Response to examiners

Response to examiner 1

List of figures and tables are missing.
I agree with the examiner’s comment and a list of figures and tables is now provided in the thesis.

Chapter 1
Primary comment
The link between COPD and lung cancer should be discussed, given the known correlation between TGF-induced EMT and cancer metastasis and the susceptibility of COPD subjects to lung cancer. Were all of the patients in the preliminary study cancer-free?
All patients were cancer free clinically, radiologically and bronchoscopically at the time of the studies, and we are not aware of any subsequent change in status. I acknowledge the examiner’s comment that there was little discussion of the potential link between COPD and lung cancer, in an attempt to avoid being over-speculative. But it is a reasonable point, and I have included some discussion on Chapter 1 page 9 and 10.

Minor comments
Re EMT in lung transplantation and investigation of EMT markers on epithelial cells: Borthwick is quoted but the previous reports by Ward 2005 and Hodge 2009 should also be referenced here.
In the introduction from Chapter 1, Ward 2005 was quoted as a reference 5 times and in the discussion 4 times. I agree with the examiner’s comment that reference from Hodge 2009 was not quoted because the study reported by Hodge and et al was mainly an in-vitro cell culture study, but was quoted in Chapter 2 (literature review), pages 77 and 78 of the thesis.

Chapter 2
Primary comments
Given the important role played by HDAC-I in TGF induced EMT (Lei rnt J Biochem Cell BioI 2010) and applicant's focus on EMT in COPD; it is puzzling why HDAC-I was not also investigated.
I agree with the examiner’s comment that HDAC1 may play an important role in TGF-induced EMT, but the aim of the study was to first confirm EMT in COPD (as this is the first study in the literature reporting active EMT in COPD), which in itself was a very comprehensive study. Now that we have confirmed that EMT is active in COPD, the next aim of this programme will be to investigate the driving mechanisms behind EMT in COPD and HDAC1 would be a very reasonable target. We already have NHMRC grants pending to take this work further. I have not added any
comment on HDAC1 to the thesis, as it is not immediately relevant to my work described in the thesis.

**P62. Figure 2.19. This is of very poor quality in my version and should be re-done.**
I thank the examiner for this and the Figure 2.19 is now re-done as suggested (Chapter I page 63).

**Minor comments**
**Page 17. What are figures in A$.**
Financial figures are now given in A$ on page 18.

**Chapter 3**
**Primary comments**

**3.1 Introduction line 15. Was this really a longitudinal study (that involves repeated observations of the same items over long periods of time)? Or a double-blind, placebo controlled randomised trial?**
It was indeed a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg/twice daily) with placebo for 6 months as explained on page number 101. It was also, of course ‘longitudinal’, but I take the point being made and have altered the title of the chapter accordingly (page number 177).

**I assume that the pharmaceutical company supported this study? Was their approval necessary or obtained?**
The original basic clinical and bronchoscopic study was supported by GSK. It covered the first phase of analysis which involving clinical and inflammatory outcomes. This work has been published (Reid et al., 2008), and formed the core of the thesis presented successfully in 2006 by Dr. Yudong Wen titled “smoking-related airway inflammation and corticosteroid responsiveness in smoking related COPD”. That funding ceased in 2004, but was a completely untied grant. Although it was always intended to go on and study airway remodelling in the context of the biopsy samples obtained, the details of this only recently emerged with my pilot work and were not at all under the direct auspices of GSK. No permission for the analysis was needed or sought from them.

**2.3.7 were the Mabs titrated for optimal staining?**
Yes, monoclonal antibodies were titrated for optimal staining. Details are given in Chapter 3, pages 104 and 105.

**3.3 The use of lignocaine should be discussed given its negative effects on epithelial cell viability (Kelsen SO Am J Respir Cell Mol Biol. 1992 (1):66). This is potentially increased in COPD where there is fragility of airway surface epithelium (Jeffery PK 2001 Novartis Found Symp. 2001; 234).**
This may be a danger for some confounding but really this would only be the case with living cells e.g. BAL samples, rather than with fixed tissue. Our group some years ago studied this phenomenon in detail (Duddridge, Kelly, Ward, Hendrick, & Walters, 1990). It is extremely unlikely that the changes we have seen are related to lignocaine, both on the basis of this work, but also because the control subjects were
treated in a exactly same way as those with COPD and, as explained in the thesis (Chapter 1), we have never found such changes in the Rbm in asthma subjects studied over the years, although again treated in the same way.

A demographic table for the subjects used in the main study is required here. In the demographic table provided for the PILOT study, there was a large difference in median ages for controls vs COPD patients: 47 vs 61. Even more concerning were the inclusion of very young subjects in the control group for the PILOT study (age 20). Can you be sure that there were no effects of increasing age on your findings?

