Chapter 3

Materials and Methods

3.1 Introduction

In preliminary observations we found that the Rbm in smokers is extensively fragmented, which can be a hallmark of EMT, as explained in previous chapters. Following our preliminary observation (Chapter 1), I designed a comprehensive cross-sectional study to test the hypotheses. I investigated current smokers with established COPD (COPD-CS) and also current smokers with normal lung function (NS), comparing them to ex-smokers with COPD (COPD-ES) and normal healthy, never-smoking controls (NC) by immuno-staining airway biopsies for different markers of EMT (S100A4, MMP-9, vimentin, cytokeratin and EGFR). To address the potential for our data to be confounded by infiltrating inflammatory cells, representative sections from the same tissue blocks were stained for neutrophil elastase (neutrophil marker), CD68 (macrophage and mature fibroblast marker) (Beranek, 2005; Inoue, Plieth, Venkov, Xu, & Neilson, 2005), CD4+/CD8+, CD19 as a B-cell marker (Bertrand et al., 1997) and CD11c for potential dendritic cells (K. F. Chung & Adcock, 2008). To confirm the extent of suppressed HDAC2 expression, airway biopsies were immuno-stained for HDAC2. In a longitudinal analysis, I used the material collected in a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months (D. W. Reid et al., 2008), and assessed the effects of ICS on Rbm fragmentation and HDAC2. Materials and methods for these studies are detailed in this chapter.

3.2 Subject recruitment

The study was approved by the Alfred and Royal Hobart Hospital Ethic Committees. All subjects (Table 3) gave written, informed consent prior to participation. Seventeen current smokers with established COPD (COPD-CS), 16 current smokers with normal lung function (NS), 15 ex-smokers with COPD (COPD-ES) and 15 normal healthy, never-smoking controls (NC) were recruited by advertisement in local newspapers and placement of posters in clinic waiting areas in the hospital, as
well as on the notice boards of social and Veterans clubs. Potential participants were interviewed and examined by a respiratory physician clinically, physiologically and ultimately pathologically.

Table 3: Demographic and lung function data for subjects.

<table>
<thead>
<tr>
<th>Groups (numbers)</th>
<th>COPD-CS (n=17)</th>
<th>COPD-ES (n=15)</th>
<th>NS (n=16)</th>
<th>NC (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GOLD I/GOLD II †</td>
<td>10/7</td>
<td>8/7</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Male/female</td>
<td>9/8</td>
<td>9/6</td>
<td>12/4</td>
<td>7/8</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61 (46-78)</td>
<td>62 (53-69)</td>
<td>50 (30-66)</td>
<td>44 (20-68)</td>
</tr>
<tr>
<td></td>
<td>(p=0.001) †</td>
<td>(p=0.001) †</td>
<td>(p=0.31)</td>
<td></td>
</tr>
<tr>
<td>Smoking (pack years)</td>
<td>45 (18-78)</td>
<td>51 (18-150)</td>
<td>32 (10-57)</td>
<td>0</td>
</tr>
<tr>
<td>FEV1% predicted (Post BD)*</td>
<td>83 (66-102) (p&lt;0.001) †</td>
<td>83 (54-104) (p&lt;0.001) †</td>
<td>99 (78-125) (p=0.01) †</td>
<td>113 (86-140)</td>
</tr>
<tr>
<td>FEV1/FVC % (Post BD)*</td>
<td>59 (46-68) (p&lt;0.001) †</td>
<td>57 (38-68) (p&lt;0.001) †</td>
<td>77 (70-96) (p=0.22)</td>
<td>82 (71-88)</td>
</tr>
</tbody>
</table>

Data expressed as median and range

†Significance difference from NC

* Post BD values after 400µg of salbutamol

† Diagnosis of COPD was made according to GOLD guidelines (GOLD, 2007)

3.2.1 Exclusion criteria

1. Subjects with a history suggestive of asthma, which includes symptoms in childhood, related atopic disorders, eczema or hay fever, substantial day-to-day variability or prominent nocturnal symptoms, or a history of wheeze rather than progressive breathlessness and any who had previously used ICS (oral or inhaled) were excluded.

