Analysis of Volatile Biomarkers of Airway Inflammation in Breath

Jack F Dummer

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Abstract

Breath analysis is non-invasive and acceptable to patients, and is an attractive method for the diagnosis and monitoring of airway inflammation in asthma and COPD. The measurement of the fraction of nitric oxide in exhaled breath (FE\textsubscript{NO}) already has clinical applications because of its association with eosinophilic airway inflammation and the clinical response to corticosteroid, but its role has not been defined in COPD. There may also be other volatile biomarkers of airway inflammation in breath, such as hydrogen sulphide (H\textsubscript{2}S) and hydrogen cyanide (HCN). These compounds can be analysed in breath using selected ion flow tube–mass spectrometry (SIFT-MS).

A study was performed to establish whether FE\textsubscript{NO} levels could predict the clinical response to oral corticosteroid in COPD. A double-blind, crossover “trial of steroid” was undertaken in 65 randomised patients with COPD. The predictive values of FE\textsubscript{NO} for clinically significant changes in six-minute walking distance (6MWD), spirometry (FE\textsubscript{V1}), and St. George’s Respiratory Questionnaire (SGRQ) were calculated. Receiver operator characteristic analysis showed the area under the curve for an increase of 0.2 litres in FE\textsubscript{V1} was 0.69 (p=0.04) with an optimum FE\textsubscript{NO} cut point of 50ppb. FE\textsubscript{NO} was not a significant predictor for changes in 6MWD or SGRQ.

Experiments were performed to characterise the accuracy, repeatability and dynamic response of the SIFT-MS instrument using acetone as a model volatile compound. Similar experiments were then performed using H\textsubscript{2}S and HCN. Using a SIFT-MS instrument synchronised with a pneumotachometer, the effects of expiratory flow and volume, and oral vs. nasal passage, on the concentration of a volatile compound in breath were investigated. Using known \textit{in vitro} acetone concentrations of 600-3000 ppb, there was an instrument measurement bias of 8\%, inter-day and intra-day CVs were 5.6\% and 0.0\% respectively, and the 10-90\% response time was 500±50 ms (mean±SE). In 12 healthy volunteers, acetone concentrations at expiratory flows of 193±18 (mean±SD) and 313±32 ml/s were 619±1.83 (geometric mean ± logSD) and 618±1.82 ppb in the fraction 70-85\% by volume of exhaled vital capacity (V\textsubscript{70-85\%}), and 636±1.82 and 631±1.83 ppb in V\textsubscript{85-100\%}. 
Abstract

For H₂S, the mean percentage deviation of SIFT-MS measurements from known concentrations was -12 to -13%. Inter-day and intra-day CVs were 13-22% and 15-25% respectively, and the 10-90% response time was 500±60 ms (mean±SE). For HCN, the mean percentage deviations of SIFT-MS measurements from the known concentrations were -3% to +11%. Inter-day and intra-day CVs were 9-12% and 4-6% respectively, and the dynamic response time was 620±50 ms (mean±SE). Higher concentrations of H₂S and HCN were observed in oral vs. nasal exhalations, and the exhaled H₂S concentration fell from rapidly after hydrogen peroxide mouthwash.

The final experiment compared the concentrations of exhaled H₂S and HCN in asthma and COPD patients with control subjects, and determined any relationship between these volatile compounds and biomarkers of airway inflammation. There was no difference in post-mouthwash, nasally-exhaled H₂S concentration in six COPD patients vs. six control subjects (2.2±0.4 vs. 2.3±0.3 ppb (mean ± SE)) or in six asthma patients vs. six control subjects (2.1±0.2 vs. 2.2±0.2 ppb). There was no difference in nasally-exhaled HCN concentration in the COPD vs. control groups (3.4±0.3 vs. 3.1±0.4 ppb) or the asthma vs. control groups (4.8±0.4 vs. 4.4±0.8 ppb). In the COPD group, there was a negative correlation between the exhaled H₂S concentration and the percentage of neutrophils in sputum (rₛ=-0.89, p=0.02), while in the control group, a positive correlation between the exhaled H₂S concentration and the percentage of neutrophils in sputum approached significance (rₛ=0.77, p=0.07). The exhaled HCN concentration was negatively correlated with sputum neutrophils in COPD patients (rₛ=-0.49 to -0.66, p=0.16 to 0.33). Positive correlations were observed between markers of eosinophilic airway inflammation in asthma patients and the concentrations of both H₂S (rₛ=0.6-1.0, p=<0.05 to 0.21) and HCN (rₛ=0.6-0.8, p=0.16-0.20) in exhaled breath.

In conclusion, FE_NO was a weak predictor of short-term response to oral corticosteroid in COPD, its utility being limited to predicting increase in FEV₁. The characteristics of the SIFT-MS analytical technique were appropriate for the on-line analysis of acetone, H₂S and HCN, in exhaled breath. On-line SIFT-MS measurement of exhaled acetone concentration required control of expiratory volume but not flow. On-line SIFT-MS measurement of exhaled H₂S and HCN concentration required nasal exhalation. While the concentrations of H₂S and HCN in exhaled breath did not differ between patient groups and their controls, there were associations between markers of airway inflammation and the concentrations of H₂S and HCN in exhaled breath that are worthy of further exploration.
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Table of Contents

1. An introduction to the analysis of volatile biomarkers of airway inflammation in exhaled breath ................................................................. 1
   1.1. Volatile biomarkers of airway inflammation in breath .............................. 1
      1.1.1. The potential for volatile biomarkers of airway inflammation in breath 1
      1.1.2. Analytical techniques .................................................................... 4
      1.1.3. Technical considerations for breath analysis .................................... 5
      1.1.4. Methodological considerations for breath analysis .......................... 6
   1.2. Nitric oxide in exhaled breath .............................................................. 10
      1.2.1. Nitric oxide and its biological roles and reactions ............................ 10
      1.2.2. Biosynthesis of nitric oxide in the respiratory tract ......................... 11
      1.2.3. Physiology of nitric oxide exhalation .............................................. 11
      1.2.4. Measurement of $F_{E\text{NO}}$ and sensor technology ....................... 13
      1.2.5. Rationale for the use of $F_{E\text{NO}}$ measurements ............................ 15
      1.2.6. Clinical applications of $F_{E\text{NO}}$ measurement ............................. 16
      1.2.7. $F_{E\text{NO}}$ measurement in COPD ..................................................... 17
   1.3. Hydrogen sulphide in exhaled breath .................................................. 20
      1.3.1. Hydrogen sulphide and its biological roles and reactions .................. 20
      1.3.2. Hydrogen sulphide in COPD ........................................................ 22
      1.3.3. Analysis of hydrogen sulphide in exhaled breath ............................ 22
   1.4. Exhaled Hydrogen Cyanide ............................................................... 23
      1.4.1. Hydrogen cyanide and its biological roles and reactions .................. 23
      1.4.2. Analysis of hydrogen cyanide in exhaled breath ............................ 25
   1.5. Selected Ion Flow Tube – Mass Spectrometry ....................................... 25
      1.5.1. Analysis of volatile compounds using SIFT-MS ............................... 25
      1.5.2. Breath analysis using SIFT-MS ...................................................... 30
   1.6. Summary and overall objectives of the thesis ...................................... 32
2. Predicting corticosteroid response in COPD using exhaled nitric oxide ......... 34
   2.1. Introduction .......................................................................................... 34
   2.2. Methods ............................................................................................. 35
### Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2.1.</td>
<td>Participants</td>
<td>35</td>
</tr>
<tr>
<td>2.2.2.</td>
<td>Study design and procedures</td>
<td>35</td>
</tr>
<tr>
<td>2.2.3.</td>
<td>Exhaled nitric oxide measurement</td>
<td>36</td>
</tr>
<tr>
<td>2.2.4.</td>
<td>Lung function testing</td>
<td>36</td>
</tr>
<tr>
<td>2.2.5.</td>
<td>Six-minute walk test</td>
<td>37</td>
</tr>
<tr>
<td>2.2.6.</td>
<td>St. George’s Respiratory Questionnaire</td>
<td>37</td>
</tr>
<tr>
<td>2.2.7.</td>
<td>Sputum induction and processing</td>
<td>37</td>
</tr>
<tr>
<td>2.2.8.</td>
<td>Statistical analysis</td>
<td>38</td>
</tr>
<tr>
<td>2.3.</td>
<td>Results</td>
<td>39</td>
</tr>
<tr>
<td>2.3.1.</td>
<td>Subject characteristics</td>
<td>39</td>
</tr>
<tr>
<td>2.3.2.</td>
<td>Relationship between baseline FE\textsubscript{NO} measurements and sputum eosinophils</td>
<td>42</td>
</tr>
<tr>
<td>2.3.3.</td>
<td>Overall response to prednisone</td>
<td>42</td>
</tr>
<tr>
<td>2.3.4.</td>
<td>Response to prednisone according to baseline FE\textsubscript{NO}</td>
<td>43</td>
</tr>
<tr>
<td>2.3.5.</td>
<td>Predicting response to prednisone using FE\textsubscript{NO}</td>
<td>48</td>
</tr>
<tr>
<td>2.4.</td>
<td>Discussion</td>
<td>50</td>
</tr>
<tr>
<td>2.5.</td>
<td>Summary</td>
<td>54</td>
</tr>
<tr>
<td>3.</td>
<td>Accurate, reproducible measurement of acetone concentration in breath using selected ion flow tube – mass spectrometry</td>
<td>56</td>
</tr>
<tr>
<td>3.1.</td>
<td>Introduction and aims</td>
<td>56</td>
</tr>
<tr>
<td>3.2.</td>
<td>Methods</td>
<td>58</td>
</tr>
<tr>
<td>3.2.1.</td>
<td>Voice100™ SIFT-MS instrument</td>
<td>58</td>
</tr>
<tr>
<td>3.2.2.</td>
<td>SIFT-MS analysis of acetone</td>
<td>59</td>
</tr>
<tr>
<td>3.2.3.</td>
<td>Instrument accuracy, repeatability and dynamic response</td>
<td>59</td>
</tr>
<tr>
<td>3.2.4.</td>
<td>Breath analysis system</td>
<td>60</td>
</tr>
<tr>
<td>3.2.5.</td>
<td>Synchronisation of the SIFT-MS instrument and the pneumotachometer</td>
<td>60</td>
</tr>
<tr>
<td>3.2.6.</td>
<td>Study design for testing of participants</td>
<td>62</td>
</tr>
<tr>
<td>3.2.7.</td>
<td>Statistical analysis</td>
<td>64</td>
</tr>
<tr>
<td>3.3.</td>
<td>Results</td>
<td>65</td>
</tr>
<tr>
<td>3.3.1.</td>
<td>SIFT-MS instrument characteristics</td>
<td>65</td>
</tr>
<tr>
<td>3.3.2.</td>
<td>Synchronisation of the SIFT-MS instrument and the pneumotachometer</td>
<td>65</td>
</tr>
<tr>
<td>3.3.3.</td>
<td>Breath analysis</td>
<td>68</td>
</tr>
<tr>
<td>3.4.</td>
<td>Discussion</td>
<td>72</td>
</tr>
<tr>
<td>3.4.1.</td>
<td>SIFT-MS instrument characteristics</td>
<td>72</td>
</tr>
<tr>
<td>3.4.2.</td>
<td>Synchronisation of the SIFT-MS instrument and the pneumotachometer</td>
<td>73</td>
</tr>
<tr>
<td>3.4.3.</td>
<td>Breath analysis</td>
<td>74</td>
</tr>
</tbody>
</table>
3.5. Summary .................................................................................................................. 76
4. The analysis of hydrogen sulphide and hydrogen cyanide in exhaled breath .......... 77
4.1. Introduction .............................................................................................................. 77
4.2. Methods ................................................................................................................... 79
  4.2.1. Voice200™ SIFT-MS instrument ...................................................................... 79
  4.2.2. SIFT-MS analysis of hydrogen sulphide ......................................................... 80
  4.2.3. SIFT-MS analysis of hydrogen cyanide.......................................................... 81
  4.2.4. Instrumental accuracy, repeatability and dynamic response ......................... 82
  4.2.5. Breath analysis system ..................................................................................... 82
  4.2.6. Synchronisation of the SIFT-MS instrument and the pneumotachometer..... 83
  4.2.7. Processing of data files .................................................................................... 83
  4.2.8. Study design for testing of participants ............................................................ 87
  4.2.9. Statistical analysis ............................................................................................ 89
4.3. Results – hydrogen sulphide .................................................................................. 89
  4.3.1. SIFT-MS instrument characteristics for the analysis of hydrogen sulphide .... 89
  4.3.2. Subject characteristics ...................................................................................... 90
  4.3.3. Concentration of hydrogen sulphide in ambient air ........................................ 90
  4.3.4. Direct sampling of hydrogen sulphide from the oral and nasal cavities ........... 90
  4.3.5. Effect of oral vs. nasal breathing manoeuvres and effect of mouthwash ......... 92
  4.3.6. Effect of expiratory flow on hydrogen sulphide concentration in nasally- exhaled breath ............................................................................................................. 94
  4.3.7. Effect of repetition of breathing manoeuvre ..................................................... 96
  4.3.8. Effect of oral vs. nasal inhalation on the concentration of H₂S in nasally- exhaled breath ............................................................................................................. 96
  4.3.9. Relationship between the concentration of H₂S in nasally-exhaled breath and sources of contamination ......................................................................................... 97
4.4. Results – hydrogen cyanide ................................................................................... 97
  4.4.1. SIFT-MS instrument characteristics for the analysis of hydrogen cyanide .... 97
  4.4.2. Subject characteristics ...................................................................................... 98
  4.4.3. Concentration of hydrogen cyanide in ambient air ........................................ 98
  4.4.4. Direct sampling of hydrogen cyanide from the oral and nasal cavities ........... 98
  4.4.5. Effect of oral vs. nasal breathing manoeuvres .................................................. 99
  4.4.6. Effect of expiratory flow on hydrogen cyanide concentration in nasally- exhaled breath ............................................................................................................. 100
  4.4.7. Effect of repetition of breathing manoeuvre ..................................................... 102
## Contents