A demographic table for the subjects is now provided in Chapter 3, page 103 as suggested by the examiner. I acknowledge the examiner’s comment that some of the controls subjects were of relatively young age for a study of COPD, but as explained in the thesis (pages 106 and 135) linear regression analysis was undertaken and age was not related to the findings we observed in the COPD or smoking populations.

3.2.2 Do you consider 6 months cessation of smoking to be enough for reversal of the Rbm changes?
The inclusion criteria stipulated that COPD ex-smokers had to have at least six months of smoking cessation, but this really was a `theoretical` minimum and as a group they had considerably more time from quitting than this (usually years). In the event, we found that S100A4 positive cells in the Rbm were decreased in these COPD ex-smokers and there was no indication that this was related to time since smoking cessation. In the near future I/we plan to analyse a specific smoking cessation study for which we have serial biopsies from 15 individuals before and after they had ceased smoking for 3 months and 12 months. This is supported by our current NHMRC grant and should give some indication to the time scale of these changes.

3.5 Presentation of the immunostaining techniques pp 109-131 is tedious and could be condensed. Details of manufacturers: city/state or city/state/country are missing or incomplete.
Considering the complex nature of the processes being investigated for the first time in COPD, details of the stains done seemed to me very important, especially if anyone else wishes to replicate the work. I would prefer for this reason to leave the section unchanged. As requested, I have updated the manufactures’ details and sources of the antibodies in the thesis (Chapter 3, pages 104 and 105).

Chapter 4
Primary comments
The inclusion criteria states 'over 18' in the controls and '40-70 years' for the COPD group (described in 3.2.2) compared to COPD subjects aged '40-70y'. However, in 4.3 'Results', COPD subjects of 78 years of age are included. These subjects should be removed and stats redone.
I thank the examiner for picking up this inconsistency, which reflects a change in recruitment policy after the original protocol was designed; and inadvertently it was this I was quoting. I should have quoted the operative inclusion criterion for age, which was for COPD subjects over 40 years old but with no upper limit as long as clinically fit enough for the procedures (page 103). This is now altered in the thesis.
Chapter 5
Primary comments

Re low numbers of-cells in biopsies. There appears to be surprisingly few T-cells in the biopsies. Do you think this could be related to severity of the COPD subjects as reported by Di Stefano 2001; 31:893? The data referred to are part of a comparison of cells positive for S100A4 in the basal epithelium and in the Rbm versus T-cells and other immune cells within these structures. This was specifically done to show that cells in the basal epithelium and Rbm are of epithelial origin and that the data are not confounded by infiltrating inflammatory and immune cells. One would not expect to see many such cells normally in these areas.

Chapter 6
Primary comments

Page 168 "it is likely that in COPD the epithelium may get damaged by cigarette smoke". There have been several studies that have very convincingly shown that cigarette smoke does damage the epithelium and/or cause remodelling: these should be discussed (e.g., Pera 2010, Zhao 2009). The specific reference has been added to the introduction mentioned on page 169; further details on epithelial damage and airway remodelling are given in detail in Chapter 2.

6.5 Discussion, last para. How exactly will you do a comprehensive longitudinal follow up study?" Won't the invasive nature of the airway biopsy procedure preclude repeated observations over long periods of time? As already discussed, in the near future we plan to analyse a specific smoking cessation study for which we already have serial biopsies collected.

What are the potential mechanisms for the effects of ICS on Rbm fragmentation- direct effect or via effects on inflammatory mediators or injury? How will this be assessed in future studies? The exact mechanisms for how ICS work in COPD are not very clear, but potential molecular mechanisms involved are described in the Chapter 2. It is very hard to say anything about the exact mechanisms involved in normalising the Rbm at this stage as the findings on Rbm fragmentation are so new. However, we already have plans, and a funding grant submitted, to investigate the role of RAGE (Receptor for advanced glycation end-products) as a driver of these ‘amplification’ remodelling changes in the context of chronic airway inflammation in COPD. We will then be able to see if this is the level of control. This itself may be under the influence of VEGF or TGF-β, and we wish to dissect these factors out from each other using the intervention studies we have done.

Chapter 7.
Primary comments

P 177 it is rather harsh to claim that the methodology used in the Barnes study were "rather questionable". Rather they were not comprehensive enough to identify the changes in total cellularity in the lamina propria. The examiner’s phraseology is probably more polite and reasonable, and I have changed the use of words in Chapter 7, page 178.
Minor comment

7.3.5 How was normality assessed?

Normality was tested using the Kolmogorov Smirnov & Shapiro Wilk test (Chapter 7 page 180).

Discussion

Primary comments

PI99 Para 2. How do you anticipate doing the “long term smoking cessation” study? Would a murine smoking model be appropriate?

As explained above, we already have serial biopsies from a smoking cessation study involving 15 individuals over 12 months. Since we have human tissue available, we do not need to revert to animal models.

In future studies, a discussion of potential future studies investigating the signaling pathways extensively discussed in pp 64-73 would be appreciated.

