you consent to the use of Study Data as described in this form.

What will happen to my samples?

Your lung tissue samples will be allocated a unique number that can be traced to you as the donor. These samples will be stored securely, processed and used for investigations at the Freeman Hospital, Newcastle Upon Tyne NHS Trust, University of Newcastle Upon Tyne and AstraZeneca. AstraZeneca may also wish to provide samples or data to third parties such as laboratories with whom they have collaborations, to perform specific analyses. All samples provided will either be completely used up or returned to AstraZeneca, and will only be used for the purposes described within a specified period. During the preparation of your samples for research it is also likely that some fragments will be waste; this is normal in the processing of tissue to ensure samples are suitable for use. No personal identifiable information will be sent outside the Freeman Hospital, Newcastle Upon Tyne NHS Trust.

Lung tissue samples and coded data will be stored for the duration of the study. The studies that we are doing on your lung tissue are not intended to suggest any clinical diagnosis or treatment for your disease. It is not the purpose of this research to provide you with test results. After your old lung has been carefully examined by a pathologist at the Freeman Hospital and also at AstraZeneca, the link between your unique code will be broken. After the link has been broken the tissue will not be traceable back to you, so no findings from this research will directly apply to you or have implications on your future health or treatments. The investigator and AstraZeneca may use your data and samples for administration purposes, research and development of pharmaceutical products, diagnostics and/or medical aids, statistical analysis, the approval, registration and marketing processes relating to its products/study medication, and carrying out safety and efficacy evaluations.

What will happen if I withdraw my consent?

Participation in any research study is completely voluntary and you can decide to withdraw from the study. You may withdraw at any time up until the link between your personal details and your old lung tissue is broken. This period will be up to 4 months following your transplant. Following this time, because no link between you and your tissue remains, you will not be able to withdraw your consent. If you withdraw your consent before the link is broken, the Sponsoring Company and any other third parties involved in the research, will arrange to have your samples destroyed. They are not obliged to destroy results of research that has already been performed. The Sponsoring Company, and any other third parties involved in the research, may continue to use your study data if you withdraw your consent, either where any study analyses are incomplete or if they need to share your study data with regulatory authorities to ensure validity of the study. Withdrawing from the study will not affect the level of care that you get from your doctors.

What rights do I have to the results of the research?

Any information derived directly or indirectly from this research, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result of this research, are the sole property of the Sponsoring Company (and their successors, licensees, and assigns) and may be used for commercial purposes. You will have no right to this property and will have no rights to any share of the profits that may be earned directly or indirectly as a result of this research. However, in signing this form and donating the samples for research, you do not give up any rights that you would otherwise have as a participant in research.

Whom should I contact if I need more information or help?

If you have any further questions about this study, or wish to contact the Sponsoring

Company, please discuss this in the first instance with:

Professor Paul Corris, Dr James Lordan or Dr Andrew Fisher, Cardiopulmonary Transplant

Unit, Freeman Hospital, High Heaton, Newcastle upon Tyne, NE7 7DN Tel: 0191 233 6161

Thank you for reading this invitation.

Study title: A Single-Centre, Open, Exploratory Research Study into the Cellular and Molecular Mechanisms in Chronic Obstructive Pulmonary Disease Using Tissue Obtained from Diseased Lungs Removed at The Time of Lung Transplantation

Name of Principal Investigator: Prof Paul Corris, Prof of Thoracic Medicine Initials box

1. I confirm that I have read and understand the information sheet dated 12 December 2006 (version 4) for the above study. I confirm that the study information and procedures have been explained to me by ……on …… during the consent process for this study. ☐

2. I confirm that I have had the opportunity to ask questions about this study and I am satisfied with the answers and explanations that have been provided. ☐

3. I consent to donate tissue that would normally be discarded as part of my operation for the purposes of this study. ☐

4. I understand that my participation is voluntary and that I am free to withdraw my consent up until the link between my personal information and tissue samples is broken, without giving any reason and without any medical care or legal rights being affected. ☐