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appendix A</td>
<td>Publications resulting from this thesis</td>
<td>167</td>
</tr>
<tr>
<td>Appendix B</td>
<td>Macro programs</td>
<td>168</td>
</tr>
</tbody>
</table>
List of Tables

Chapter 1
1-1 Concentrations of volatile compounds in the exhaled breath of 30 healthy volunteers quantified by SIFT-MS ................................................................. 30

Chapter 2
2-1 Schedule of study visits and procedures. .......................................................... 36
2-2 Baseline subject characteristics after withdrawal of inhaled corticosteroid. ............ 41
2-3 Outcomes before and after treatment with oral prednisone and placebo in 62 patients with COPD ......................................................................................... 44
2-4 Mean change in outcomes after prednisone compared to placebo for subjects stratified by \( \text{FE}_{\text{NO}} \) tertiles .................................................................................................. 45
2-5 Sensitivities, specificities, positive and negative predictive values (PPV and NPV respectively) and accuracy of cut-points for \( \text{FE}_{\text{NO}} \) as a predictor for an increase in \( \text{FEV}_1 \) of (A) 0.2 litres or greater in response to prednisone and (B) for an increase in \( \text{FEV}_1 \) of 20% or greater ........................................................................................................ 48

Chapter 3
3-1 Subject characteristics .......................................................................................... 69
3-2 Expiratory flows and volumes ............................................................................. 70
3-3 Mean acetone concentrations in exhaled breath at breath fractions of 70-85% and 85-100% by volume, at target expiratory flows of 170 and 330 ml/s .............................................. 71

Chapter 4
4-1 Example of processed exhalation data presented after adjustment for difference in transit time between the SIFT-MS instrument and pneumotachometer and after extraction from raw data file ........................................................................................................ 85
4-2 Example of processed exhalation data presented as exhalation characteristics and expiratory flow and analyte concentration at various breath volume fractions .......... 86
4-3 Four breathing manoeuvres were performed in random order ............................... 88
Table 4-4 Subject characteristics ........................................................................................................ 91
Table 4-5 Subject characteristics ........................................................................................................ 98

Chapter 5

5.1 Characteristics of patients with COPD and control subjects .................................................... 116
5.2 Characteristics of patients with asthma and control subjects ..................................................... 117
5.3 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of $H_2S$ in exhaled breath and the percentage of eosinophils in the sputum and the $FE_{NO}$ measurement in patients with COPD and control subjects ........................................... 120
5.4 Spearman’s rank correlation coefficients ($r_s$) for the correlations of FEV$_1$ parameters with the concentration of $H_2S$ in exhaled breath and the sputum neutrophil percentage in patients with COPD and control subjects ........................................................................................................... 121
5.5 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of $H_2S$ in exhaled breath and the percentage of eosinophils in the sputum and the $FE_{NO}$ measurement in patients with asthma and control subjects .................................................... 125
5.6 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of $H_2S$ in exhaled breath and the percentage of neutrophils in the sputum in patients with asthma and control subjects ........................................................................................................... 125
5.7 Spearman’s rank correlation coefficients ($r_s$) for correlation of the concentration of $H_2S$ in exhaled breath with FEV$_1$ and FVC in patients with asthma and control subjects ........ 126
5.8 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of neutrophils in the sputum in patients with COPD and control subjects ........................................................................................................... 129
5.9 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum and the $FE_{NO}$ measurement in patients with COPD and control subjects ........................................... 130
5.10 Spearman’s rank correlation coefficients ($r_s$) for correlation of the concentration of HCN in exhaled breath with FVC in patients with COPD and control subjects .................... 131
5.11 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum and the $FE_{NO}$ measurement in patients with asthma and control subjects ........................................... 135
5.12 Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of neutrophils in the sputum in patients with asthma and control subjects ........................................... 135
Tables

5-13 Spearman’s rank correlation coefficients ($r_s$) for correlation of the concentration of HCN in exhaled breath with FEV$_1$ and FVC in patients with asthma and control subjects .......... 136
List of Figures

Chapter 1
1-1 Illustration of the response to a step change in acetone concentration at the inlet of a SIFT-MS analyser and its effect on quantification of acetone concentration in breath.............. 7
1-2 Two-compartment model for the exhalation of nitric oxide ........................................ 12
1-3 Schematic diagram of SIFT-MS .................................................................................. 26

Chapter 2
2-1 Study profile .............................................................................................................. 40
2-2 Correlation between FE_{NO} measurements and percentage sputum eosinophils. .... 42
2-3 Mean (SE) changes in 6MWD, FEV_{1}, and SGRQ for each tertile after prednisone compared to placebo. .......................................................................................... 46
2-4 Changes in (A) FE_{NO} and (B) percentage sputum eosinophils in response to placebo and prednisone for subjects stratified by baseline FE_{NO} tertile .......................................... 47
2-5 Receiver operator characteristic curves demonstrating the utility of FE_{NO} and % sputum eosinophils for predicting response to prednisone ................................................................... 49

Chapter 3
3-1 Schematic diagram of the breath analysis system ...................................................... 60
3-2 Scheme of the SIFT-MS signal, at m/z 19, to an input of humidified air ...................... 62
3-3 Single-Breath Nitrogen Washout (SBN_{2}) .................................................................. 63
3-4 Bland-Altman plots showing acetone concentrations from the custom permeation system measured by the SIFT-MS instrument vs. expected concentrations ........................................ 66
3-5 SIFT-MS instrument dynamic response times for acetone in humid air ................. 67
3-6 Example of SIFT-MS trace for m/z 19 and acetone concentration in response to the discharge of a syringe containing a mixture of humid air and acetone ............................. 67
3-7 Example of the acetone concentration plotted against exhaled volume in six exhalations from one volunteer ................................................................. 69
3-8 Intra-day and inter-day variation in acetone concentration in the exhaled breath of three volunteers ........................................................................................................ 72
Chapter 4
4-1 Diagram of the breath analysis system. .......................................................... 80
4-2 Median and individual concentrations of H\textsubscript{2}S measured by direct sampling from the mouth and nose before and after rinsing the mouth with 3\% H\textsubscript{2}O\textsubscript{2} mouthwash .............. 91
4-3 Mean (SE) H\textsubscript{2}S concentrations for (1) 2 min of pre-test tidal breathing then inhalation to TLC via mouth or nose, followed by, (2) exhalation of vital capacity, via mouth or nose .... 93
4-4 (A) Mean (SE) H\textsubscript{2}S concentrations for exhalations performed at target expiratory flows of 170 and 330 ml/s. (B) Mean (SE) actual expiratory flows at target expiratory flows of 170 and 330 ml/s. ........................................................................................................... 95
4-5 Median and individual concentrations of HCN measured by direct sampling from the mouth and nose. ........................................................................................................... 99
4-6 Mean (SE) HCN concentrations for (1) 2 min of pre-test tidal breathing then inhalation to TLC via mouth or nose, followed by, (2) exhalation of vital capacity, via mouth or nose. .. 100
4-7 (A) Mean (SE) HCN concentrations for exhalations performed at target expiratory flows of 170 and 330 ml/s. (B) Mean (SE) actual expiratory flows at target expiratory flows of 170 and 330 ml/s. ........................................................................................................... 101
4-8 Mean (SE) HCN concentrations in exhaled breath after 2 minutes of tidal breathing via the nose, then inhalation to TLC via the mouth or nose, followed by exhalation of vital capacity, at a target expiratory flow of 10 l/min, via the nose ........................................................................ 103