As already mentioned we are now intending to go on and investigate such mechanisms. We will focus on RAGE and TGF-β1 systems and their second messengers. To assess the drivers of airway inflammation and remodelling, we will immunostain airway biopsies for expression of RAGE in epithelium, endothelium and inflammatory cells. We find that TGFβ1 is highly and indiscriminately expressed in airway biopsies from all groups, so for this core mediator we will use quantitative PCR (qPCR) on extracted biopsy mRNA for the growth factor itself and use both immunostaining and molecular quantitation for its strategic transcription factors SMAD-2,-3 and -7. We will also stain for the downstream major pathogenic trigger TWIST and quantitate it by molecular means; and will also perform these measures on matched cell pellets (mainly macrophages) from BAL (bronchoalveolar lavage; essentially small airway washings) which have been stored for each individual.

However, I feel that these studies are beyond the scope of my thesis as planned and written. The work is already rather long, as noted by the examiner, and to do adequate justice to these complex issues would require a whole new set of areas to discuss at length, and properly reference etc (there is now a substantial literature on this) if we were to go down this avenue, which I would prefer to avoid.

Typographical errors

All the specific typing errors picked up by the examiner have been corrected.
Response to examiner 2

Minor points
Figure 1.1 Assume the asthmatic was a non-smoker
I acknowledge examiner’s comment. This asthma subject was a non-smoker and the figure legend is changed accordingly (Chapter 1, pager 4).

In the study reported in chapter 1.2.1 and associated table, clarity on how the asthma phenotype was excluded in the COPD patients and excluded from the controls would help ensure no overlapping phenotyping effects. The age was different and no discussion about the effects of age on Rbm clefts/fragmentation was mentioned.
As explained in Chapter 3, page 101 & 102, diagnosis were made by a respiratory physician clinically, physiologically and ultimately pathologically. Subjects with a history suggestive of asthma, which included 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 were excluded. Any previous medical diagnosis of asthma was also an exclusion. None of our COPD subjects had an airway histological picture that looked like asthma.

I acknowledge the examiner’s comment that some of the control subjects were of young age, there was a wide spectrum of age in the control group and in reality the differences between smokers/COPD did not depend on the mean age difference. As explained in the thesis, linear regression analysis was undertaken within the COPD group and age was not related to the findings.

In chapter 3.2.1 exclusion criteria including other co-existing respiratory disorders was loosely described ie was CT routinely used to exclude fibrosis and bronchiectasis as these are often incidentally found to co-exist in COPD.
CT scanning was not performed, but clinical and physiological examination was done, and subsequently obtained BAL fluid had to be free of bacterial growth/colonisation. Thus, there was no clinical (e.g. crackles on auscultation) or radiological evidence of fibrosis; and no clinical, radiological or microbiological evidence of bronchiectasis.

Discussion in 4.4 should acknowledge that the (a) smoking controls are younger and have lower pack years exposure and that adjustment for this does not effect the findings which and (b) that changes consistent with EMT was tracking with smoking per se and the specific association with COPD and SiOAA4 cells was significantly only after correction for Rbm length but not reflected in the cleft number. (pg 151).
The issue of age variations has already been dealt with above.

I agree that the changes I have concluded are related to EMT are related to smoking per se, but the data also strongly suggest that the changes are especially marked in COPD current smokers. This was extensively discussed in Chapter 4, pages 152-155.

The use of normal controls for comparative purposes in the chapter 6 study is problematic as they are younger with less smoking history. I'm sure this
A researcher would have preferred to have examined changes in biopsies in the same patients before and after treatment as a more rigorous means of comparing effects of steroid vs. placebo on EMT characteristics in equally matched groups. If the placebo group is smaller then any effects may be lost due to under-powering.

The realities of doing intervention studies with bronchoscopic outcome measures include the fact that one needs to optimise the material obtained. Our group has made the point on many previous occasions that the control data is frequently largely wasted; it is there to try and re-assure the reader that no change is spontaneously occurring. In the study included in the thesis there is just no suggestion of a consistent change in the control group, with no realistic chance of a Type-2 statistic error. Further, although the sample size was small in this study, as explained in Chapter 6, I did perform power and sample size calculations for this analysis, which showed that a number of subjects smaller than 15 was adequate to find differences for Rbm fragmentation before and after treatment with an alpha error 5% and beta error 80%. This is quite consistent with previous work from our group published some years ago by Richmond et al (Richmond, Booth, Ward, & Walters, 1996).

I would have liked to have seen a stronger argument that supported the hypothesis that ICS might affect EMT through some mechanism explained in chapter 2.

This issue was dealt with in detail in response to examiner 1.

Typographical errors

All the typing errors mentioned by the examiner have now been corrected in the thesis. I am grateful to the examiner for such a careful and helpful reading of the text.

REFERENCES

