Chapter 7
HDAC2 in COPD

7.1 Introduction
As already described in detail in Chapter 2, COPD is characterized by progressive inflammation of the airways (and destruction of the lung parenchyma) mediated by increased expression of inflammatory genes, in response to noxious particles, gases and especially tobacco smoke (Barnes, 2003). In COPD a specific pattern of airway inflammation is mainly characterized by increased numbers of neutrophils, macrophages and T-lymphocytes, predominantly cytotoxic (CD8+) cells (Barnes et al., 2005; Saetta et al., 1993; Saetta et al., 1998; Saetta et al., 1997; Saetta, Turato, Maestrelli, Mapp, & Fabbri, 2001). The increased expression of these inflammatory genes is regulated by acetylation of core histones around which DNA is wound, allowing access of pro-inflammatory transcription factors to transcription-regulatory sites. On the other hand these activated genes are switched off by deacetylation of these histones (Chapter 2) (Barnes et al., 2005). Examples of the transcription factors involved in this regulatory system in airway diseases are nuclear factor-Kappa B (NF-κB), activator protein-1 (AP-1) and also the activated glucocorticoid receptors (Barnes, 2006d). These are all at least partly controlled by this histone acetylation/deacetylation mechanism.

COPD responds relatively poorly to therapeutic corticosteroids, even though steroids have been shown to have effects both in the short term and long term use. As discussed in Chapter 2 there is emerging evidence that corticosteroids resistance in COPD is due to decrease in histone deacetylase -2 (or HDAC-2) (Barnes, 2006c; Ito et al., 2001). Though exact mechanisms are still not clearly understood, it is suggested that it may involve modulation of HDACs by nitrosylation on distinct tyrosine residues in response to tobacco smoke (Barnes et al., 2005).

Since 1960, it has been known that acetylation of DNA-associated histone proteins and remodelling of the tightly packed chromatin structure is associated with
induction of genes (Littau et al., 1965). Histone and chromatin remodelling is central to gene expression and regulation through the process of acetylation, deacetylation and also methylation, though this is even less understood (Barnes et al., 2005; Rice & Allis, 2001). Research regarding the exact role of histone acetylation and deacetylation in chronic inflammatory disease is only in its infancy, and even more so in COPD, where the picture has probably been made rather over-simplistic, as indeed the degree of absolute ICS insensitivity has been exaggerated. However, there is significant body of literature (as explained in Chapter 2) suggesting that expression and activity of anti-inflammatory HDAC2 are reduced in COPD lungs, airways and alveolar macrophages and becomes worse with severity of the disease (Barnes, 2006c; Ito et al., 2001).

The methodology that has been used was not comprehensive enough to identify the changes in total cellularity in the lamina propria and largely dependent on molecular RNA quantitation and protein analysis which could not take into account relative differences in cellular profiles in airway tissues in different disease and control groups, where biases could arise due to differences in total and differential cellularity. There has been a serious lack of comprehensive biopsy studies to confirm the extent of suppressed HDAC2 levels by immunostaining within the airways of COPD; which can take into account cell numbers and type, its reversibility with ICS or smoking cessation. Therefore, I designed a detailed cross-sectional study and used material collected in a longitudinal study (D. W. Reid et al., 2008), for looking in detail at the status of HDAC2 expression in COPD airways.
7.2 Hypothesis
I hypothesized that the current literature is correct in that HDAC2 is down-regulated in COPD airways, and that these reduced HDAC2 levels are normalised by aggressive ICS therapy and also smoking cessation in patients with COPD.

7.2.1 Aims
1. To confirm the extent of suppressed HDAC2 by immunostaining airway biopsies from COPD current smokers and also from normal lung function smokers to understand whether changes in HDAC2 are solely related to COPD or smoking or both.

2. To confirm the potential of smoking cessation to raise HDAC2 expression by comparing airway biopsies from COPD ex-smokers *versus* current smokers with COPD, and the potential for HDAC2 expression to be normalised by aggressive ICS therapy.