5. I agree to allow research staff and doctors at the Freeman Hospital, Newcastle Upon Tyne NHS Trust to record personal data and information relevant to my disease from my medical records and agree to allow the information to be kept in the future after I have donated tissue. ☐

6. I understand that some tissue and analysis data, but no personal information such as name, address or telephone number to identify me, may be provided to other organisations approved by the collaboration to assist in the research. ☐

7. I understand that some tissue and analysis data, but no personal information such as name, address or telephone number to identify me, may be transferred to countries outside the United Kingdom to assist in the research. ☐

8. I agree to take part in the above study and I agree that my GP can be informed. ☐

9. I understand that my personal data such as name, address and telephone number will NOT be provided to AstraZeneca OR to third parties and that all information about me is treated in the strictest confidence. ☐

10. By signing this information and consent form I agree that my personal data, including data relating to my physical or mental health or condition, and race or ethnic origin, may be used as described in this consent form. ☐

11. I confirm that I have received a signed copy of this information and consent form to keep. **YES/NO**

Name of Subject (BLOCK CAPITALS)

……...…….

Signature…….....…….. Date…...……..…

Name of Investigator (BLOCK CAPITALS)……..

Signature…….....…….. Date…...……..…

9.1.3 Midlands lung consortium form – applicable to samples N1-N6

27.4.2007:Version 1

Subject Information Leaflet and Consent Form

Study Title: Molecular and Functional Mechanisms of Human Lung Disease

Principle Investigator:(*Please insert name of PI)*

Study Funded by: Midlands Lung Tissue Consortium.

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

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.

What is the purpose of the study?

In order to find the causes of lung disease such as COPD and lung cancer and to find new ways of treating these diseases we have to do more research. It is ideal to do the research on tissue from human lungs because we are investigating a human disease. The lung research teams at Glenfield Hospital, Leicester Birmingham Heartlands Hospital, Walsgrave Hospital, Coventry and AstraZeneca (a pharmaceutical company) have joined together in order to collaborate on studies into lung disease using human lung tissue. A numbers of diseases will be studied and the lung tissue will be used in a number of different laboratory studies. AstraZeneca will use the lung tissue in the understanding of lung disease and development of new treatments for lung disease. These experiments will be done on lung tissue that has been removed from patients as part of their medical treatment, which would otherwise be destroyed. In some cases we may also wish to take a blood sample to compare the findings in the lung tissue and blood.

Why have I been chosen?

You have been chosen because your doctor has said that you may need to have some of your lung removed to treat your disease.

Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I take part?

Your surgeon has recommended having an operation that should help you with your current condition. Some samples of lung tissue removed as l part of your operation would normally be destroyed. If you are willing to take part in our research, your surgeon will provide the collaborating hospitals and AstraZeneca with some of the lung tissue that has been removed as a normal part of your operation. The surgeon will not remove extra lung tissue for this research. We will also record some information about your recent medical history, medicines taken and reason for the operation from your medical records. In terms of your operation, stay in hospital and subsequent follow up there will be no difference to what will happen to you whether you take part in the study or not except that in a few cases we may wish to take an extra blood sample of approximately 15mL (about three tablespoons full) before your operation.

What happens if I don't want to take part?

Nothing, you simply don't sign this form. This will not affect your medical care or your legal rights in any way.

What rights do I have to the results of the research?

Any information derived directly or indirectly from this research by the collaborating hospitals or by AstraZeneca, as well as any patents, diagnostic tests, drugs, or biological products developed directly or indirectly as a result, are the sole property of the company (or their successors, licensees, and assigns) and may be used for commercial purposes. You have no right to this property or to any share of the profits that may be earned directly or indirectly as a result of this research. However, in signing this form and donating a blood sample, you do not give up any rights that you would otherwise have as a participant in research.

What do I have to do?
There is nothing extra to do as a result of being part of this study
What are the possible disadvantages and risks of taking part?
There are no disadvantages or risks to taking part in the study over and above the normal risks associated with this surgery, which you require as part of your care. If you are asked to donate an additional blood sample there may be some discomfort of the needle being inserted into a vein in your arm and the possibility of bruising developing afterwards around the area that the needle was inserted. This should disappear in a few days
What are the possible benefits of taking part in the study?
There are no direct benefits. 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.
What if something goes wrong?