Chapter 5
5-1 Mean-exhaled and end-exhaled concentrations of H\textsubscript{2}S in the post-mouthwash, nasally-exhaled breath of patients with COPD and control subjects. ........................................ 118
5-2 (A) Mean (SE) H\textsubscript{2}S concentrations in the post-mouthwash, nasally exhaled breath of the COPD and control groups. (B) Mean (SE) expiratory flow for the COPD and control groups at a target expiratory flow of 170 ml/s. .............................................................. 118
5-3 (A) Mean-exhaled and (B) end-exhaled concentrations of H\textsubscript{2}S plotted against percentage sputum neutrophils in six patients with COPD and six control subjects ...................... 119
5-4 (A) Mean-exhaled and (B) end-exhaled H\textsubscript{2}S concentration plotted against ambient H\textsubscript{2}S concentration in six patients with COPD and six control subjects ...................................................... 121
5-5 Mean-exhaled and end-exhaled concentrations of H\textsubscript{2}S in the nasally-exhaled breath of patients with asthma and control subjects after mouthwash ..................................... 122
5-6 (A) Mean (SE) H\textsubscript{2}S concentrations in the exhaled breath of the asthma and control groups. (B) Mean (SE) expiratory flow for the asthma and control groups at a target expiratory flow of 170 ml/s. ................................................................. 123
5-7 (A) Mean-exhaled and (B) end-exhaled concentrations of H$_2$S plotted against percentage sputum eosinophils in four patients with asthma and four control subjects. .......................... 124
5-8 (A) Mean-exhaled and (B) end-exhaled concentrations of H$_2$S plotted against Fe$_{NO}$ in six patients with asthma and six control subjects. ................................................ 124
5-9 (A) Mean-exhaled and (B) end-exhaled H$_2$S concentration plotted against ambient H$_2$S concentration in six patients with asthma and six control subjects. ......................... 126
5-10 Mean-exhaled and end-exhaled concentrations of HCN in the nasally-exhaled breath of patients with COPD and control subjects ............................................................. 127
5-11 (A) Mean (SE) HCN concentrations in the exhaled breath of the COPD and control groups.  (B) Mean (SE) expiratory flow for the COPD and control groups at a target expiratory flow of 170 ml/s .................................................................................. 128
5-12 (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against percentage sputum neutrophils in six patients with COPD and six control subjects .......... 129
5-13 (A) Mean-exhaled and (B) end-exhaled HCN concentration plotted against ambient HCN concentration in six patients with COPD and six control subjects .......................... 131
5-14 Mean-exhaled and end-exhaled concentrations of HCN in the nasally-exhaled breath of patients with asthma and control subjects ........................................................................ 132
5-15 (A) Mean (SE) HCN concentrations in the exhaled breath of the asthma and control groups.  (B) Mean (SE) expiratory flow for the asthma and control groups at a target expiratory flow of 170 ml/s. .................................................................................. 133
5-16 (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against percentage sputum eosinophils in four patients with asthma and four control subjects ...... 134
5-17 (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against Fe$_{NO}$ in six patients with asthma and six control subjects ......................................................... 134
5-18 (A) Mean-exhaled and (B) end-exhaled HCN concentration plotted against ambient HCN concentration in six patients with asthma and six control subjects ................. 136
List of Abbreviations

AECOPD  Acute exacerbation of chronic obstructive pulmonary disease
AHR   Airway hyper-responsiveness
BMI   Body mass index
°C   Degrees celcius
CF   Cystic fibrosis
CI   Confidence interval
cm   Centimetres
cm H₂O   Centimetres of water
COPD  Chronic obstructive pulmonary disease
eNOS  Endothelial nitric oxide synthase
FE₉₀  Fraction of nitric oxide in exhaled breath
FEV₁  Forced expiratory volume in one second
FVC   Forced vital capacity
GC-MS Gas chromatography – mass spectrometry
GOLD Global initiative for chronic obstructive lung disease
HCN   Hydrogen cyanide
H₂O₂  Hydrogen peroxidase
H₂S   Hydrogen sulphide
Hz    Hertz
ICS   Inhaled corticosteroid
IFN-γ Interferon – gamma
iNOS Inducible nitric oxide synthase
IQR   Inter-quartile range
l     Litres
LOD   Limit of detection
m     Metres
MCID Minimum clinically important difference
mL    Millilitres
MPO   Myeloperoxidase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate, oxidised form</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NPV</td>
<td>Negative predictive value</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>Peroxynitrite</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>ppt</td>
<td>Parts per trillion</td>
</tr>
<tr>
<td>PPV</td>
<td>Positive predictive value</td>
</tr>
<tr>
<td>PTR-MS</td>
<td>Proton transfer reaction – mass spectrometry</td>
</tr>
<tr>
<td>RI</td>
<td>Reference interval</td>
</tr>
<tr>
<td>rₛ</td>
<td>Spearman’s rank correlation coefficient</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>SIFT-MS</td>
<td>Selected ion flow tube – mass spectrometry</td>
</tr>
<tr>
<td>SGRQ</td>
<td>St. George’s Respiratory Questionnaire</td>
</tr>
<tr>
<td>SPME</td>
<td>Solid phase microextraction</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical package for the social sciences</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operator characteristic</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor – alpha</td>
</tr>
<tr>
<td>μg</td>
<td>Micrograms</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometres</td>
</tr>
<tr>
<td>6MWD</td>
<td>Six-minute walk distance</td>
</tr>
<tr>
<td>6MWT</td>
<td>Six-minute walk test</td>
</tr>
</tbody>
</table>
Chapter One

1.

An Introduction to the Analysis of Volatile Biomarkers of Airway Inflammation in Exhaled Breath

1.1. Volatile biomarkers of airway inflammation in breath

1.1.1. The potential for volatile biomarkers of airway inflammation in breath

Airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD), involve chronic inflammation and oxidative stress. Subgroups of patients with these diseases demonstrate patterns of airway inflammation, or inflammatory phenotypes, with different therapeutic responses (Pavord et al., 1999; Brightling et al., 2000). The use of biomarkers to differentiate between inflammatory phenotypes in clinical practice would allow improved targeting of therapy, and might permit titration of therapy to the inflammatory response in individual patients.

A biomarker is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson et al., 2001). Currently, biomarkers of inflammation are not routinely used in airway diseases. Cell counts from bronchial tissue biopsy and bronchoalveolar lavage are inappropriate as routine procedures because they are too invasive for widespread clinical use, and present risks to the patient (Pue and Pacht, 1995). A less invasive procedure is the analysis of induced sputum for inflammatory cells and mediators. This is performed in some specialist centres (Pizzichini et al., 1996), but is also impractical for routine clinical use (Wark et al., 2001). Some patients cannot expectorate a satisfactory sample, and inhalation of nebulised saline can cause bronchoconstriction, cough, nausea and may provoke transient airway neutrophilia, introducing diagnostic uncertainty (Nightingale et
al, 1998). All of these procedures are time-consuming, require skilled staff and are unsuitable for repeated sampling. Furthermore, the results are not immediately available, thus reducing their practical use in a clinical setting.

In the absence of inflammatory biomarkers, we use physiological measures, such as spirometry, to identify airflow obstruction and reversibility to support clinical findings (Bateman et al, 2008). Given that the physiological changes in airway diseases are secondary to, and therefore a step removed from, the underlying inflammatory pathology, it is unsurprising that physiological measures behave imperfectly for the diagnosis and assessing the severity of airways disease. For example, variable airflow obstruction is one of the defining characteristics of asthma (Bateman et al, 2008), but persistent airflow obstruction is frequently observed in severe asthma (ten Brinke et al, 2001). Conversely, COPD is characterised by fixed airflow obstruction, but reversibility occurs in a significant minority (Anthonisen et al, 2005). Diagnostic labelling on the basis of spirometry can therefore be unreliable, and the introduction of biomarkers that are more closely related to underlying disease processes has the potential to improve the current situation (Pavord et al, 2008).

The need to monitor airway inflammation has led to the investigation of trace substances in exhaled breath. These substances may be divided into volatile compounds that are mixed with the other gaseous components of exhaled breath, and non-volatile compounds present in the exhaled breath condensate. This thesis is restricted to the investigation of volatile compounds; the analysis of non-volatile compounds in the exhaled breath condensate is reviewed elsewhere (Mutlu et al, 2001; Horvath et al, 2005).

The principal gaseous components of breath are nitrogen, oxygen, carbon dioxide, water vapour and the inert gases. The remainder is a mixture of trace volatile compounds occurring in concentrations in the parts per billion (ppb) to parts per trillion (ppt) range (Miekisch et al, 2004). Breath analysis of volatile compounds using modern techniques began in the 1970s, with the identification of over 200 of these compounds in exhaled breath (Pauling et al, 1971). Since then, improvements in the sensitivity of analytical techniques have led to over 500 different compounds being identified, both endogenous and exogenous in origin. Some of these compounds, such as nitric oxide, carbon monoxide, ethane and pentane, are related to inflammatory and oxidative processes within the lungs, and are therefore potential biomarkers of airway inflammation (Kharitonov and Barnes, 2001).
The analysis of volatile biomarkers of airway inflammation in breath is an attractive concept because breath analysis is non-invasive, agreeable to patients, takes little time and allows for repeated sampling (Cao and Duan, 2007). However, its development has been slow. For example, the US FDA approved the first nitric oxide analyser for clinical use in 2003 (Silkoff et al, 2004) – ten years after the discovery of elevated nitric oxide in the exhaled breath of asthmatic patients (Alving et al, 1993) – and the full clinical role of exhaled nitric oxide measurements (FE\textsubscript{NO}) is still being debated (Taylor, 2009). Other potential biomarkers remain in the early stages of development. Such slow progress is due to the complex and interacting issues involved in achieving adequate performance characteristics for an exhaled marker. Development issues include the instrumentation required for measurement, the specificity of the analytical technique for an individual volatile compound, the physiology of exhalation and how this affects the concentration of the compound at various stages of the exhalation manoeuvre, the relationship between the proposed biomarker and the underlying disease, and the interpretation of results in relation to the clinical symptoms (Miekisch et al, 2004; Stockley, 2007).

The measurement of the fraction of nitric oxide in exhaled breath (FE\textsubscript{NO} ) has already been shown to have some clinical utility in the assessment of airway inflammation (Pavord et al, 2008). FE\textsubscript{NO} measurement has proven to be of value because of its association with eosinophilic airway inflammation, and because eosinophilic airway inflammation responds to treatment with corticosteroid (Taylor et al, 2006). An elevated FE\textsubscript{NO} level (>50ppb) predicts corticosteroid response, while a low normal level (<25ppb) predicts the absence of a response (Pavord et al, 2008). The development of FE\textsubscript{NO} measurement required an analytical technique that was selective and specific for nitric oxide, and that could quantify nitric oxide on-line with appropriate sensitivity, dynamic response, accuracy and reproducibility (Silkoff et al, 2004). It was also necessary to gain an understanding of the physiology of exhalation of nitric oxide; the concentration measured at the mouth depending on the expiratory flow and the exclusion of the high concentrations of nitric oxide present in the nose and sinuses (Silkoff et al, 1997).

While FE\textsubscript{NO} has some clinical utility, it is not a perfect predictor of airway inflammation (Berry et al, 2005). Therefore, there is the need to explore other exhaled biomarkers of airway inflammation, whose performance characteristics may improve on those of FE\textsubscript{NO}. The successful transition of FE\textsubscript{NO} measurement from bench to bedside provides a template for their development (Bates and Silkoff, 2003).
1.1.2. Analytical techniques

A number of competing technologies are available for the analysis of trace gases in exhaled breath. The most commonly used analytical technique is gas chromatography, usually coupled with mass spectrometry (GC-MS) (Cao and Duan, 2007). More recently developed techniques include selected ion flow tube – mass spectrometry (SIFT-MS), proton transfer reaction – mass spectrometry (PTR-MS), optical spectroscopy, and “electronic noses”. Each has its advantages and disadvantages, but few of these modalities are able to detect and quantify compounds present at ppb or ppt concentrations, and even fewer techniques are capable of performing real time analyses of exhaled breath because they require sample collection into bags and/or onto traps (Smith and Spanel, 2007).

Gas chromatography – mass spectrometry requires the collection of trace gases from relatively large volumes of breath onto an adsorption trap (Phillips and Greenberg, 1992). The sample is then desorped, injected onto the head of a chromatographic column and transported through the column in a mobile inert gas phase. The sample constituents pass through the column at different speeds depending on the interaction of each constituent with the stationary phase in the column. A detector at the end of the column identifies analytes according to their retention time within the column and the order in which they emerge (Harris, 1999). The effluent from the column then passes into a mass spectrometer that fragments and ionises the analyte, which can then be identified by the fragmentation pattern and quantified by measuring the number of daughter ions (Cheng and Lee, 1999). Substantial work has been undertaken in the field of breath analysis using this technique, but it does have some disadvantages (Smith and Spanel, 2007): the technique cannot be performed in real time, quantification is reliant on calibration using known compounds, and solid phase microextraction (SPME) is often used, which improves sensitivity but diminishes accuracy because of uncertainties in collection and desorption efficiencies.