7.3 Methods and materials

7.3.1 Subjects and study design
The studies involved both detailed cross-sectional and longitudinal analyses. In the cross-sectional study, 17 current smokers with established COPD (CS), 16 current smokers with normal lung function (NS), 17 ex-smokers with COPD (ES) and 15 normal healthy, never-smoking controls (NC) were recruited for bronchoscopy and airway biopsy. In longitudinal analysis the COPD subjects further entered a double blind randomised controlled trial comparing fluticasone propionate (0.5 mg inhaled twice daily) with placebo for 6 months (details given in Chapter 3).

7.3.2 Bronchoscopy
Details are given in Chapter 3.
7.3.3 Processing of biopsies and immunostaining

Sections were stained with monoclonal antibodies: anti-HDAC2, together with a horseradish peroxidase (HRP) conjugated DAKO Envision + reagent for secondary antibody binding and colour resolution using diaminobenzidine (DAB) (details given in Chapter 3).

7.3.4 Biopsy analysis

Details are given in Chapter 3.

7.3.5 Statistical analysis

As data were not normally distributed (tested by kolmogorov smirnov & Shapiro Wilk test), a non-parametric ANOVA (Kruskal Wallis Test) was undertaken, and specific group differences then explored using the Mann Whitney U test. 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. Statistical analyses were performed using SPSS 15.0 for Windows, 2003, with a two-tailed P-value $\leq 0.05$ being considered statistically significant (details are given in Chapter 3).
7.4 Results (cross-sectional analysis)

The group demographics of subjects who participated in the study are the same as presented Chapter 4.

7.4.1 HDAC2 expression in the airway epithelium

HDAC2 expression in the airway epithelium (as measured by percentage area of epithelium stained for HDAC2) was not different between the groups (p=0.7, Figure 7.1 and 7.2).

7.4.2 Total cellularity of the lamina propria

There was a significant reduction in total number of cells in lamina propria in COPD-CS compared to NC (p=0.04) (Figure 7.3 and 7.1). This total cell change strongly negatively correlated to smoking history (R = -0.8, p<0.003) (Figure 7.6). No association was found with lung function measurements.

7.4.3 HDAC2 positive cells in the lamina propria

Compared to NC there was a significant reduction in HDAC2 positive cells in the lamina propria in COPD-CS (p=0.02). Interestingly, normal lung function smokers had significantly more HDAC2 positive cells in the lamina propria compared to COPD-CS (p<0.001), ie smoking actually seems to be stimulating anti-inflammatory HDAC2. There were significantly more HDAC2 positive cells in COPD-ES compared to COPD-CS (p=0.04) (Figure 7.4 and 7.1); ie COPD-CS smokers were essentially normalised. No association was found with lung function measurements.

7.4.4 Percentage cell HDAC2 staining in the lamina propria

For percentage cell HDAC2 staining (ie taking into account total cell numbers) normal lung function smokers were significantly different from all other groups (p<0.001, with smoking again apparently stimulating anti-inflammatory HDAC2), but COPD-CS, ES and NC were quite similar (p=0.42) (Figure 7.5). No association was found with lung function measurements.
Figure 7.1: Bronchial biopsy section from (A) normal control; (B) normal lung function smoker; (C) COPD current smoker; and (D) COPD ex-smokers: (black arrows) brown staining indicates HDAC2 positive cells in the epithelium and in the lamina propria, black arrows, also indicating decreased cellularity in the LP in (B) and (C). Original magnification, x400.
Figure 7.2: Percentage of area of epithelium stained for HDAC2 in COPD current smokers and ex-smokers, normal lung function smokers compared to normal controls with no significant difference between groups. Horizontal bars represent the median for each group.
Figure 7.3: Total number of cells in the lamina propria per Sqmm of lamina propria;
†significant difference from NC (p=0.04).
Figure 7.4: Number of HDAC2 positive cells in the lamina propria per Sqmm of lamina propria; † significant difference from NC (p=0.02); ◊ significant difference from COPD-CS (p<0.001); * significant difference from COPD-CS (p=0.04).
Figure 7.5: Percentage cell staining for HDAC2 in the lamina propria per Sqmm of lamina propria; †significant difference from COPD-CS, ES and NC (p<0.001).
Figure 7.6: Correlation between total number of cells per Sqmm of lamina propria in COPD-CS and smoking (pack years).
7.5 Longitudinal ICS intervention study

The group demographics of subjects who participated in the study are the same as presented Chapter 6.