2. Significant uncontrolled comorbidities such as diabetes, angina or cardiac failure, and other coexisting respiratory disorders including pulmonary fibrosis, lung cancer and bronchiectasis.
3. Subjects with inability to give written informed consent were also excluded.

3.2.2 Inclusion criteria

1. Current-smokers with COPD aged at least 40 years with smoking history equal to or more than 15 pack-years and subsequently obtained BAL fluid had to be free of bacterial colonisation; FEV1 40% to 80% predicted, with FER (ratio of FEV1 to FVC) $\leq 70\%$ post bronchodilator with definite scalloping out of the descending limb of flow-volume loop on spirometry. COPD ex-smokers with at least six months of smoking cessation were included.

2. Normal healthy never smoking controls and current smokers with normal lung function recruited also underwent bronchoscopic examination and physiological evaluation. They were at least 18 years old and FEV1/FVC ratio of 70% or higher and FEV1% predicted of 80% or higher.

3. None of the never-smoking controls individuals had any history of respiratory illness or smoking. For normal lung function current smokers the inclusion criteria were; a minimum 10 pack-year history of cigarette smoking with spirometry within normal limits (FEV1 (forced expiratory volume) >80% of predicted, and FEV1/FVC (forced vital capacity) >70%) and no scalloping out of the expiratory descending limb of the flow-volume curve, suggesting small airway dysfunction.

3.3 Bronchoscopy

Fiberoptic bronchoscopy was performed, subjects were pre-medicated with nebulized salbutamol (5 mg) approximately 15–30 min before the bronchoscopic examination and sedation was achieved with intravenous administration of midazolam (3–10 mg) and fentanyl (25–100 µg) to COPD current smokers and ex-smokers and normal healthy non-smokers and smokers with normal lung function. Lignocaine (4%) was applied topically to the nose, pharynx and larynx, and 2 % lignocaine was administered below the cords in 2 ml aliquots as required up to a maximum dose of 6ml. Subjects were monitored by pulse oximetry and administered oxygen during the
procedure. Endobronchial biopsies were taken from subsegmental carinae of the right lower pulmonary lobe of each patient, using alligator forceps (FB-15C; Olympus, Tokyo, Japan). At each fiberoptic bronchoscopy performed, up to eight good biopsy specimens were obtained.

### 3.4 Processing of biopsies

Two biopsies were immersed with ornithine carbamyl transferase (OCT) and snap frozen in liquid nitrogen-chilled isopentane slurry, and stored at -80°C for immunohistochemistry and two more were snap frozen in liquid nitrogen for molecular studies (not presented here). The other four were fixed in 10% neutral buffered formalin for two hours, and then transferred into 50% ethanol until they were processed using a Leica ASP 200 tissue processor. Fixed tissue blocks were progressively dehydrated in graded ethanol followed by clearing in xylene before being infiltrated with wax under vacuum pressure. Two 3 µm paraffin embedded sections, separated by at least 50 microns, were cut for staining and mounted on a slide (Figure 3.1).