We do not think there is any significant risk of any harm occurring as a result of participating in this study. However if you are harmed by taking part in this research project, there are no special compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms would be available to you.'

Will my taking part in this study be kept confidential?

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. In all instances, your confidentiality will be maintained, in accordance with the Data Protection Act or as local laws permit.

AstraZeneca and Regulatory authorities may wish to check that this research has been done properly, they may have access to your files and know your identity, but they are under a duty of confidentiality not to disclose details to others.

What will happen to the samples that I have donated?

The samples will be processed by the research team and used in a range of experiments into the causes of lung disease. Samples may be transported to AstraZeneca or other hospitals in the collaborating group to do further experiments including tests to develop new drugs. Those samples that are not fully used up in experiments may be stored by the research team or by AstraZeneca, for use in future experiments, for up to 20 years.
Who is organising and funding the research?
The research is a collaboration between the lung research teams at the hospitals in Leicester, Coventry, Birmingham and AstraZeneca. AstraZeneca will be contributing to costs of the research at the collaborating hospitals. The income obtained from AstraZeneca will only be used to support the work carried out as pat of this project.

Can I Withdraw my consent?

You may withdraw your consent to the use of your data and samples at any time. If you withdraw your permission consent before your donated tissue and data are used, we will not use the data and the samples will be destroyed. If you withdraw your consent after your tissue sample has been sent for analysis we will ensure that your sample(s) are destroyed. However, if analysis has already been performed neither AstraZeneca nor ourselves are obliged to destroy results of this research.
Who has reviewed the study?

The study has been reviewed by the research teams within the consortium and by the members of the Department of Respiratory Medicine, Allergy and Thoracic Surgery. Individual research projects where we use the lung tissue have been reviewed by a variety of charities and funding organisations

If you have any further questions about this study please do discuss them with:(***Insert name and contact details of PI)***

This document must be kept in the investigator's study file and retained for a minimum period of 20 years after completion of the study.

Study Title: Molecular and Functional Mechanisms of Human Lung Disease

Initials

1. I confirm that 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 *(Insert name of relevant hospital)* and AstraZeneca. ☐

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 *(Insert Name of PI)*, if I need further information. ☐

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: ……..

9.2.1 Pathological criteria

The pathological criteria recorded and noted for each patient are listed according to anatomical location in Table 6 of section 2.5.1 in the methods.

Table 9.1Tissue blocks & numbers selected for the panels used within this study

Pat no:	Total	Full	Sub	1-10	Key features
1	20	2	0	2	
2	34	3	0	3	
3	45	2	0	3	
4	34	3	1	3	Pleural
5	55	3	1	3	Adventitia
6	52	2	1	3	Adventitia
7	43	2	0	3	
8	49	3	0	3	
9	60	2	0	3	
10	40	2	0	3	
11	58	3	1	0	Focal lesions
12	17	2	0	0	
13	42	3	1	0	
14	40	3	0	0	
15	47	2	0	0	
16	59	3	0	0	
17	69	2	0	0	
18	61	3	1	0	Alveolar
19	52	2	1	0	Pleural
20	46	3	2	0	Hilar & adventitia
21	72	4	2	0	Pleural & adventitia
22	38	3	1	0	Focal lesions
23	25	2	1	0	Focal lesions
24	50	2	1	0	Fibrosis
25	37	2	1	0	Pleural
26	21	2	1	0	Fibrosis
27	57	2	1	0	Pleural
28	95	3	1	0	Hilar
29	46	2	0	0	
30	31	2	2	0	Hilar & fibrosis
31	74	4	2	0	Alveolar & fibrosis
32	58	4	2	0	Alveolar & fibrosis
33	48	2	2	0	Hilar & alveolar
34	73	3	2	0	Adventitia & focal lesions
35	15	2	0	0	
36	12	2	1	0	Focal lesions
37	24	3	1	0	Alveolar
38	8	3	1	0	Pleural
39	58	4	0	0	
40	19	2	0	0	
41	28	3	1	0	
42	19	1	0	0	
43	41	2	0	0	
44	28	3	0	0	
45	29	1	0	0	
46	34	2	1	0	Adventitia
47	26	1	0	0	
48	49	1	1	0	Fibrosis
49	35	3	2	0	Pleural & Adventitia
50	30	2	0	0	