Optical spectroscopic methods are usually selective for a single small molecule; the detection and quantification of exhaled nitric oxide using ozone chemiluminescence being the most prominent example (see Section 1.2.4, Page 13). These methods are particularly useful once a specific molecule has been identified in association with a specific pathological process, and are capable of on-line real time analysis at the ppb level, as demonstrated by ozone-chemiluminescence nitric oxide analysers (Silkoff et al, 2004). In contrast, electronic noses are non-selective chemical sensor arrays coupled with complex pattern recognition techniques such as partial least squares-discriminant analysis (Gardner and Bartlett, 1999). Whilst
unable to identify an individual volatile compound, the electronic nose can be “trained” to recognise a profile in exhaled breath associated with a disease state (Machado et al, 2005; Fens et al, 2009).

Techniques based on mass spectrometry, including SIFT-MS and PTR-MS show potential in the identification of volatile compounds in breath (Smith and Spanel, 2005; Smith and Spanel, 2007). Both techniques can be used for on-line real time breath analysis of a range of volatile compounds at ppb levels (Smith and Spanel, 2005b). They employ the chemical ionisation of analytes, in samples of air or exhaled breath, using ions that do not react with molecules making up the bulk matrix of air. In PTR-MS, H$_3$O$^+$ reagent ions transfer protons to volatile compounds as they pass down a drift tube, and a downstream detector counts the number of ions at each mass-to-charge ratio (Lagg et al, 1994). Because the PTR-MS technique characterises analytes only according to their mass-to-charge ratio, chemical identification is not possible and must be confirmed by other techniques (Amann et al, 2004). SIFT-MS is a related technique, but has a number of reagent ions at its disposal, allowing the identification of an analyte (Smith and Spanel, 2007). The SIFT-MS technique is discussed more fully in Section 1.5.1 (Page 25) of this thesis.

1.1.3. Technical considerations for breath analysis

Ideally, an analytical technique for breath analysis would identify and accurately quantify a volatile compound in exhaled breath in real time without the need for sample collection into bags or onto traps that can compromise the sample and delay analysis. This would permit the exploration of the compound’s exhalation physiology (Amann et al, 2007). Given that the trace gases of interest are present in breath at ppb levels or lower, this is a challenging task (Miekisch et al, 2004). The development of Fe$_{50}$ measurement required an analytical technique that that could quantify nitric oxide on-line, at the ppb level, with appropriate accuracy, reproducibility and dynamic response (Silkoff et al, 2004).

The analytical technique must be capable of detecting the small quantities of trace compounds in exhaled breath, with the limit of detection (LOD) being defined as the smallest quantity of a compound distinguishable from the absence of that compound (MacDougall et al, 1980). The quantification of a volatile compound also relies on the accuracy and precision of the analytical technique (Amann et al, 2007). The accuracy of a measurement is the fractional error in a measurement compared to the true value. The precision of a measurement is the fractional error between repeated measurements of an identical sample, and is unrelated to the
true value but instead measures the spread of the data: the more tightly clustered the data, the more precise the measurement (Grubbs, 1948; Rodriguez, 2008).

If performing breath analysis on-line in real time, the dynamic response of the instrument or technique must be appropriate (see Figure 1-1): the 90% response time should be $\lt 10\%$ of the total duration of an exhalation to accurately measure the analyte concentration (Bates et al., 1983). This response time is relevant to techniques such as SIFT-MS and the analysis of nitric oxide by ozone chemiluminescence.

1.1.4. Methodological considerations for breath analysis

Breathing manoeuvre

The collection of a breath sample for analysis must be performed with normal pulmonary physiology in mind, because the composition of the exhaled breath changes during the course of exhalation (Risby and Sehnert, 1999). The concentration of a specific volatile compound in breath may vary depending on whether a sample is taken from the whole exhalation, or from a fraction of the breath originating from the airways or the alveoli. In addition, the concentration of the volatile compound may vary with expiratory flow and volume (Silkoff et al., 1997; Hlastala, 2003).

In some previous studies, samples of breath originating from the alveoli have been sampled on the premise that the concentration of volatile compounds in alveolar samples is in equilibrium with blood in the systemic circulation (Spanel and Smith, 2001; Miekisch et al., 2004; Cao and Duan, 2007). The alveolar fraction of breath can be collected off-line using fractionating methods that only collect the portion of breath in which the concentration of CO$_2$ is consistent with alveolar gas (Schubert et al., 2001). Using on-line real time monitors, alveolar gas can be sampled by measuring the analyte concentration at end-exhalation (Risby and Solga, 2006). The concentration of some volatiles is 2-3 times higher in alveolar gas than in whole breath samples (Schubert et al., 2001), and alveolar samples have a lower concentration of exogenous contaminants than whole breath samples (Miekisch et al., 2008).
Figure 1-1  (A) Illustration of the response to a step change in acetone concentration at the inlet of a SIFT-MS analyser. Output (red line) rises more slowly than the input (blue line) due to the dynamic response characteristics of the analyser.  (B) A 0-90% response time of 0.5s is too long to allow quantification of acetone concentration in tidal breaths <5s duration (first four peaks), but is short enough to allow quantification in a slow exhalation of vital capacity (>5s) (plateau).

A.

![Acetone concentration diagram](image)

Despite this apparently helpful information, the simple collection and analysis of alveolar samples may not be enough to provide accurate, reproducible and clinically useful results. For example, the exhalation of a molecule whose passage from the blood to the alveoli is diffusion-limited, may not be maximal at end-exhalation (Risby and Sehnert, 1999). There is also evidence that, in the case of highly soluble volatile compounds, gas exchange occurs in the airways rather than the alveoli, and that the concentration of such a compound in a sample of end-exhaled breath may not be in equilibrium with pulmonary blood (Hlastala, 2003). The site of production of the volatile compound may influence its concentration in a sample of
exhaled breath, and this may be of particular relevance to volatiles associated with airway inflammation. In the case of exhaled nitric oxide, for example, the major source of the compound appears to be the airways rather than the alveoli (Dweik et al, 1998) and, because of this, the concentration measured at the mouth is dependent on the expiratory flow and is not in equilibrium with the pulmonary blood (Silkoff et al, 1997). In the case of exhaled ammonia, the contribution from saliva and bacteria in the mouth leads to a higher concentration in exhaled breath measured in an oral versus a nasal sample (Kleinberg and Westbay, 1990; Smith et al, 2008). Conversely, the concentration of nitric oxide in the nasal passages is higher than in the mouth, leading to a lower concentration in orally exhaled breath (Silkoff et al, 1997). Such data have led some to suggest that a standardised method of breath collection should be used for the collection of volatile compounds in exhaled breath, in which a subject performs a single oral exhalation, based on the manoeuvre for measurement of FE\textsubscript{NO}, at a controlled flow against a resistance (Risby and Solga, 2006). However, this manoeuvre will not be appropriate for all volatile compounds. For example, if an expiratory manoeuvre is to be developed to assess the systemic concentration of ammonia, a nasal manoeuvre may be preferable to an oral one. A better approach may be to tailor the exhalation manoeuvre to the individual volatile compound (O'Hara et al, 2008).

**Control of ambient conditions**

When the concentration of a volatile compound is similar in the exhaled breath and the ambient air, correction for the background level in the ambient air can be difficult (Risby and Sehnert, 1999). If the concentration of the analyte in the inspired air is greater than 25% of the concentration in breath, results should be treated with caution, because the subject may not be in steady state with the local environment, thus introducing significant error into the results (Risby and Solga, 2006). The simplest solution is to subtract the concentration in the ambient air from that of the exhaled breath (Phillips, 1997), but this may not account for the sometimes complex exhalation physiology of trace volatile compounds (Miekisch et al, 2004). An alternative solution is to eliminate the ambient concentration of a volatile compound by asking a subject to breathe purified air: four minutes of pre-test breathing of purified air is sufficient to displace ambient air from the lungs of a healthy subject, but a longer period may be required before lipid soluble volatile compounds in the body equilibrate with the purified air (Risby and Sehnert, 1999). This may be impractical for routine clinical use.
External factors

The concentration of a volatile compound in exhaled breath may vary according to a number of external factors unrelated to the disease for which it is a potential biomarker. For example, a transient elevation in $\text{FE}_{\text{NO}}$ is observed after ingestion of nitrate-rich foods, and a reduction is seen after caffeine (Olin et al., 2001; Bruce et al., 2002). Smoking reduces the $\text{FE}_{\text{NO}}$ level, while respiratory tract infections may increase it (Kharitonov et al., 1995a; Kharitonov et al., 1995b; Robbins et al., 1997). There may also be diurnal or day-to-day variation in the concentration of the volatile compound. In the case of $\text{FE}_{\text{NO}}$ measurement, there is no significant diurnal or day-to-day variation (Kharitonov et al., 2003), whereas significant day-to-day variation has been observed in the concentration of exhaled acetone (Turner et al., 2006a). The clinical application of breath analysis for any volatile compound requires that such factors are understood and quantified (Risby and Sehnert, 1999).

Sampling considerations

There may be inter-relationships between the analytical technique, the analyte and the sampling method adopted for breath analysis. For example a technique such as GC-MS dictates that the sample be collected off-line rather than analysed on-line (Phillips and Greenberg, 1992). Sometimes the analyte may be unsuitable for collection and storage. For example, the sample integrity of acetone, ammonia and ethanol in Tedlar™ bags may be affected by the humidity and storage temperature (Neilsen, 2006), and only 65% of the original concentration of hexanal is recovered from a breath sample stored in a Tedlar™ bag for ten hours (Beauchamp et al., 2008), whereas exhaled nitric oxide may be stored in Mylar™ balloons for up to nine hours (Bodini et al., 2003). At times, it may be appropriate to modify a technique according to the concentration of the analyte in exhaled breath. For example, the sensitivity of the SIFT-MS technique is dependent on the time allowed for data acquisition through ion counting: in a second of ion counting, the instrument may have a limit of quantification of a few ppb; in ten seconds, the same analyte may be quantified at the 100 ppt level (Freeman and McEwan, 2002). In order to achieve this, off-line rather than on-line breath analysis would be necessary.

Once all of the above methodological issues have been addressed, the role of a specific volatile compound as a biomarker can then be investigated. A number of requirements have been suggested for a biomarker of chronic airways or lung diseases such as COPD (Stockley, 2007). It must be central to the pathophysiological process or must be a clear surrogate of that process. It must vary only with events known to relate to disease progression, and must
predict progression. Those individuals with a higher value at baseline must have either an increased risk of disease onset or greater disease severity. The biomarker must also be sensitive to interventions that are known to be effective. To fulfil these requirements, clinical studies similar to those performed in the development of $\text{FE}_{\text{NO}}$ measurement are necessary (Bates and Silkoff, 2003; Lim and Mottram, 2008; Sandrini et al).

1.2. **Nitric oxide in exhaled breath**

1.2.1. **Nitric oxide and its biological roles and reactions**

Nitric oxide (NO) is a free radical (i.e. it has an unpaired electron), but compared to other free radicals it is relatively stable (Braker and Mossman, 1975). NO is poorly soluble in water, and exists as a gas at room and body temperature. As a biological signalling molecule, its small size facilitates cell entry, and it avidly binds to transition metals such as iron (Henry et al, 1991), which are central to the function of many cytochromes and oxidases.