### 3.5 Immunostaining with staining antibodies used

Sections were stained with the following monoclonal antibodies: anti-MMP-9 (R&D systems, cat no. MAB911, Gymea, NSW, Australia), anti-EGFR (Chemicon, cat no. CBL417, Billerica, United States), anti-S100A4 polyclonal antibody (Dako, cat no. A5114, Glostrup, Denmark), anti-Cd11c monoclonal antibody (Abcam, cat no. ab52632, Waterlo, NSW, Australia), anti-CD4/CD8 monoclonal antibodies (Novocastra, cat no. NCL-CD4-IF6 / cat no. NCL-CD8-4B11, Newcastle Upon Tyne, UK) were used for CD4/CD8 T lymphocytes, anti-CD68 monoclonal antibody for macrophages (Dako, cat no. M0814, Glostrup, Denmark) and mature fibroblasts, anti-CD19 monoclonal antibody for B-cells (Abcam, cat no. Ab31947, Waterloo, NSW, Australia), neutrophil elastase, a monoclonal antibody for neutrophils (Dako, cat no. Ab31947, Glostrup, Denmark) and for HDAC2, sections were stained with monoclonal antibody: anti-HDAC2 (Abcam cat no.ab12169, Waterloo, NSW, Australia), together with a horseradish peroxidase (HRP) conjugated DAKO Envision plus reagent for secondary antibody binding and colour resolution using diaminobenzidine (DAB). Double staining with vimentin and cytokeratin was
elaborated using an anti-vimentin monoclonal antibody (Dakocytomation, cat no. M7020 clone 3B4 at 1:1000 for two hours, Glostrup, Denmark) and a pan-cytokeratin monoclonal antibody for cytokeratin (cytokeratin AE1/AE3 at 1/40 in diluent Abcam ab 27988, Waterloo, NSW, Australia) and then secondarily bound using Dakocytomation “Real” (cat no K5005) alkaline phosphatase reagents and visualised using Dakocytomation liquid permanent red (Cat no. K0640, Glostrup, Denmark). The primary antibody was replaced using a species appropriate IgG1 at equivalent dilutions and conditions on a sequential section as negative controls. Details of general immunostaining procedure for different tissue markers used are given below. All tissues were stained with Haematoxylin before immunostaining. All monoclonal and polyclonal antibodies were titrated to maximal sensitivity and to minimise non-specific staining using standard methods (Immunocytochemistry solutions and Histo-techniques).

3.5.1 Measurements and biopsy analysis

Computer-assisted image analysis was performed with a Leica DM 2500 microscope (Leica Microsystems, Germany), Spot insight 12 digital camera and Image Pro V5.1 (Media Cybernetics, USA) software (Figure 3.2). On each slide two biopsy sections were fixed (Figure 3.1). Firstly, as many pictures as possible were taken of the tissue from the area of interest (for this study it was mainly epithelium, Rbm and lamina propria) avoiding overlapping of tissue. All image analyses were done using the above described image analyser. Five randomly selected images from the total number taken were used for desired measurements. Cellular and other changes, e.g. clefts/cracks in the Rbm, (Figure 3.3) were normalised for purposes of comparisons in terms of the total length of basement membrane assessed. The length of the individual “clefts” was measured, summed and results expressed as percentage of the total Rbm length to give a comparable measure of the degree of Rbm fragmentation for each individual. EGFR was measured as percentage of epithelium stained for EGFR over total basement membrane length. Using the image analyser HDAC2 positive and total number of cells were counted up to 50µ deep into the lamina propria and results presented as cells per sqmm of lamina propria and in the epithelium HDAC2 was measured as percentage of epithelium stained for HDAC2 over total basement membrane length. All slides were coded and randomised by an
independent person and then counted in a single batch by me blinded to subject and
diagnosis, with quality assurance on randomly selected slides provided by a
professional academic pathologist (Professor H.K. Muller).