N1	5	1	1	0	Normal
N2	3	1	1	0	Normal
N3	6	1	1	0	Normal
N4	6	1	1	0	Normal
N5	6	1	1	0	Normal
N6	4	1	1	0	Normal

Table 3 The 'Total' column shows the total number of paraffin blocks available for the LVRS sample. The 'full' column shows how many blocks were included in the full-panel for this piece of work, there are representative blocks from all patients included here. The 'sub' panel has samples representative of all the different lesions but does not necessarily include all patients. 1-10 is the first 10 patients in the cohort that were used as a pilot set for consideration. The key features are the most prominent anatomical lesion used as criteria for inclusion into the 'sub' panel.

Table 9.2 Pathology overview of the COPD cohort

Pat	Overview of the pathology found within each patient sample	Cart/Gland
1	Pleura fibrosis, mild, lymphocytic aggregates. Parenchyma, ectopic fibrosis & muscle. Arteries medial & adventitial hypertrophy. Airways muscularisation, inflammation, epithelial hypertrophy.	
2	Pleura one sclerotic area, lymphocyte aggregates. Parenchyma sub pleural fibrosis, alveolar remodelling, microvascular leak & macrophage aggregates. Small airway only, muscularisation, luminal mucus, epithelial hypertrophy.	
3	Pleural fibrosis, moderate. Parenchyma, sub pleural fibrosis. Arteries fibrosis, moderate & medial hypertrophy. Airways, fibrosed & muscularised, thickened basement membrane, inflammation, epithelium pyknotic & hypertrophic.	
4	Pleura fibrosis, moderate, with tar. Parenchyma, alveolitis, minimal fibrosis & inflammation. Arteries medial hypertrophy. Airways, muscularised, cells & mucus in lumen, epithelium hypertrophic.	
5	Pleura OK. Parenchyma, hyperinflation, parenchyma, loss, microvascular leak, localised fibrosis. Vasculature OK. Airways muscularised, small airways inflamed, cells & mucus in lumen, epithelium hypertrophic & goblet cell hypertrophy.	C, G
6	Pleura acute inflammation. Parenchyma, tissue loss, microvascular leak. Vasculature OK. Airways muscularised, thickened basement membrane, inflammation, marked, bronchiolitis, epithelium, hypertrophic, metaplasia & pyknosis areas.	C, G
7	Pleura OK. Parenchyma tissue loss, moderate, alveolitis. Arteries, medial hypertrophy. Airways, muscularised, epithelium, some hypertrophic, others basal cell hyperplasia.	C, G
8	Pleural fibrosis, inflammation & focal necrosis. Parenchyma, sub pleural fibrosis, lymphocytic aggregates, fibrotic clubbing, bronchiolisation. Arteries medial hypertrophy. Airways, muscularised, fibrosis, moderate, peribronchiolar inflammation, epithelium mainly hypertrophic with basal cell hyperplasia.	
9	Pleura OK. Parenchyma, fibrotic clubbing, microvascular leak & hyaline. Arteries, medial hypertrophy. Airways where present, muscularised, peribronchiolar inflammation, epithelium basal cell hyperplasia or goblet cell hypertrophy.	
10	Pleura lymphocytic aggregates. Sub pleura hyperinflated, bullae. Parenchyma ectopic fibrosis. Muscularised area has epithelial lining. Vasculature muscularised, mild. Airway fibrosis, muscularisation, epithelium goblet cells hypertrophy.	C, G
11	Pleura lymphocytic aggregates. Parenchyma tissue loss moderate, leak & macrophage aggregates. Arteries medial hypertrophy & myolysis. Airway muscularised, inflamed, luminal mucus & epithelium hypertrophic.	
12	Pleura OK. Parenchyma alveolitis macrophages & lymphocytes. Vasculature adventitial odema, arteries medial hypertrophy. Airways fibrosis epithelium goblet cell hypertrophy.	C
13	Pleura fibrosis. Parenchyma minimal clubbing. Arteries medial hypertrophy. Airway fibrosis, luminal cells & mucus. Epithelium goblet cell hypertrophy.	C, G
14	Pleural fibrosis. Parenchyma fibrosed, inflammation, leak, bronchiolisation, epithelial lined cysts. Vasculature intravascular leak. Airways fibrosed, inflammation, epithelium pyknotic.	
15	Pleura fibrosis, mild. Parenchyma alveolitis, fibrosis moderate. Arteries medial hypertrophy. Airways muscularised, fibrosed, luminal mucus. Epithelium hypertrophic, goblet cell hyperplasia, basal cell hyperplasia.	
16	Pleura, fibrosed. Parenchyma tissue loss, fibrosis. Vasculature vasculitis, arteries, medial hypertrophy, myolysis. Airways fibrosis, thickened basement membrane, inflammation.	