Nitric oxide has a role as a signalling molecule in a number of biological processes. Its actions are mediated by the interaction of nitric oxide with the haem prosthetic group of soluble guanylate cyclase in target cells, increasing the concentration of cyclic GMP (Moncada and Higgs, 1993). Via this biochemical pathway, NO causes vasodilation (Waldman and Murad, 1988); has a role in neurotransmission; regulates various gastrointestinal, respiratory, and genitourinary tract functions (Moncada and Higgs, 1993); contributes to the control of platelet aggregation and cardiac contractility (Moncada and Higgs, 1993); and performs some immunoregulatory functions (Bogdan, 2001).

NO also plays a role in host defence via the formation of reactive nitrogen species. Immune cells including phagocytic cells, such as neutrophils and eosinophils, are capable of synthesising NO, which then reacts with superoxide anion or via the hydrogen peroxide ($\text{H}_2\text{O}_2$) / peroxidase-dependent nitrite oxidation pathway to produce peroxynitrite ($\text{ONOO}^-$) and nitrogen dioxide ($\text{NO}_2$) (Bogdan, 2001). Peroxynitrite is a very powerful oxidant with a wide range of damaging effects, including lipid peroxidation and nitration of tyrosine residues. Thus it may be effective against infectious agents but, in excess, may also cause damage to host tissues (Szabo et al, 2007; Sugiura and Ichinose, 2008).
1.2.2. Biosynthesis of nitric oxide in the respiratory tract

The formation of nitric oxide is catalysed by nitric oxide synthase (NOS): L-arginine, NADPH and oxygen are converted to citrulline, NADP and NO. Four isoforms of the enzyme are recognised: constitutive neuronal NOS (nNOS) predominates in neuronal tissue, constitutive endothelial NOS (eNOS) is found in endothelial cells, and an inducible isoform (iNOS), is found in a wide variety of cells and tissues (Alderton et al., 2001).

The neuronal, endothelial and inducible NOS isoforms are expressed in the respiratory system. nNOS is found in nerve fibres that innervate airway smooth muscle, where NO is the major mediator for neural smooth muscle relaxation causing bronchodilation (Belvisi et al., 1992). eNOS is expressed in the epithelium of the nasal mucosa (Kawamoto et al., 1998), and the bronchial epithelium (Shaul et al., 1994), where it may modulate ciliary beat frequency (Jain et al., 1993), and in type II pneumocytes (Pechkovsky et al., 2002a). Expression of iNOS has been reported in alveolar macrophages (Pechkovsky et al., 2002b), type II pneumocytes, lung fibroblasts, airway and vascular smooth muscle cells, airway epithelial cells, mast cells, endothelial cells, neutrophils, and chondrocytes (Ricciardolo et al., 2004). iNOS is up-regulated by cytokines such as tumour necrosis factor – alpha (TNF-α) and interferon – gamma (IFN-γ) as well as bacterial toxins, viral infection, allergens and environmental pollutants (Ricciardolo et al., 2004). Airway epithelial iNOS is the main determinant of NO in the exhaled breath in both healthy and asthmatic subjects (Lane et al., 2004), and is up-regulated in the airway epithelium of asthmatic subjects, and down-regulated with corticosteroid treatment (Redington et al., 2001).

1.2.3. Physiology of nitric oxide exhalation

Nitric oxide levels vary throughout the respiratory tract: concentrations of up to 30,000ppb are observed in the nasal cavity and sinuses, whereas levels vary between 0 and 500ppb in the lower respiratory tract (Lundberg et al., 1995; Kharitonov et al., 1996a; Chatkin et al., 1999). The reason for high levels in the nasal cavity and sinuses is unclear, but may relate to sinus sterility, enhancement of ciliary motion, or modulation of lung V/Q relationship after being inhaled from the nose (Silkoff, 2008).

While the level of exhaled nitric oxide measured at the nose is a reflection of the nitric oxide concentration in the nasal cavity and sinuses (Silkoff et al., 1999), the level of exhaled nitric oxide measured at the mouth correlates with the concentration of nitric oxide in the lower
airways (Kharitonov et al, 1996a). Models of nitric oxide generation and distribution in the lower airways have steadily increased in complexity as understanding has improved.

The simplest model is a two-compartment one comprising alveolar and airway compartments (see Figure 1-2) (Tsoukias and George, 1998; George et al, 2004). The alveolar nitric oxide concentration is probably very low because of avid binding by haemoglobin in the pulmonary capillaries (Dweik et al, 1998). On exhalation, nitric oxide diffuses from the airway walls, down a concentration gradient, into the gas passing through the lumen. FE\textsubscript{NO}, measured at the outlet of the compartment, is dependent on the expiratory flow: as the flow approaches zero, nitric oxide concentration in the airway lumen increases towards that of the airway wall; as expiratory flow approaches infinity, nitric oxide concentration in the airway lumen decreases towards that of the alveolar compartment. A helpful analogy is that of a fluid passing through a section of heated pipe: at a high flow rate, there is less transit time for the fluid to be warmed than at a low flow rate. Hence, at the high flow rate, the temperature of the fluid exiting the pipe will be cooler than that of the fluid at the low flow rate.

**Figure 1-2** Two-compartment model for the exhalation of nitric oxide at a concentration of CE\textsubscript{NO} (or FE\textsubscript{NO}). Alveolar gas with NO concentration of CA\textsubscript{NO} passes through the airway compartment at flow V’. Nitric oxide in the airway wall, at a fixed concentration of Caw\textsubscript{NO}, diffuses down a concentration gradient into the airway lumen, where the NO concentration is C\textsubscript{NO}. The ease with which diffusion occurs is determined by the diffusing capacity of the airways (Daw\textsubscript{NO}). At a fixed expiratory flow, the flux of NO between the tissue and gas phase in the airway (Jaw\textsubscript{NO}), is the product of the diffusing capacity of the airways and the difference in NO concentration between the airway wall and lumen: Jaw\textsubscript{NO} = Daw\textsubscript{NO} x (Caw\textsubscript{NO} – C\textsubscript{NO}).
Modifications to the two-compartment model include the additional modelling of axial diffusion. At the low flows seen in the peripheral airways (on account of their large cross-sectional area in comparison to the proximal airways), nitric oxide diffuses against the direction of expiratory flow and into the alveolar compartment (Shin and George, 2002). The effect increases with a decrease in expiratory flow.

Further refinements to the model include the substitution of a trumpet-shaped airway compartment instead of a cylinder, to reflect the increasing surface area and nitric oxide contribution of the peripheral airways (Condorelli et al, 2007), and division of the lungs into three or four compartments to better reflect the heterogeneity of nitric oxide production (Condorelli et al, 2004; Kerckx and Van Muylem, 2009). The most recent of these suggests that airway generations 0 to 1, and airway generations 14 to 17, contribute 20 and 80% of nitric oxide production respectively, with no contribution from any other airway generations (Kerckx and Van Muylem, 2009).

1.2.4. Measurement of FE\textsubscript{NO} and sensor technology

The technique for online measurement of nitric oxide exhaled from the lower respiratory tract is described in American Thoracic Society / European Respiratory Society recommendations (ATS/ERS, 2005). The subject is seated comfortably, inserts a mouthpiece, and then inhales NO-free air over 2 to 3 seconds to TLC. The subject then exhales against an expiratory resistance of 5 to 20 cm H\textsubscript{2}O, at a constant expiratory flow of 50 ml/s for a minimum of 6 seconds. The subject self-regulates expiratory flow by biofeedback: the expiratory flow is presented in real time on a computer screen and the subject regulates expiratory effort to achieve the target flow. A profile of NO concentration over time is obtained, from which a 3 second plateau in concentration is measured. Repeated exhalations are performed to obtain at least two NO plateau measurements within 10% of each other.

The recommended inspiratory / expiratory manoeuvre is designed to eliminate the effects of variable and high concentrations of nitric oxide in the ambient air and nasal cavity, and to control the effect of variable expiratory flow. NO-free air is recommended for the inspiratory phase because, when inhaling NO-rich ambient air, an early NO peak in the exhalation profile is observed, probably because of ambient NO in the device and the NO present in the subject’s dead space (Silkoff et al, 1997). Exhalation against an expiratory resistance causes closure of the velopharyngeal aperture, thus excluding the nasal compartment from the exhalation, and minimising nasal NO contamination (Silkoff et al, 1997). Exhalation at a
standardised expiratory flow controls for the effect of flow on the concentration of exhaled NO (Silkoff et al, 1997).

Analysis of nitric oxide in exhaled breath is performed using an ozone chemiluminescence assay. Ozone reacts with nitric oxide in the gas phase to generate light (see reaction below). The luminescence, measured by a photomultiplier tube, is in direct proportion to the concentration of nitric oxide (Archer, 1993).

\[ \text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2 + \text{photon} \]

Using this assay, it is possible to perform on-line analysis of nitric oxide in exhaled breath. There are several chemiluminescence analysers, including the NIOX device (Silkoff et al, 2004) (NIOX; Aerocrine, Stockholm, Sweden). This measures nitric oxide concentration in the 2-200ppb range. It samples at a frequency of 20Hz, and has a suitably short lag time of <0.8s, and 10-90% response time of <0.7s. Its accuracy and precision are adequate for clinical applications. When measuring the concentration of NO in calibration gas, below 50ppb it has an accuracy (deviation from the mean value) of ±2.5ppb and a precision (expressed as SD) of <2.5ppb; above 50ppb, it has an accuracy of ±5% and a precision (expressed as coefficient of variation) of <5%. Regular recalibration is required for inherent drift of less than 3ppb over 14 days. The device was approved for clinical use by the US Food and Drug Administration in 2003.

While the instrument is appropriate for the measurement of nitric oxide in exhaled breath, its use is limited to specialist centres, because of its size and rigorous calibration requirements. Smaller, portable devices, using alternative analytical techniques that do not require recalibration, have recently become available. One such device employs an electrochemical sensor using the amperometric technique – the production of a current when a potential is applied between two electrodes (Hemmingsson et al, 2004) (NIOX-MINO; Aerocrine, Stockholm, Sweden). Real-time analysis is not possible because of a dynamic response time approaching 15s. However, a buffering unit allows storage of the last portion of the exhalation, which is then transferred to the sensor via a pump and valve system for analysis. Results using this analyser are comparable to those obtained using the ozone chemiluminescence assay (Menzies et al, 2007). A second portable analyser is the Apieron Insight (Apieron, Menlo Park, CA, USA), which uses a solid-state gel detection device suitable for office-based practice (Awabdy et al, 2010). Nitric oxide molecules in the exhaled
breath attach to highly specific protein molecules within a glass matrix. Light is passed through the matrix and an optical signal is produced, which is proportional to the concentration of nitric oxide. These smaller devices offer the possibility of office-based and even home-based \( \text{FE}_{\text{NO}} \) monitoring in the future.