3.5.2 Statistical analyses
Results are presented as medians and ranges. A non-parametric ANOVA (Kruskal
Wallis Test) was undertaken, and specific group differences then explored using the
Mann Whitney U test. Associations between variables were assessed using
Spearman's rank test. Statistical analyses were performed using SPSS 15.0 for
Windows, 2003, with a two-tailed $P$-value $\leq 0.05$ being considered statistically
significant. Wilcoxon two related-samples test was used to test the effect of ICS and
placebo in the longitudinal study. Associations between variables were assessed
using Spearman's rank test. Linear regression analysis for potential confounders was
undertaken and differences between groups in gender balance, age, and atopy were
found to be non-contributory.
Figure 3.1: Showing two biopsy sections fixed on a slide with black circles around them.
Figure 3.2: Computer-assisted image analyser (Leica DM 2500 LB microscope, Leica Microsystems, Germany; Dage-MTI DC200 one-chip video camera; Image Pro V4.5, media Cybernetics, USA software)
Figure 3.3: Bronchial biopsy specimen from a COPD current smoker, black arrows showing Rbm fragmentation with many “clefts” evident which frequently contain cells. Stain: haematoxylin and eosin (H & E). Original magnification, x630
3.5.3 MMP-9 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Slides were sorted out and had positive tissue control and a negative reagent control for each section being stained.
5. Circles were made at the back of the slide around the sections with xylene resistant pen and labelled with date of staining.
6. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
7. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
8. Sections were rinsed well in running tap water (2 minutes).
9. Then placed in 3% H2O2 in distilled water for 15 minutes.
10. Washed in running water for 2 minutes.
11. Placed in 500ml of dist water in bottom of pressure cooker.
12. Added 500ml of Dako S1700 solution from the fridge, in plastic incubation vessel (or diluted to concentration 1 in 10 and reused).
13. Slides were placed in S1700 solution and then heated in pressure cooker for 6 minutes on high then 14 minutes on about 6.5 to maintain steam.
14. Allowed pressure cooker to cool and then removed the lid and placed vessel in the running water until the solution had reached 35 degrees C. Then placed sections in running water for 1 minute.
15. Covered slides with PBS pH 7.4.
16. Applied primary antibody to sections for appropriate time at appropriate concentration and temperature (1:50 for 2 hours). Also applied negative control sera (normal sera from same species as primary antibody, mouse IgG1) to control section and if possible used reagent control antibody that is
well demonstrated in the tissue under investigation. Made primary antibody using Dako diluent.

17. Washed sections well using PBS 3 x changes 5 mins each change.
18. Applied DAKO Envision + (Dako k4001) reagent to sections for 30 mins.
19. Washed sections well using PBS 3 x changes 5 mins each change.
20. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
21. Applied DAB to sections for 10 mins.
22. Washed sections well using PBS 5 mins.
23. Sections were rinsed in running water.
24. Sections were placed in Mayer’s haematoxylin to elaborate nuclei for 1 min.
25. Then rinsed in running water.
26. Placed in approx 400ml of water with 6-8 drops of ammonia 30 seconds.
27. Rinsed well in running water.
28. Then Dehydrated sections in clean fresh 95% ethanol, and then changed twice with 100% ethanol (2 minutes each change) (made sure that 95% is not pink, if so, it was changed).
29. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
30. Then finally coversliped using machine with 54 mm coverslips.
31. Dried on hotplate overnight.
3.5.4 EGFR immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Then placed in 3% H2O2 in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied primary antibody to sections (chemicon CBL417 clone F4) 1/40 hr rt made up using diluent with 3% BSA. Also applied negative control sera (normal sera from same species as primary antibody) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied DAKO Envision + (Dakok4001) reagent to sections for 30 mins.
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 mins.
16. Washed sections well using PBS 5 mins.
17. Rinsed sections in running water.
18. Sections were placed in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiated with three dips in 1% acid alcohol).
19. Rinsed in running water again.
20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
21. Rinsed well in running water.
22. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and sections were mounted in Depex (or similar).
24. Then finally coversliped using machine with 54 mm coverslips.
25. Dried on hotplate overnight.
3.5.5 S100A4 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Then placed in 3% H2O2 in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Slides were placed in pressure cooker 1 for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of s1700 dako buffer solution with 500mls of distilled water in pressure cooker.
10. Washed in running tap water for 2 minutes.
11. Covered slides with PBS pH 7.4 for 1 minute.
12. Applied primary antibody to sections for appropriate time (90 mins rt) at appropriate concentration and temperature. Also applied negative control sera (normal sera from same species as primary antibody) to control section and if possible used reagent controlled antibody that was well demonstrated in the tissue under investigation, (S100 A4 (Dako polyclonal antibody code A 5114 protein concentration 0.72g/l) at dilution of 1/2500 (2.88x10^-4 g/l) made from stock of 1 in 100 diluted in dako diluent and normal rabbit immunoglobulin as negative control at dilution of 1/70000(2.85 x 10^-4 g/l) (from stock Dako X0903 protein concentration 20g/l).
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Applied DAKO Envision + (rabbit polyclonal) (Dako K4003) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change.
16. Made DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.