7.5.1 HDAC2 expression in the airway epithelium

Six months of treatment with fluticasone propionate (active arm; p=0.2, placebo; p=0.5) made no difference on HDAC2 expression in the airway epithelium (Figure 7.7).

7.5.2 HDAC2 positive cells in the lamina propria

There was no difference observed before versus after treatment for HDAC2 positive cells in the lamina propria (active arm, p=0.5 and placebo; p=0.3, Figure 7.8). I also observed that there was no change in the total number of cells in the lamina propria before and after treatment (active arm, p=0.5 and placebo; p=0.8, Figure 7.9). Thus, percentage cell HDAC staining was also not different before and after treatment (active arm, p=0.5 and placebo; p=0.8, Figure 7.10).
Figure 7.7: The percentage change in HDAC2 in the epithelium on treatment with (A) fluticasone propionate and (B) placebo, the horizontal bars represent the medium. There was no significant difference comparing before and after treatments.
Figure 7.8: Number of HDAC2 positive cells in the lamina propria per Sqmm of lamina propria on treatment with (A) fluticasone propionate and (B) placebo: the horizontal bars represent the mediums. There was no significant difference comparing before and after treatments.
Figure 7.9: The total number of cells in the lamina propria per Sqmm of lamina propria on treatment with (A) fluticasone propionate and (B) placebo: the horizontal bars represent the mediums. There was no significant difference between before and after treatments.
Figure 7.10: Percentage cell staining for HDAC2 in the lamina propria per Sqmm of lamina propria; (A) with fluticasone propionate and (B) with placebo: the horizontal bars represent the mediums. There was no significant difference comparing before and after treatments.
7.6 Discussion

To the best of my knowledge, this is the first detailed biopsy immunostaining study looking at the status of HDAC2 expression in COPD and its potential reversibility with ICS or/and smoking cessation (albeit the latter in a cross-sectional study only at this stage).

The airway epithelium showed strong positivity for HDAC2 staining/expression but, interestingly this was not different between the groups. Although COPD current smokers showed a slight decrease in HDAC2 staining in the epithelium (as percentage of HDAC2 area of staining in the epithelial area measured), this was not statistically significant when compared to normal controls. However the difference between groups, even if real, hardly suggests that this is a major effect and HDAC2 is more variable between individuals than groups. The fact that HDAC2 expression in the epithelium is well preserved in smokers (even if little less in current smokers COPD subjects) suggest that ICS may still be effective in this tissue compartment, as the CS-GCR complex should be able to access its transcription sites as CS-sensitive genes.

In the lamina propria the data were very striking. I found that there was a decrease in the total number of cells in the lamina propria in COPD current smokers compared to normal controls. This total cell change was strongly related to smoking history and is an effect not previously described. Normal lung function smokers also showed a slight decrease in total cellularity whilst the COPD-ES group showed a normalisation in total cellularity.

In the COPD subjects the plot clearly indicates that smoking is affecting the total cellularity of the lamina propria. Notably, if I had used molecular techniques as used by the Barnes group, etc I would have come to a false conclusion as I would not be aware that there is a dramatic change in total cellularity in the lamina propria, largely causing the apparent down regulation of HDAC2 mRNA and decreased protein content. For example (as explained in Chapter 2 as well), Ito et al (Ito et al., 2001), showed by using western blotting that HDAC2 protein content was reduced in bronchial biopsies from normal lung function smokers compared to normal controls,
but looking now at the images they actually published in their paper (but which they
did not comment on), it is quite clear that total cellularity of the lamina propria in
their normal lung smokers was also substantially less than in their normal controls.
But, as stated above, their analysis did not take into account the overall decrease in
cellularity and therefore the consequential total protein available. ICS made no
difference to the total number of cells in lamina propria.