	Epithelium hypertrophic.	
17	Pleura, fibrosed. Parenchyma focal pneumonitis, ectopic muscle, tissue loss. Vasculature OK. Airways muscularised, inflammation. Airways cells & luminal mucus. Epithelium squamous/basal cell hypertrophy.	
18	Pleura fibrosis, marked, inflammatory aggregates. Parenchyma tissue loss, ectopic fibrosis, epithelial lined cysts. Arteries medial hypertrophy. Airways muscularised, fibrosed, inflammation, luminal cells & mucus. Epithelium hyperplasia & hypertrophy, goblet cell hypertrophy.	C, G
19	Pleura fibrosis, mild. Parenchyma tissue loss, fibrosis, inflammatory aggregates, bronchiolisation. Arteries medial hypertrophy. Airway fibrosis, inflammation, luminal cells & mucus. Epithelium goblet cell hyperplasia.	C
20	Pleura fibrosis, moderate, odema. Parenchyma leak, gas trapping, pneumonitis. Vasculature intravascular leak, inflammation, arteries medial hypertrophy, myolysis. Airways muscularised, fibrosed inflammation, luminal mucus. Epithelium hypertrophy, goblet cells hypertrophy.	
21	Pleura odema, inflammation. Parenchyma honeycombing, blood, leak, macrophages. Arteries medial hypertrophy. Airway if present, fibrosis, moderate, inflammation. Epithelium pyknotic & goblet cell hypertrophy.	C
22	Pleura OK. Parenchyma alveolitis & ectopic muscle. Vasculature muscularised, adventitial hypertrophy, arteries medial hypertrophy with myolysis. Airways muscularised, inflammation. Epithelium is variable some basal cell hyperplasia or pyknosis others goblet cells hyperplasia.	
23	Pleura fibrosis moderate. Parenchyma sub pleural fibrosis & ectopic fibrosis. Arteries, medial hypertrophy. Airways muscularisation & mucus plugging. Epithelium hypertrophic,	C, G
24	Pleura fibrosis, mild, lymphocytic aggregates. Parenchyma fibrosis, moderate, loss of tissue. Arteries medial hypertrophy with myolysis. Airway muscularisation, thickened basement membrane & mucus plugs. Epithelium goblet cell hypertrophy with some pyknotic cells.	
25	Pleura lymphocytic aggregates. Parenchyma fibrosis, muscularisation & lymphocytic aggregates. Arteries medial hypertrophy; myolysis. Airway inflammation & mucus. Epithelial goblet cell hypertrophy & pyknosis.	C
26	Pleura fibrosis moderate, inflammation. Parenchyma fibrosis, inflammation & ectopic muscle. Large arteries, medial hypertrophy. Airways inflammation, mucus plugs. Epithelium is damaged & friable.	
27	Pleura fibrosis, mild, inflammation. Vasculature OK. Airways muscularisation & fibrosis. Epithelium is normal or basal cell hyperplasia.	
28	Pleura OK. Parenchyma shows some fibrosis & loss of tissue. Vasculature smaller vessels, vasculitis. Airways muscularisation. Epithelium is hyperplastic & goblet cell hyperplasia.	C, G
29	Pleura OK. Parenchyma tissue loss, moderate, leak. Arteries medial hypertrophy. Airways muscularisation, thickened basement membrane, luminal mucus, moderate. Epithelium pyknosis & goblet cell hyperplasia.	
30	Pleura fibrosis, moderate. Parenchyma tissue loss, marked, fibrosis, inflammation. Arteries medial hypertrophy, inflammation. Few intact airways fibrosis. Epithelium OK.	
31	Pleura fibrosis. Parenchyma has fibrosis, lymphocytic aggregates, ectopic fibrosis, bronchiolisation. Arteries medial hypertrophy, marked. Vasculature, inflammation. Airways muscularised, inflammation, luminal plugging. Epithelium some normal, others obliterative.	
32	Pleura fibrosis, focal necrosis, sclerosis, inflammation. Parenchyma fibrosis, hyperinflation, bronchiolisation. Airways inflammation, luminal plugging. Epithelium hypertrophic or obliterated.	
33	Pleura fibrosis, odema. Parenchyma remodelled, inflammation, ectopic fibrosis. Arteries medial hypertrophy. Airways fibrosis, mucus plugs. Epithelium goblet cell hypertrophy or metaplasia.	
34	Pleura fibrosis, marked. Parenchyma tissue loss, remodelling, inflammation, bullae. Arteries, medial hypertrophy. Airways muscularised, inflammation, mucus. Epithelium, some pyknotic/metaplastic/normal.	C, G
35	Pleura fibrosis, marked. Parenchyma remodelling, loss of tissue, inflammation, ectopic fibrosis. Vasculature OK. Airways fibrosis, inflammation, mucus. Epithelium goblet cell	