17. Applied DAB to sections for 10 mins.

18. Washed sections well using PBS 5 mins.

19. Rinsed sections in running water.

20. Sections were placed in Mayer’s haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiated with three dips in 1% acid alcohol).

21. Rinsed in running water.

22. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.

23. Rinsed well in running water.

24. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).

25. Cleared in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar).

26. Then finally coversliped using machine with 54 mm coverslips.

27. Dried on hotplate overnight.
3.5.6 Vimentin immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Slides were placed in pressure cooker1, for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of Citrate Buffer pH6 solution with 500mls of distilled water in pressure cooker.
8. Washed in running tap water for 2 minutes.
9. Applied Dako monoclonal anti-vimentin antibody at 1/1000 for 2 hours at room temp (diluted in Dako diluent).
10. Washed three times with tris buffer for three minutes each.
11. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20mins at room temperature (RT).
12. Washed three times with tris buffer for three minutes each.
13. Incubated in REAL streptavidin (also known as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate for twenty minutes at RT.
14. Washed three times with tris buffer for three minutes each change.
15. Made up chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubate sections for ten minutes.
16. Washed in running tap water for 2 minutes.
17. Sections were placed in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiated with three dips in 1% acid alcohol).
18. Rinsed in running water.
19. Placed in approx 400ml of water with 6-8 drops of ammonia for 30 secs.
20. Rinsed well in running water.
22. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
23. Then finally coversliped using machine with 54 mm coverslips.
24. Dried on hotplate overnight.
3.5.7 S100A4 and vimentin double-immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Slides were placed in pressure cooker1, for 6 minutes on high then 15 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
8. Washed in running tap water for 2 minutes.
9. Applied Dako monoclonal anti-vimentin M7020 clone 3B4 antibody at 1/1000 for 2 hours at room temp (diluted in Dako diluent).
10. Washed three times with tris buffer for three minutes each.
11. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20 minutes RT.
12. Washed three times with tris buffer for three minutes each.
13. Incubated in REAL streptavidin (also know as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate twenty minutes room temperature.
14. Washed three times with tris buffer for three minutes each change.
15. Made chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubated sections for ten minutes.
16. Washed in running tap water for 2 minutes.
17. Placed in 3% H2O2 in distilled water for 15 minutes.
18. Washed in running tap water 2 minutes.
19. Applied primary antibody to sections for appropriate time (60 mins RT) at appropriate concentration and temperature. Also applied negative control sera
(normal sera from same species as primary antibody) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation. Use S100 A4 (Dako polyclonal antibody code A 5114 protein concentration 0.72g/l) at dilution of 1/3000 (2.4x10^-4 g/l) made from stock of 1 in 100 diluted in dako diluent. Used normal rabbit immunoglobulin as negative control at dilution of 1/70000 (2.9 x 10^-4 g/l) (from stock Dako X0903 protein concentration 20g/l).