1.2.5. **Rationale for the use of \( \text{FE}_{\text{NO}} \) measurements**

The measurement of \( \text{FE}_{\text{NO}} \) is clinically useful because it correlates with eosinophilic airway inflammation, and in turn, this inflammation is associated with a positive response to treatment with corticosteroid (Taylor *et al.*, 2006). In steroid-naïve asthma, \( \text{FE}_{\text{NO}} \) measurements correlate significantly with absolute sputum eosinophil counts and the percentage of eosinophils in induced sputum \( (r_s = 0.45-0.48) \) (Jatakanon *et al.*, 1998; Berlyne *et al.*, 2000). Levels of \( \text{FE}_{\text{NO}} \) also correlate significantly with the percentage of eosinophils in BAL fluid in asthmatic patients \( (r_s = 0.54-0.78) \) (Warke *et al.*, 2002; Lex *et al.*, 2006). \( \text{FE}_{\text{NO}} \) measurements and airway mucosal eosinophilia from endobronchial biopsy are elevated in both asthma and eosinophilic bronchitis compared to normal controls (Brightling *et al.*, 2003), and \( \text{FE}_{\text{NO}} \) measurements correlate with airway mucosal eosinophilia in asthma \( (r_s = 0.54) \) (Payne *et al.*, 2001). \( \text{FE}_{\text{NO}} \) levels rise and fall respectively with worsening and improving eosinophilic airway inflammation (Jatakanon *et al.*, 2000; Jones *et al.*, 2001; Leuppi *et al.*, 2001; Van Den Berge *et al.*, 2001; Covar *et al.*, 2003), and the relationship between \( \text{FE}_{\text{NO}} \) levels and airway eosinophilia is independent of the clinical diagnosis (Gratziou *et al.*, 1999; Henriksen *et al.*, 1999; Rutgers *et al.*, 1999; van den Toorn *et al.*, 2001; Brightling *et al.*, 2003; Fabbri *et al.*, 2003; Jouaville *et al.*, 2003).

It should be noted that some authors have argued that the correlation between \( \text{FE}_{\text{NO}} \) measurements and eosinophilic airway inflammation is inconsistent, and that elevated \( \text{FE}_{\text{NO}} \) levels may not reflect eosinophilic inflammation (Stick and Franklin, 2009). In a recent study, \( \text{FE}_{\text{NO}} \) levels were elevated in relation to the presence of airway hyper-responsiveness and response to bronchodilator even in the absence of significant sputum eosinophilia (Cowan *et al.*, 2010). Certainly, the correlation between \( \text{FE}_{\text{NO}} \) and eosinophilia is imperfect, with a number of other factors influencing \( \text{FE}_{\text{NO}} \) levels, including age, gender, height, atopy, respiratory tract infection and smoking status, and even these factors only explain around 10% of the variance in healthy subjects (Olin *et al.*, 2007; Dressel *et al.*, 2008). With a sensitivity and specificity of approximately 70% in predicting sputum eosinophilia (Berry *et al.*, 2005), the performance of \( \text{FE}_{\text{NO}} \) is similar in predicting elevated BAL eosinophils (sensitivity 70%,}
specificity 79%) and bronchial mucosal eosinophilia (sensitivity 60%, specificity 59%), and can only be described as “fair” (Lex et al, 2006).

Nevertheless, the relationship between F_{ENO} and eosinophilic airway inflammation is important because eosinophilic inflammation is associated with a positive response to corticosteroid treatment. Treatment with corticosteroid reduces the number of eosinophils seen on bronchial biopsy and in the induced sputum of asthmatic patients, and results in a simultaneous clinical improvement (Djukanovic et al, 1997; Lim et al, 1999). Conversely, asthmatic patients with a low percentage of eosinophils in the sputum show a poor response to corticosteroid (Pavord et al, 1999; Green et al, 2002b). The relationship between eosinophilic airway inflammation and corticosteroid response is similar in COPD: a subgroup of COPD patients with airway eosinophilia demonstrates an improvement in airflow obstruction and symptoms in response to corticosteroid, while the majority of COPD patients show little or no response (Pizzichini et al, 1998; Brightling et al, 2000). An assessment of eosinophilic airway inflammation is therefore potentially clinically useful in differentiating between those patients with airway diseases who require corticosteroid therapy and those who do not.

Elevated F_{ENO} levels have been shown to predict the response to corticosteroid in patients with asthma and chronic cough: at optimum F_{ENO} cut-points, positive and negative predictive values were 83-90% and 72-85% respectively (Little et al, 2000; Szefer et al, 2005; Hahn et al, 2007). In a study of patients with undiagnosed respiratory symptoms, baseline F_{ENO} was superior to physiological measures including spirometry, bronchodilator response and airway hyperresponsiveness, for predicting the response to inhaled corticosteroid (ICS) (Smith et al, 2005a). The introduction of ICS treatment results in a fall in F_{ENO} in asthmatic patients (Kharitonov et al, 1996b; Pijnenburg et al, 2005a; Malerba et al, 2008), while withdrawal of ICS results in an increase in F_{ENO} (Jones et al, 2001). Importantly, there is a dose-dependent relationship between the two, with higher doses of ICS resulting in faster and greater falls in F_{ENO} levels (Jones et al, 2002; Kharitonov et al, 2002), and this dose-response relationship shows good within-patient reproducibility (Silkoff et al, 2001).

1.2.6. Clinical applications of F_{ENO} measurement

F_{ENO} measurements are useful for predicting the response to corticosteroid in steroid-naïve patients. In symptomatic patients, high levels (>50ppb) predict a response (Pijnenburg et al, 2005b; Smith et al, 2005a), while low levels (<25ppb) predict the absence of a response (Zacharasiewicz et al, 2005). F_{ENO} measurements between 25 and 50ppb are difficult to
interpret because of the overlap between the $\text{FeNO}$ range seen in healthy subjects (Olin et al., 2007) and the range seen in patients with airway inflammation (Kostikas et al., 2008).

Two proof of concept studies showed that using either airway hyper-responsiveness (AHR) (Sont et al., 1999) or sputum eosinophils (Green et al., 2002a) to guide therapy with inhaled corticosteroids, the frequency of asthma exacerbations could be reduced. Accordingly, a number of studies have been carried to evaluate whether the regular monitoring of $\text{FeNO}$ levels might be beneficial in the management of chronic eosinophilic airway inflammation such as that seen in eosinophilic asthma (Pijnenburg et al., 2005a; Smith et al., 2005b; Fritsch et al., 2006; Shaw et al., 2007; Szefler et al., 2008; de Jongste et al., 2009). However, the results of studies comparing treatment algorithms with and without $\text{FeNO}$ measurements have not been definitive. Using $\text{FeNO}$ measurement as part of a treatment algorithm has been shown to reduce the maintenance dose of ICS without detriment to asthma control (Smith et al., 2005b), and to improve airway hyperresponsiveness (Pijnenburg et al., 2005a). However, its addition does not improve symptoms over and above standard management (de Jongste et al., 2009), and no study has yet shown a significant decrease in exacerbation rates.

In order to determine the role of $\text{FeNO}$ measurement in the monitoring of asthma, some aspects of study design and methodology may need to be addressed. Foremost amongst these, to discern any benefit of $\text{FeNO}$ measurement, a treatment algorithm incorporating $\text{FeNO}$ measurement must result in sufficiently different management decisions from an algorithm based on current best practice (Gibson, 2009). At present, the role of $\text{FeNO}$ measurement in asthma monitoring remains uncertain.

### 1.2.7. $\text{FeNO}$ measurement in COPD

COPD is a very common respiratory disease, giving rise to substantial morbidity and mortality, and its incidence is increasing (Mannino and Buist, 2007). The principal symptoms are breathlessness, cough and sputum production and, in end-stage disease, chronic respiratory failure and disability. The disease is characterised by airflow limitation that is not fully reversible and this is usually progressive. It is caused by chronic inflammation of the airways and lung parenchyma, usually secondary to tobacco smoke exposure (GOLD, 2008). The typical profile of inflammatory cells present in the airways of patients with COPD includes neutrophils, macrophages and T-helper 1 cells, which is in contrast to the eosinophilic inflammation commonly seen in asthma (Barnes, 2008).
The neutrophilic inflammation seen in COPD is poorly responsive to corticosteroid (Keatings et al., 1997). Nevertheless, ICS treatment is widely used in patients with COPD. ICS are recommended for reducing exacerbation frequency in severe disease (GOLD, 2008), but their effectiveness remains controversial (Suissa and Barnes, 2009): they do not reduce overall mortality (Drummond et al., 2008), and overall, have only borderline effects on lung function or quality of life (Suissa and Barnes, 2009). One of the challenges in the treatment of COPD is to identify potential “steroid responders”. This has always been a challenge, and early studies were unable to demonstrate a relationship either between bronchodilator response and steroid response or between short term trials of steroid and outcomes during long term treatment (Yang et al., 2007). COPD is heterogeneous (Marsh et al., 2008), and there is a recognised subgroup of patients who may potentially benefit from inhaled corticosteroid treatment (Weir et al., 1990; Weir and Burge, 1993). Against a background of concerns that treatment with ICS may predispose to pneumonia in at-risk patients (Drummond et al., 2008; Singh et al., 2009), identifying and treating responders selectively is potentially important in improving the overall risk-benefit ratio for ICS therapy.

Eosinophilic airway inflammation is present in a subgroup of stable COPD patients (Chanez et al., 1997; Brightling et al., 2000; Leigh et al., 2006), and also in some patients with acute exacerbations (Zhu et al., 2001; Fujimoto et al., 2005). There is evidence that “steroid responders” are more likely to be characterised by the presence of eosinophilic airway inflammation. Studies have shown that sputum eosinophilia in patients with COPD is associated with a short-term response to corticosteroid, demonstrated by increased airway calibre and improved health-related quality of life (Pizzichini et al., 1998; Brightling et al., 2000; Brightling et al., 2005; Leigh et al., 2006). As a surrogate for eosinophilia, measurement of FE\textsubscript{NO} may have a useful role in COPD for detecting the presence of steroid-responsive eosinophilic airway inflammation.

FE\textsubscript{NO} levels in patients with stable COPD, measured according to ATS/ERS recommendations, are only occasionally elevated compared to controls (Delen et al., 2000; Fabbri et al., 2003; Beg et al., 2009). However, a significant correlation between FE\textsubscript{NO} levels and the percentage of eosinophils in sputum has been reported in patients with COPD ($r_s = 0.65$), in contrast to healthy subjects, in whom no correlation was seen (Rutgers et al., 1999). Similarly, even though FE\textsubscript{NO} levels may be within the “normal” range, there is a significant relationship between baseline FE\textsubscript{NO} and the subsequent increase in FEV\textsubscript{1} after bronchodilator (Papi et al., 2000) or inhaled corticosteroid (Ferreira et al., 2001; Zietkowski et al., 2005). COPD patients
with partial reversibility of their airflow limitation (increase in FEV\textsubscript{1} of <12% but >200 ml after 200 mcg of inhaled salbutamol) have been shown to have a higher Fe\textsubscript{NO} than patients with no reversibility (increase in FEV\textsubscript{1} of <12% and <200 ml after 200 mcg of inhaled salbutamol): 24 (15.3 to 32) ppb (median (interquartile range)) versus 8.9 (4.6 to 14.7) ppb, p < 0.01 (Papi \textit{et al}, 2000). An inverse correlation between changes in Fe\textsubscript{NO} and changes in FEV\textsubscript{1} after a two-week course of ICS has been demonstrated ($r = -0.50$, $p = 0.02$) (Ferreira \textit{et al}, 2001) and, importantly, another study has shown that the baseline corticosteroid-naïve level of Fe\textsubscript{NO} correlates with the increase in post-bronchodilator FEV\textsubscript{1} after two months of ICS therapy ($r = 0.73$, $p < 0.001$) (Zietkowski \textit{et al}, 2005). While unable to demonstrate any correlation between baseline Fe\textsubscript{NO} and the increase in FEV\textsubscript{1} after four weeks of ICS therapy, a further study, in a post-hoc analysis, showed that baseline Fe\textsubscript{NO} predicted clinically significant increase in FEV\textsubscript{1} in response to ICS: the area under the receiver operator characteristic curve was 0.72 (95% CI: 0.53 to 0.91) (Kunisaki \textit{et al}, 2008). These studies suggest that Fe\textsubscript{NO} may be useful as a predictor of response to corticosteroid in stable COPD.