	hypertrophy & pyknotic.	
36	Pleura leak, odema. Parenchyma loss of tissue, remodelling, leak. Medial hypertrophy, intravascular leak. Airways muscularisation, fibrosis, inflammation. Epithelium hypertrophic & goblet cell hyperplasia.	
37	Pleura OK. Parenchyma, tissue loss, inflammation. Airways inflammation. Small airway obliterative. If present epithelium goblet cell hypertrophy.	
38	Pleura, fibrosis. Parenchyma loss of tissue, ectopic muscle. Airways if present, muscularised, fibrosis. Epithelium normal.	
39	Pleura OK. Parenchyma fibrosis, muscularisation, macrophages & lymphocytes, marked. Small airways obliterated, bronchiolisation, epithelial lined cysts. Arteries medial hypertrophy, inflammation. Airways if present, hypertrophy or goblet cells hypertrophy	
40	Pleura OK. Parenchyma variable leak, clubbing, tissue loss, inflammation. Vasculature intravascular leak. Airway muscularisation. Epithelial goblet cell hypertrophy.	
41	Pleura OK. Parenchyma pneumonitis marked. Arteries medial hypertrophy. Airways muscularised, fibrosed, thickened basement membrane, inflammation. Epithelium variable metaplasia or goblet cells hypertrophy.	C, G
42	No pleura. Parenchyma hyaline, moderate. Arteries medial hypertrophy. Epithelium is hypertrophic.	C, G
43	Pleura fibrosis, minimal. Parenchyma pneumonitis, type II hyperplasia, macrophages & lymphocytic aggregates. Arteries medial hypertrophy, vasculitis & activated endothelium. Airways muscularisation, thickened basement membrane & inflammation. Epithelium hypertrophic, some goblet cell hypertrophy or metaplasia or obliterative.	
44	Pleura fibrosis, moderate. Parenchyma pneumonitis, type II hyperplasia, hyaline & lymphocytic aggregates, bronchiolisation. Arteries medial hypertrophy, marked, myolysis. Airways muscularisation, thickened basement membrane & lymphocytic aggregates. Small airways obliterative. Large airway epithelial hyperplasia, goblet cells hyperplasia, damage.	C, G
45	Pleura OK. Parenchyma remodelling, alveolitis. Arteries medial hypertrophy. Airways where present muscularised, inflammation. Epithelium obliterative.	
46	Pleura odema. Parenchyma hyaline deposition, alveolitis, macrophages. Arteries adventitial & medial hypertrophy, myolysis. Airways inflammation, marked, muscularisation, thickened basement membrane & mucus. Epithelium hyperplasia or goblet cell hypertrophy or metaplasia.	C, G
47	Pleura odema, blood, focal fat. Parenchyma, fibrosis, alveolitis. Vasculature vasculitis, arteries medial hypertrophy. Airways muscularised, marked, thickened basement membrane, lymphocyte aggregates. Metaplastic epithelium.	C, G
48	Pleura OK. Parenchyma hyperinflation, alveolitis, bronchiolisation. Vasculature inflammation, arteries adventitial & medial hypertrophy. Airways muscularised, thickened basement membrane. Small airways obliterated. Large airway epithelium hypertrophic.	
49	Pleura, fibrosis. Parenchyma, pneumonitis, type II hyperplasia, macrophages ectopic fibrosis, bronchiolisation. Vasculature vasculitis, arteries medial hypertrophy, marked, occlusion. Airways muscularised chronic & acute inflammation, mucus. Small airways obliterated, large airways epithelium hyperplastic, goblet cell hyperplasia.	
50	Pleura fibrosis. Parenchyma tissue loss, alveolitis, bronchiolisation. Vasculature vasculitis. Arteries medial hypertrophy, myolysis. Airways where present fibrosed, inflammation. Small airways obliterated. Large airway epithelium squamous metaplasia.	
N1	Pleura fibrosis, mild, lymphocytic aggregates. Parenchyma fibrotic clubs. Arteries medial hypertrophy. Airways muscularisation & luminal mucus. Epithelium metaplastic.	C
N2	Pleura odema. Parenchyma fibrosis, mild. Large arteries medial hypertrophy. Airways fibrosis, small airways obliterative, large airways metaplastic, basal cell hypertrophy.	C, G
N3	Pleura fibrosis, acute inflammation. Parenchyma fibrosis, moderate. Vasculature vasculitis. Airway fibrosis, small airway: obliterative, epithelium hyperplastic.	
N4	Pleura fibrosis, moderate. Parenchyma sub pleural fibrosis, fibrosis, moderate, hyaline. Vasculature OK. Airways muscularisation & fibrosis, small airways obliterative, epithelium metaplastic.	
N5	Pleura fibrosis, mild. Parenchyma fibrosis, mild. Vasculature OK. Airways fibrosis, epithelium hypertrophy & basal cell hypertrophy.	
N6	Pleura fibrosis, mild. Parenchyma fibrosis, mild. Vasculature vasculitis. Airways fibrosis, inflammation. Epithelium small airways obliterative, larger airways squamous or pyknotic.	