20. Washed sections well using PBS pH 7.4 3 x changes 5 mins each change.
21. Applied DAKO Envision + (rabbit polyclonal) (Dako K4003) reagent to sections for 30 mins.
22. Washed sections well using PBS 3 x changes 5 mins each change.
23. Made up DAKO DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
25. Rinsed sections in running water.
26. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiate with three dips in 1% acid alcohol).
27. Rinsed in running water.
28. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
29. Rinsed well in running water.
30. Dehydrated on hotplate.
31. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
32. Then finally coversliped using machine with 54 mm coverslips.
33. Dried on hotplate overnight.
3.5.8 Vimentin and cytokeratin double-immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Placed in 3% H2O2 in distilled water for 15 minutes.
8. Washed in running tap water 2 minutes.
9. Slides were placed in pressure cooker1, for 6 minutes on high then 6 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
10. Allowed to cool to below 35 degrees C before washing in running tap water 2 minutes.
11. Washed sections well using PBS pH 7.4.
12. Applied Dako monoclonal anti-vimentin M7020 clone 3B4 antibody at 1/500 for 2 hours 20 mins at room temp (diluted in Dako diluent).
13. Washed sections well using PBS pH 7.4 3x 3mins each change.
14. Applied Dako Envision + (monoclonal) (Dako K4001) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change.
16. Made Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
17. Applied DAB to sections for 10 mins.
18. Rinsed sections in running water.
19. Placed sections in Harris’s haematoxylin to elaborate nuclei, 5 mins then differentiated with three dips in 1% acid alcohol.
20. Rinsed in running water.
21. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
22. Rinsed well in running water and check nuclei (adjusted further if necessary).
23. Washed with tris buffer for three minutes.
24. Applied primary antibody (cytokeratin AE1/AE3 at 1/40in diluent Abcam ab 27988) to sections for appropriate time (2hrs RT) at appropriate concentration and temperature. Also applied negative control sera (normal sera from same species as primary antibody) to control sections and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
25. Washed three times with tris buffer for three minutes each.
26. Covered all sections in link reagent (Dako REAL K5355 or K5005), for 20mins RT.
27. Washed three times with tris buffer for three minutes each change.
28. Incubated in REAL streptavidin (also know as AP enzyme enhancer) (Dako K5355) with ALK phos conjugate twenty minutes at RT.
29. Washed three times with tris buffer for three minutes each change.
30. Made up Chromogen by adding 20 microlitres of chromogen to each ml of substrate and then incubated sections for ten minutes.
31. Washed in running tap water 2 minutes.
32. Rinsed in distilled water.
33. Dehydrated on hotplate.
34. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
35. Then finally coversliped using machine with 54 mm coverslips.
36. Dried on hotplate overnight.
3.5.9 CD11c immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Sections were rinsed well in running tap water (2 minutes).
7. Placed in 3% H2O2 in distilled water for 15 minutes.
8. Washed in running tap water 2 minutes.
9. Slides were placed in pressure cooker1, for 6 minutes on high then 6 minutes at about 6.5 on dial till just expelling steam using: 500ml of citrate buffer pH6 solution with 500mls of distilled water in pressure cooker.
10. Allowed to cool to below 35 degrees C before washing in running tap water 2 minutes.
11. Washed sections well using PBS pH 7.4.
12. Applied CD11c Abcam ab52632 clone ep1347Y for 2 hours mins at room temp (dilate in dako diluent) and rabbit IgG1 at 1/20,000 for two hours at RT on serial sections.
13. Washed sections well using PBS pH 7.4 3x 3mins each change
14. Applied Dako Envision + (anti rabbit) (Dako K4003) reagent to sections for 30 mins.
15. Washed sections well using PBS 3 x changes 5 mins each change
16. Made up Invitrogen DAB plus solution using 20 micro litres of DAB to 1 ml of substrate buffer.
17. Apply DAB to sections for 10 mins.
18. Rinsed sections in running water
19. Placed sections in mayers haematoxylin to elaborate nuclei, 2 mins.
20. Rinsed in running water.
21. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
22. Rinsed well in running water and check nuclei (adjusted further if necessary).
23. Washed in running tap water 2 minutes.
24. Rinsed in distilled water.
25. Dehydrated in 95% then 2 x 100% ethanol for 1 minute each change.
26. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
27. Then finally coversliped using machine with 54 mm coverslips.
3.5.10 CD4/CD8 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. Dried overnight at 37 degrees.
4. Dried on hotplate for a further hour at 56 degrees just prior to sectioning.
5. Dewaxed sections in “xylene” 2 x 5 mins each in fume hood.
6. Hydrated to water using 100% ethanol followed by 95% then 70%, 3 mins each change.