Although airway NO and Fe\textsubscript{NO} may be normal in stable COPD, there is an increase in peripheral NO that is related to disease severity (Hogman \textit{et al}, 2002; Brindicci \textit{et al}, 2005). Because of this, it has been suggested that a measure of the alveolar concentration of nitric oxide, using multiple expiratory flows, may be a more useful biomarker than Fe\textsubscript{NO} (Barnes \textit{et al}, 2006). However, previous calculations of peripheral levels of NO using the multiple-flow technique have been based on the two-compartment model of nitric oxide exhalation (Hogman \textit{et al}, 2002; Brindicci \textit{et al}, 2005) (see Figure 1-2), and recent, more sophisticated models suggest that the apparently elevated alveolar concentration of nitric oxide in COPD may be an artefact caused by increased axial back-diffusion of nitric oxide in the airway lumen during exhalation (Kerckx and Van Muylem, 2009; Malinovschi \textit{et al}, 2009; Gelb \textit{et al}, 2010). The role of the multiple-flow technique in COPD is therefore uncertain at present.

Fe\textsubscript{NO} is known to be elevated in acute exacerbations of COPD (Maziak \textit{et al}, 1998; Bhowmik \textit{et al}, 2005; Antus \textit{et al}, 2010), and an elevated Fe\textsubscript{NO} level predicts a greater response to treatment of the acute exacerbation (Antus \textit{et al}, 2010). The underlying cause of increased Fe\textsubscript{NO} measurements in acute exacerbations is uncertain, but may be related to eosinophilic inflammation secondary to viral infection. Acute exacerbations are frequently precipitated by the advent of acute viral infection (Mohan \textit{et al}, 2010) and, in a study demonstrating the utility of sputum eosinophilia as a predictor of viral aetiology in acute exacerbations, Fe\textsubscript{NO} and sputum eosinophil percentage correlated significantly in the viral exacerbation group ($r_s =$
0.67) (Papi et al, 2006). The relationship between $\text{FE}_{\text{NO}}$, sputum eosinophilia and viral infection in acute exacerbations is intriguing, and raises the question of the underlying cause of eosinophilic airway inflammation in stable COPD. Whether eosinophilic inflammation in stable COPD is simply a manifestation of disease at an intermediate point on the spectrum between asthma and COPD (as per the “Dutch hypothesis” (Orie et al, 1961)), or whether it has a separate cause, such as viral persistence (Sikkel et al, 2008), remains unknown, and is beyond the scope of the present work.

$\text{FE}_{\text{NO}}$ measurement can predict response to corticosteroid in diseases of the airways (Pavord et al, 2008), but its predictive utility in COPD has not yet been systematically assessed. If it were to fulfil a role as a predictor of corticosteroid response in COPD, $\text{FE}_{\text{NO}}$ measurement would be of benefit to patients and their physicians in deciding whether to initiate ICS therapy.

1.3. Hydrogen sulphide in exhaled breath

1.3.1. Hydrogen sulphide and its biological roles and reactions

Hydrogen sulphide ($\text{H}_2\text{S}$) is a colourless gas at room temperature, somewhat soluble, with a characteristic smell of rotten eggs. Its odour is offensive above concentrations of 10 to 100ppb (Shusterman, 1992) and, if inhaled at concentrations of above 200 parts per million (ppm), the gas can be lethal within minutes (Woodall et al, 2005). Its toxicity is mediated by the inhibition of the mitochondrial enzyme, cytochrome c oxidase, preventing respiration (Nicholls and Kim, 1982; Khan et al, 1990).

$\text{H}_2\text{S}$ has recently received increasing attention, as mounting evidence suggests that it is the third endogenous “gasotransmitter” to be discovered after nitric oxide and carbon monoxide (Wang, 2002; Wang, 2010). Whilst toxic at higher concentrations, these molecules have biological roles at physiological levels (Wang, 2003). The gasotransmitters are endogenous gaseous transmitters with a number of features that differentiate them from other classical transmitters and humoral factors (Wang, 2003): they are gaseous molecules; they are freely permeable to membranes and do not rely on membrane receptors; they can have endocrine, paracrine and autocrine effects; they have defined and specific functions at physiological concentrations; and their cellular effects may or may not be mediated by second messengers, but they have specific cellular and molecular targets. Furthermore, haemoglobin may be the
common “sink” for the three molecules, with NO, CO and H₂S forming nitrosyl haemoglobin, carboxyhaemoglobin and sulphhaemoglobin respectively (Park and Nagel, 1984; Wang, 1998).

In mammalian cells, H₂S is mostly synthesised from L-cysteine by the enzymes cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Wang, 2002). Production of H₂S in the human brain has been attributed to CBS (Boehning and Snyder, 2003; Kimura and Kimura, 2004), while CSE has been detected in vascular smooth muscle and endothelial cells (Pryor et al, 2006). The distribution of the two enzymes elsewhere in the body is not well-characterised (Lefer, 2007). H₂S can be detected in the blood of normal human subjects at concentrations of 30–40 μmol/l (Chen et al, 2005).

A number of biological roles for H₂S are emerging. The most closely studied of these is its role as a vasorelaxant. Production of H₂S in vascular endothelial cells is triggered by muscarinic cholinergic activation in a similar manner to NO (Wang, 2009). The mechanism of action of H₂S in smooth muscle cells differs from that of NO, in that it opens smooth muscle K⁺ATP channels, whereas NO acts via guanylyl cyclase (Wang, 2009). However, there is growing evidence that the two gasotransmitters interact in regulating vasorelaxation (Whiteman et al, 2006; Kubo et al, 2007b). In its role as a smooth muscle relaxant, H₂S may also regulate movement of material through the small intestine (Fiorucci et al, 2006), and cause bronchodilation in the lungs (Kubo et al, 2007a).

In various animal models of inflammation, increased H₂S-synthesising enzyme activity and plasma H₂S levels have been observed. In addition, prophylactic treatment with an enzyme-inhibitor has been shown to attenuate the inflammatory response (Bhatia et al, 2005; Li et al, 2005; Zhang et al, 2006). H₂S may affect the inflammatory process by regulation of leukocyte function, trafficking and survival (Zhang and Bhatia, 2008), and also by stimulating the release of pro-inflammatory neuropeptides such as substance P and calcitonin gene-related peptide (Patacchini et al, 2004; Trevisani et al, 2005; Zhang et al, 2007). Despite this evidence, the role of H₂S in inflammation is not yet clear, and other studies in animal models have demonstrated that H₂S may have an anti-inflammatory effect. For example, H₂S appears to protect the gastric mucosa against aspirin-induced injury (Fiorucci et al, 2005), and endogenous H₂S reduces airway inflammation and remodelling in a rat model of asthma (Chen et al, 2009a). In this last study, generation of endogenous pulmonary H₂S was downregulated in rats with ovalbumin (OVA)-induced asthma, while exogenous administration of
NaHS (an H₂S donor) improved airway inflammation. There was some evidence that the anti-inflammatory effect was mediated by iNOS inhibition, again suggesting an interaction between H₂S and NO.

1.3.2. Hydrogen sulphide in COPD

Altered levels of serum H₂S have been demonstrated in patients with COPD (Chen et al., 2005). Investigators showed that serum H₂S levels in patients with stable COPD were higher than in healthy subjects, and that levels were higher in stable COPD than during acute exacerbations. Interestingly, the same pattern was observed for serum NO levels. Furthermore, serum H₂S levels were positively correlated with those of serum NO, and with the percentage of predicted FEV₁, and negatively correlated with the proportion of sputum neutrophils. While these findings suggest that H₂S levels may be associated with disease activity and severity, they may or may not be associated with the inflammatory response. Alternatively, levels of H₂S in COPD may change in relation to hypoxic pulmonary arterial vasoconstriction as has been established in other mammals (Olson et al., 2006; Olson et al., 2010).

1.3.3. Analysis of hydrogen sulphide in exhaled breath

Measurement of H₂S levels in exhaled breath might be a feasible method for the measurement of systemic and/or airway levels. For a long time, it has been known that intravenous injection of a solution of H₂S gas results in its exhalation within seconds (Bernard and Tripier, 1857), and this has been confirmed in more recent experiments (Insko et al., 2009). The measurement of levels of H₂S originating from the lower respiratory tract in humans has recently been attempted (Furne et al., 2008). Ten normal volunteers inhaled and performed a breath-hold for 15 seconds, with their mouths open to reduce the accumulation of oral, bacterially-produced H₂S. They then rapidly exhaled through a tube from which 20 ml of end-exhaled air was aspirated. The sample was then analysed using a gas chromatograph and chemiluminescence sulphur detector. H₂S levels in end-exhaled breath were around 1.5 ppb, compared with 1.2 ppb in the ambient air. These results must be interpreted with caution, however, because the contribution from oral bacteria is difficult to quantify.

Because oral bacteria produce H₂S, and this can be detected in exhaled breath (Rosenberg and McCulloch, 1992; Suarez et al., 2000), eliminating this source of contamination presents a challenge. One possibility is the temporary elimination of the bacteria themselves: rinsing the
mouth with 3% H\textsubscript{2}O\textsubscript{2} for one minute can reduce the H\textsubscript{2}S levels in the oral cavity from around 500 ppb to less than 30 ppb (Suarez et al., 2000). A second possibility is the collection of nasally-exhaled breath for analysis: a small study of two subjects showed that nasally-exhaled levels of H\textsubscript{2}S were around ten times lower than those in orally-exhaled breath (Pysanenko et al., 2008). H\textsubscript{2}S in humid air can be quantified by SIFT-MS (Spanel and Smith, 2000b), and the technique presents an opportunity to explore breathing manoeuvres that minimise the contamination of exhaled breath by H\textsubscript{2}S from oral bacteria, and to assess the potential of H\textsubscript{2}S measurement in exhaled breath as a biomarker of airway inflammation.

1.4. Exhaled Hydrogen Cyanide

1.4.1. Hydrogen cyanide and its biological roles and reactions

Hydrogen cyanide (HCN) is a colourless chemical compound, miscible in water, with a boiling point of 26°C making it volatile at the temperature of exhaled breath. It is best known for its toxicity: the cyanide ion inhibits the mitochondrial enzyme, cytochrome c oxidase, in a similar manner to H\textsubscript{2}S (Albaum et al., 1946). Inhalation at a concentration of around 3000ppm is lethal to mammals within minutes (Ballantyne, 1983).

Despite its toxicity, there is evidence that HCN is produced in humans. In the 1950s, the formation of cyanide in blood was demonstrated in humans following administration of thiocyanate, with the subsequent discovery of conversion of thiocyanate to cyanide by an erythrocytic enzyme (Goldstein and Rieders, 1951; Goldstein and Rieders, 1953; Goldstein et al., 1953). At a similar time, excretion of HCN in trace amounts in the exhaled breath was also discovered (Boxer and Rickards, 1952). Further study has shown that the concentration of HCN in exhaled breath is higher than would be expected from the blood concentration, and that the additional HCN is generated from oxidation of thiocyanate by salivary peroxidase in the oropharynx (Lundquist et al., 1988).