Table 9.3 Summary of VHL & CA IX staining

AT II=type II cell, AT I=type I cells

Pat	Pathology Code	Airways	Parenchyma	Metaplasia
4 VHL	Airway changes & fibrosis	No airways	AT II, occ AT I +, Few ++ cells	SP mix of ++ to – macrophage-like weaker
CA IX		-	Wash across	-
5 VHL	Airway changes & fibrosis	++	AT II, occ AT I +,	Adv regional ++ to -
CA IX		+ Mucus	Generally low	-
6 VHL	Goblet cell airway changes	+	AT II, occ AT I +,	Adv & fib most ++ few +
CA IX		-	-	-
11VHL	Goblet cell airway changes	+	AT II, occ AT I +,	Focal clusters +
CA IX		-	Fib & vasc +	-
13VHL	Airway changes & fibrosis	+	Lots +++	+++, SP squamous +/-
CA IX		Mucus +, few ++ basal	+ Musc & endo	-
18VHL	Airway changes & fibrosis	+	AT II, occ AT I +,	Adv 70%- rest+, sp few +, - where leaky, hilar ++
CA IX		-	Wash in musc	-
19VHL	Airway changes & fibrosis	Weak,	AT II, occ AT I +,	Adv +, SP +
CA IX		Mucus +	Musc +	-
20VHL	Airway changes & fibrosis	+	AT II, occ AT I +, Patchy, weak at pleura	Alv, SP, hilar mainly-, few +
CA IX		-	-	
21VHL	Goblet cell airway changes	+	AT II, occ AT I ++	Adv mix +/-, SP ++/+
CA IX		-	Strong + musc blocks, fibres in pleura +	-
22VHL	Goblet cell airway changes	+	AT II, occ AT I +,	Hilar, SP few +
CA IX		-	-	-
23VHL	Airway changes & fibrosis	+	AT II, occ AT I +, Weak at pleura	SP +
CA IX		-	Strong near pleura	None observed
24VHL	Airway changes & fibrosis	+	AT II, occ AT I +,	None observed
CA IX		-	-	SP macrophage-like +
25VHL	Airway changes & fibrosis	+	AT II, occ AT I +,	SP, hilar ++ adv patchy
CA IX		-	Weak across section	None observed
26VHL	Airway changes & fibrosis	+	Lots AT II, occ AT I +, weak near pleura	None observed
CA IX		Some +	Vasc some +	None observed
27VHL	Fibrosis	+	AT II, occ AT I +,	SP few +
CA IX		-	Weak across section	None observed
28VHL	Airway changes & fibrosis	Airways +	AT II, occ AT I +,	+/-