7. Rinsed sections well in running tap water (2 mins).
8. Placed in 0.5% H2O2 in water for 15 minutes. (5 ml of 30% H2O2 in 295ml of Water).
9. This was an important step since the antigens are destroyed if harsh peroxidase treatment was used. Use only 0.5% in water.
10. Washed in running water 2 minutes.
11. Added 500ml 1mm EDTA pH 8.00 in round plastic medium staining jar and placed in plastic autoclave container that has 250ml of distilled water in it (0.186g of EDTA in 400ml of distilled water, adjust pH to 8 with 1 molar sodium hydroxide and then make up to 500ml with distilled water).
12. Heated solutions without slides in microwave in autoclave (with lid in open position but rubber bung in place) for 10minutes on high.
13. Heated for 3 minutes on high in microwave with autoclave in closed position and yellow button pressed down.
14. Removed autoclave from microwave and cool rapidly in running water until yellow button drops (5 mins, care was required here, gloves or cloth was used to remove autoclave and do not open until temperature returns to RT).
15. Removed plastic staining jar from autoclave and further cool sections in water bath until temperature reached about 35 degrees C.
16. Placed slides in PBS pH 7.4, 2 minutes.
17. Applied primary antibody to sections for 2hr RT diluted to appropriate
dilution in Dako antibody Diluent. (1:15) Also applied negative control sera
(normal sera from same species as primary antibody) to control sections and
if possible used reagent control antibody that is well demonstrated in the
tissue under investigation.
18. Washed sections well using PBS 3 x changes 5 mins each change.
19. Applied Dako Envision + (Dako 003355) reagent to sections for 30 mins.
20. Washed sections well using PBS 3 x changes 5 mins each change.
21. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1
   ml of substrate buffer.
22. Applied DAB to sections for 10 mins.
23. Washed sections well using PBS 5 mins.
24. Rinsed sections in running water.
25. Placed sections in Mayers haematoxylin to elaborate nuclei, 1 min (or in
   Harris’s 2 mins then differentiate with three dips in 1% acid alcohol).
26. Rinsed in running water.
27. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
28. Rinsed well in running water.
29. Dehydrated in 95% ethanol, then twice in 100% ethanol (2 minutes each
   change)
30. Cleared in three changes of xylene (2 minutes each change) and mounted
   sections in Depex (or similar).
31. Dried on hotplate overnight.
3.5.11 Neutrophil elastase immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H$_2$O$_2$ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied Neutrophil elastase (Dako M 0752) 1/500 1hr RT (no protease).
    Also applied negative control sera (normal sera from same species as primary antibody) to control section at similar concentration.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied Dako Envision + (Dako k4001) reagent to sections for 30 mins.
13. Washed sections well using PBS 3 x changes 5 mins each change.
14. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 mins.
16. Washed sections well using PBS 5 mins.
17. Rinsed sections in running water.
18. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiate with three dips in 1% acid alcohol).
19. Rinsed in running water.
20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
21. Rinsed well in running water.
22. Dehydrated in 95% ethanol then changed twice with 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
24. Dried on hotplate overnight.
3.5.12 CD19 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. The slides were dried for 2 hours in slide drying oven.
4. Sections were dewaxed in “xylene” 2 x 5 mins each in fume hood.
5. Then hydrated to water using 100% ethanol followed by 95% then 70%, 3 minutes each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H\textsubscript{2}O\textsubscript{2} in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Applied primary antibody to sections for 2hours at 1/8000 dilution at room temperature. Also applied negative control sera (normal mouse sera at 1/5000) to control section and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
11. Washed sections well using PBS 3 x changes 5 mins each change.
12. Applied Dako Envision + (Dako k4001 or k4003) reagent to sections for 30 minutes.
13. Washed sections well using PBS 3 x changes 5 minutes each change.
14. Made up Invitrogen DAB solution (cat no 882014) using 20 micro litres of DAB to 1 ml of substrate buffer.
15. Applied DAB to sections for 10 minutes.
16. Washed sections well using PBS 5 minutes.
17. Rinsed sections in running water.
18. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 minutes
19. Rinsed in running water.
20. Placed in approx 400ml of water with 6-8 drops of ammonia 30 seconds.
21. Rinsed well in running water.
22. Dehydrated in 95% ethanol, then two changes of 100% ethanol (2 minutes each change).
23. Cleared in three changes of xylene (2 minutes each change) and mounted sections in Depex (or similar).
24. Dried on hotplate overnight.
3.5.13 CD68 immunostaining procedure