Our data for HDAC2 positive cells in the lamina propria was quite interesting as
well. It suggested that HDAC2 positive cells decrease in the lamina propria of
current smokers with COPD compared to normal controls and on the other hand
there was an increase in HDAC2 positive cells in the lamina propria of COPD ex-
smokers compared to COPD current smokers; essentially returning to normal. Data
on normal lung function smokers was quite remarkable; paradoxically they had
significantly higher numbers of HDAC2 positive cells in the lamina propria. This
suggests that smoking is actually stimulating anti-inflammatory HDAC2 levels,
although ICS made no difference to HDAC2 positive cells in lamina propria.

The data on percentage cell staining for HDAC2 further confirmed the above data on
normal lung function smokers that the percentage HDAC2 staining was also
significantly high in normal lung function smokers compared to other groups, but
COPD-CS, COPD-ES and NC were quite similar to each other. This strongly
confirms at least the possibility of smoking stimulating anti-inflammatory HDAC2
expression in chronic smokers. However, the decrease observed in COPD in relation
to HDAC2 does seem most likely due to changes in cellularity, and not HDAC2
itself. ICS made no difference to percentage cell HDAC2 staining.

Taken together, these data suggest that smoking itself stimulates the HDAC2
expression while COPD does not seems to be affecting the HDAC2 status itself, but
the apparent decrease in HDAC2 observed in COPD current smokers is due to
confounding by changes in total cellularity, which itself is strongly negatively related
to smoking history. Quitting does seem to have a potential for upregulating HDAC2
at a cell level as shown by an increase in number of cells positive for HDAC2 in the
lamina propria of COPD ex-smokers and further supported by slight increase in
percentage cells staining in COPD ex-smokers, although this was not statistically
significant when compared to COPD current smokers (type 2 error). Most of the effect seems related to change in total cell numbers. However, we need long term smoking cessation studies with larger cohorts to confirm and tease out these findings.

More precisely, the key finding is the difference in HDAC2 expression in normal lung function smokers and decrease in cells in COPD current smokers. It probably suggests that smoking stimulates anti-inflammatory HDAC2 in non-COPD smokers, but in those who develop COPD then HDAC2 goes down but mainly due to decrease in cellularity of lamina propria and not HDAC2 expression itself. Moreover the status of HDAC2 is not different in the epithelium in either smokers or COPD. So essentially smoking is stimulating HDAC2, but decreasing the total lamina propria cellularity in COPD current smokers. It is possible that this difference between smokers with and without COPD may be important in the aetiology of COPD ie if in an individual smoking decreases cells more than stimulating anti-inflammatory HDAC2, then COPD might ensue.

I cannot say at this stage which particular cell type(s) is decreased in the lamina propria in smokers. For more information I would need double staining studies for different cells in the lamina propria ie HDAC2 plus specific cell type marker. It is also suggested that in COPD there is increased apoptosis but this is controversial and is not clearly established. However, increased oxidative stress produced by tobacco smoke and protease–antiprotease imbalance, and also genetic susceptibility, may contribute to increased apoptosis in COPD airways (Park, Ryter, & Choi, 2007; Plataki et al., 2006). In another parallel study in our group (Soltani A, 2009) we found that there is a significant reduction in total number of vessels in the lamina propria in smoking/COPD, so decreased vascular supply might be contributing to decreased cellularity in COPD airways, but we need further studies to confirm that.

In summary, our data suggest that HDAC2 expression is increased in normal smokers but reduced in current smokers with COPD, though the latter seems largely due to confounding by changes in cellularity in the lamina propria but not in HDAC2 itself. Quitting does seem to have a real effect on up-regulating HDAC2 at a cell level, but it is not affected by ICS. We need comprehensive immunohistochemical studies to fully understand cellular changes in the lamina propria and prospective
long term smoking cessation studies with larger cohorts to confirm these findings. Molecular methods must take such changes in the cellular environment into account and cannot be taken on simple face value; indeed the current published data is likely to be misleading because this was not taken into account.