CA IX		-	Weak inf, rest of section -	None observed
30VHL	Fibrosis	+	AT II, occ AT I patchy ++to-	++/-
CA IX		-	-	SP +

Pat	Pathology Code	Airways	Parenchyma	
31VHL	Fibrosis	+	AT II, occ AT I +,	SP, hilar, fib +/-
CA IX		-	Patchy, stenosed areas +	Few +
32VHL	Airway & pleural change	+	AT II, occ AT I +, patchy	++/- mix alv +
CA IX		-	Some vasc +	Some SP +
33VHL	Airway changes & fibrosis	+	++ Across AT II, occ AT I, weak at pleura	Adv ++/+, hilar +
CA IX		-	-	SP weak +, fib ++, hilar-
34VHL	Fibrosis	+, Focal ++	AT II, occ AT I ++,	++/+
CA IX		Few + goblet	Vasc +	-
36VHL	Goblet cell airway changes	Focal ++	AT II, occ AT I +,	++/-
CA IX		-	Patchy	None observed
37VHL	Goblet cell airway changes	Mainly ++	AT II, occ AT I ++,	SP, alv ++
CA IX		-	Vasc ++	-
38VHL	Fibrosis	+	AT II, occ AT I +,	SP few +, rest -
CA IX		-	Weak across slide	None observed
41VHL	Airway changes & fibrosis	+	AT II, occ AT I +,	Adv ++, SP+/-
CA IX		-	Vasc +	Adv -, SP some +
46VHL	Airway & parenchymal change	Mix ++to-	AT II, occ AT I +,	Adv +
CA IX		-	Inf, vasc, paren +	-
48VHL	Widespread ongoing damage	++	Lots AT II, occ AT I +,	Adv, hilar, SP, fib +
CA IX		-	Weak across section	None observed
49VHL	Airway & parenchymal change	++	AT II, occ AT I ++,	Alv++/+ Adv, SP, hilar ++
CA IX		-	Weak, strong near Pl	-
N1VHL	Normal	Low	Some AT II, occ AT I +	None observed
CA IX		Some +	Some +	None observed
N2VHL	Normal	++/+	Some AT II, occ AT I +,	None observed
CA IX		-	Patchy strong	None observed
N3VHL	Normal	++	AT II, occ AT I +,	Adv +
CA IX		-	Vasc & macs +	None observed
N4VHL	Normal	+	AT II, occ AT I ++,	None observed
CA IX		-	+ Vasc & macs	None observed
N5VHL	Normal	++	AT II, occ AT I +, patchy	None observed
CA IX		-	Paren & vasc +	None observed
N6VHL	Normal	++	AT II, occ AT I ++/- patchy	None observed
CA IX		-	+ Macs	None observed

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