1. Paraffin blocks were sectioned at 3 microns after cooling in a -20 freezer or on ice blocks for 5-10 minutes.
2. Two sections separated by 40-50 microns (approximately 10 sections) were placed on each slide on 3 Aminopropyl triethoxy-silane APTS (Sigma A-3648) coated slides (in milli Q water bath with one drop of Tween 20 (ICN 103168) in water bath at approx 50 degrees centigrade).
3. Dried on hotplate at 56 degrees one hour and overnight at 37 degrees.
4. Dewaxed sections in “xylene” 2 x 5 mins each in fume hood.
5. Hydrated to water using 100% ethanol followed by 95% then 70%, 3 mins each change.
6. Rinsed sections well in running tap water (2 mins).
7. Placed in 3% H₂O₂ in distilled water for 15 minutes.
8. Washed in running water 2 minutes.
9. Rinsed in working phosphate buffered saline (diluted from Tubbs stock buffer 1 in 25) 2 mins.
10. Pre-incubated them in PBS at 37 degrees in water bath for 5 mins.
11. Incubated in 0.025% protease type VIII (Sigma P5380) in PBS for three minutes at 37 degrees in water bath.
12. Washed in running tap water 5 minutes.
13. Covered slides with PBS pH 7.4.
14. Applied primary antibody to sections for 30 mins at room temp diluted to 1/500 in Dako antibody diluent. Also applied negative control sera (normal sera from same species as primary antibody) to control sections and if possible used reagent control antibody which was well demonstrated in the tissue under investigation.
15. Washed sections well using PBS 3 x changes 5 mins each change.
16. Applied DAKO Envision + (Dako k4001) reagent to sections for 30 mins.
17. Washed sections well using PBS 3 x changes 5 mins each change.
18. Made up Dako DAB plus solution (K3468) using 20 micro litres of DAB to 1 ml of substrate buffer.
19. Applied DAB to sections for 10 mins.
20. Washed sections well using PBS 5 mins.
21. Rinsed sections in running water.
22. Placed sections in Mayers haematoxylin to elaborate nuclei, 2 mins (or in Harris’s 2 mins then differentiate with three dips in 1% acid alcohol).
23. Rinsed in running water.
24. Placed in approx 400ml of water with 6-8 drops of ammonia 30 secs.
25. Rinsed well in running water.
26. Dehydrated in 95% ethanol then changed twice in 100% ethanol (2 minutes each change).
27. Cleared in three changes of xylene (2 minutes each change) and mount sections in Depex (or similar).
3.5.14 General procedure for Haematoxylin and Eosin (H&E) stain

1. Paraffin blocks were sectioned at 5 microns and were picked on APTS coated slides.
2. Dried overnight at 37 degrees or on hotplate at 56 degrees two hours.
3. Dewaxed sections in fresh “xylene substitute (Shandon- kept in flame cupboard)” 2 x 5 mins each in fume hood.
4. Hydrated to water using 100% ethanol followed by 95% then 70%, 2 mins each change.
5. Rinsed sections well in running tap water (2 minutes).
6. Placed sections in Mayer’s haematoxylin to elaborate nuclei, 5 minutes.
7. Rinsed in running water.
8. Placed in approx 400ml of water with 8 drops of ammonia 30 seconds.
9. Rinsed well in running water.
10. Checked nuclei, if they are sufficiently stained using microscope.
11. Placed in eosin solution 2 minutes.
12. Rinsed quickly in running water to remove excess eosin and place into 95% ethanol 30 seconds with agitation.
13. Further dehydrated with 3 changes of 100% ethanol 1 min each change.
14. Cleared sections using 3 changes of fresh substitute xylene 2 minutes each change.
15. Placed sections in Xylene for 1 min prior to coverslapping with xylene based mountant (Depex or similar).
16. Dried on hotplate overnight.