Hydrogen cyanide is produced by leukocytes, making the compound a possible biomarker of inflammation and infection. Cyanide has been detected in a mixture of thiocyanate, myeloperoxidase (MPO) and hydrogen peroxidase (Sorbo and Ljunggren, 1958), and there is evidence that thiocyanate is a major substrate of myeloperoxidase at physiological concentrations (van Dalen et al., 1997). It was initially thought that HCN was a product of the oxidation of thiocyanate by hydrogen peroxide, but this theory has since been challenged, and
alternative reactions of thiocyanate, with minimal generation of HCN, have been suggested. However, HCN generation depends on the reaction conditions, therefore its formation via this reaction pathway in vivo remains plausible (Wilson and Harris, 1961; Figlar and Stanbury, 2000). In support of in vivo HCN generation by leukocytes, HCN formation has been noted in the action of MPO/H$_2$O$_2$/Cl$^-$ on peptides (Stelmaszynska and Zgliczynski, 1978), and in the action of MPO/H$_2$O$_2$/Cl$^-$ on phagocytosed bacteria in neutrophils (Stelmaszynska, 1985). In this last study, the authors noted that chlorination of S epidermidis resulted in larger amounts of HCN than chlorination of E coli, and suggested that HCN was liberated as a result of chlorination of polyglycyl peptides present in the cell wall of S epidermidis but not in E coli (Stelmaszynska, 1985). The same group demonstrated the creation of HCN from thiocyanate by leukocytes challenged with S epidermidis (Stelmaszynska, 1986).

While the role of thiocyanate oxidation products from leukocytes has not been fully elucidated (van Dalen et al, 1997), some evidence of their function is emerging (Wang et al, 2006). Any biological role of hydrogen cyanide remains unclear, but one possible function is the stimulation of the respiratory burst that accompanies phagocytosis in order to degrade internalised particles and bacteria (DeChatelet et al, 1977). Little work has been done on the role of HCN in leukocyte function since the mid-1980s and further studies are needed (Ryall et al, 2008).

HCN is also produced by P aeruginosa, a known respiratory pathogen. This organism manufactures HCN from a membrane-bound HCN synthase, its production possibly giving it an advantage over competing organisms in soil (Goldfarb and Margraf, 1967; Blumer and Haas, 2000). Hydrogen cyanide has been detected in the headspace above cultures of P aeruginosa (Carroll et al, 2005), and cyanide has been found in the sputum of cystic fibrosis (CF) patients infected with P aeruginosa (Ryall et al, 2008; Sanderson et al, 2008). While cyanide was undetectable in the sputum of normal controls and eight out of nine CF patients without P aeruginosa infection, measurable levels were found in the sputum of all seven patients infected with P aeruginosa (Sanderson et al, 2008). In another similar study, cyanide was detected in the sputum of 15 out of 25 CF and non-CF bronchiectasis patients with P aeruginosa infection, and not detected in any of 10 patients without this organism (Ryall et al, 2008).
1.4.2. Analysis of hydrogen cyanide in exhaled breath

HCN has been quantified at values of between 1 and 60 ppb in the orally exhaled breath of healthy normal subjects (Spanel et al, 2007; Stamyr et al, 2009). Measurements from nasally exhaled breath have since been found to be 2-14 times lower than those from oral exhalations (Wang et al, 2008), consistent with the known source of contamination from HCN production by salivary peroxidase in the oral cavity (Lundquist et al, 1988).

HCN in exhaled breath has recently been examined as a biomarker of respiratory tract infection or colonisation with *P aeruginosa* (Enderby et al, 2009b), and elevated levels of HCN have been demonstrated in children with CF compared to children with asthma (13.5 vs. 2.0 ppb, \( p < 0.001 \)). However, measurements were taken from oral exhalations, and whether these results reflect levels of HCN in the lower airways is therefore uncertain.

The question of whether HCN in exhaled breath might be a biomarker for airway inflammation remains unexplored. The detection and quantification of HCN in humid air and breath has previously been demonstrated using SIFT-MS (Spanel et al, 2004; Spanel et al, 2007), and this technique presents an opportunity to explore HCN measurement in exhaled breath as a biomarker of airway inflammation and *P aeruginosa* infection.

1.5. Selected Ion Flow Tube – Mass Spectrometry

1.5.1. Analysis of volatile compounds using SIFT-MS

Selected ion flow tube – mass spectrometry is an analytical technique that can be used for real-time quantification of trace gases in a gas mixture at concentrations as low as a few parts per billion. The technique was originally developed to study the reactions between ions and neutral molecules that are thought to occur in interstellar gas clouds, but also lends itself to the detection and quantification of trace gases present in air and breath (Freeman and McEwan, 2002).

In conventional mass spectrometry, a gas sample is ionised by passing through an electron beam, to create an ion source. Ions from this source are then passed through a mass analyser, which applies an electromagnetic field to the sample and sorts the ions according to their mass-to-charge ratios. The ions then pass into a detector that records either the current
produced or the charge induced when an ion hits a surface or passes by. Data from the detector is then analysed to calculate the abundance of each ion present (Sparkman, 2000). Analysis of trace gases in air or breath using conventional electron ionisation mass spectrometry is difficult because of excessive gas loading of the ion source by the constituents of the bulk matrix of air or exhaled breath, such as N₂, O₂ and water vapour (Smith and Spanel, 2005b). Furthermore, traditional mass spectrometry requires an interpretation of many mass fragments for each compound (Smith and Spanel, 2005b). SIFT-MS overcomes these problem by chemical ionisation of the gas sample using reagent ions (for example, H₃O⁺, O₂⁺ and NO⁺) that do not react with the major constituents of air and breath (Smith and Spanel, 2005b). This ‘soft’ chemical ionisation greatly reduces the fragmentation of trace gases in the sample when compared to traditional mass spectrometry (Smith and Spanel, 2005b).

**Figure 1-3** Schematic diagram of SIFT-MS.

A schematic diagram of a SIFT-MS instrument is shown in Figure 1-3. A mix of positive ions is created when air or water vapour passes through a microwave resonator. These ions are then discharged into a quadrupole mass spectrometer that selects reagent ions (usually H₃O⁺, O₂⁺ or NO⁺) by their mass-to-charge ratio and injects them into a fast-flowing stream of inert carrier gas via a Venturi-type orifice. The stream of inert gas (for example, helium at a pressure of approximately 1 Torr) passes down a flow tube of around 30-100 cm length, at a velocity of around 100 m/s. The gas sample enters the flow tube near the up-stream end via a heated calibrated capillary at a known flow rate. The reagent ion and the sample react with
each other as they pass down the flow tube, and characteristic product ions are formed from
the reactions of each reagent ion with each volatile compound. The reagent and product ions
are sampled from the down-stream end of the flow tube, via a pinhole orifice, into a
differentially-pumped quadrupole mass spectrometer and ion-counting system for detection
and analysis. The instrument may be operated in either Mass Scan mode (in which the
detector quadrupole mass spectrometer scans a predetermined mass range to obtain a
spectrum of product ions) or Selected Ion Monitoring mode (in which product ions of interest
are pre-selected by the operator). More detailed descriptions of the SIFT-MS technique are
available elsewhere (Spanel and Smith, 1996; Freeman and McEwan, 2002; Smith and
Spanel, 2005b). An example of a reaction of the reagent ion, H$_3$O$^+$, with trace gas, M, is
shown in equation (1). A proton transfer reaction takes place to create the product ion, MH$^+$:

$$H_3O^+ + M \rightarrow MH^+ + H_2O \quad (1)$$

The loss of H$_3$O$^+$ reagent ions and the production of MH$^+$ ions are dependent on the
concentration of M in the carrier gas, [M]. The count rates of the H$_3$O$^+$ ions and the MH$^+$ ions
at the downstream ion-counting system are given by the relationship in equation (2):

$$[MH^+]_t = [H_3O^+] k [M] t \quad (2)$$

Quantification of the analyte is possible because the rate coefficient ($k$) for the reaction
between the analyte and the reagent ion is known, as is the flow velocity and integration time
($t$). Hence, [M] can be determined. Quantification can be achieved in near-real time using
onboard software and databases of previously determined rate coefficients (Smith and Spanel,
2005b).

Exhaled breath is saturated with water vapour, and this complicates the analysis of trace
gases. Cluster ions such as H$_3$O$^+$(H$_2$O)$_{1,2,3}$ form in the carrier gas, and may react with the
trace gas species, M, to form ions like MH$^+$(H$_2$O)$_{1,2,3}$. While adding to the number of product
ions to be monitored, and increasing the complexity of calculation, these additional product
ions must be included for the accurate quantification of M (Smith and Spanel, 2005b). This
has been done for a number of analytes (Spanel et al, 1997b; Spanel and Smith, 2000b; Spanel et al, 2004).
Detection

The detection limit of a SIFT-MS instrument depends on the count rate of the reagent ions at the downstream ion-counting system, the rate reaction coefficient, the integration time (see reaction (2)) and the instrumental background signal associated with the analyte (Milligan et al., 2007). The integration time (t) can be altered depending on the application of the SIFT-MS technique. For example, when performing on-line selected ion monitoring of an exhalation, a sampling period of around 1 second or less may be required, whereas a longer sampling period may be used when very low limits of detection and/or quantification are required (Milligan et al., 2007). Using a 10 second integration time, a limit of detection of 200 ppt has been reported (Milligan et al., 2007), but for on-line breath analysis, limits are typically 1-2 ppb (Smith and Spanel, 2005b; Enderby et al., 2009a).

Accuracy

The accuracy of the SIFT-MS technique for the analysis of trace gases in dry air has been established for a number of gases, including ethanol, acetone, benzene, toluene and xylene, using known concentrations of the gases ranging from 10 ppb to 20 ppm, prepared by both the syringe injection and permeation tube methods. The accuracy of measurement was within 10% of the true value for these organic compounds (Spanel et al., 1997a; Smith et al., 1998). The precision of the SIFT-MS technique depends on the number of product ions counted during a single measurement: the standard error of each measurement is determined from the square root of the total number of product ions counted by the downstream ion-counting system (Smith and Spanel, 2005a). Typical values for the standard error of measurement of ammonia and acetone concentrations in a single exhalation have been reported at ±5% to ±20% (Smith and Spanel, 2005b). To date, instrumental accuracy and reproducibility data have not been routinely given in SIFT-MS breath analysis studies (Turner et al., 2006a; Turner et al., 2006d; Turner et al., 2006c; Turner et al., 2006b; Enderby et al., 2009b). However, the repeatability of SIFT-MS analysis has been investigated in a recent study (Boshier et al., 2010): single exhalations were collected into sample bags, then analysed once for 60 seconds and then immediately reanalysed for a second time, for a further 60 seconds. A number of breath analytes were studied. The instrument-specific coefficients of variability were low (1%) for breath analytes at relatively high concentrations, such as acetone (500-1000ppb), but higher for analytes present at concentrations less than 10ppb, – for example, 19% in the case of hydrogen cyanide. Analyte concentration and consequent product ion count rate were identified as key determinants of measurement variation. The authors recommended further
investigation of repeatability using accurately known gas standards, and also further investigation of intra-day and inter-day repeatability.

**Dynamic response**

The dynamic response time for the SIFT-MS technique has previously been reported as 20 ms (Spanel et al., 1996; Smith and Spanel, 2005b). However, this figure is at variance with our own early work using an instrument adapted for breath analysis, during which we found the 0-90% response time for acetone at physiological concentrations, to be 500 ms (Dummer et al., 2007). Whether this discrepancy is related to differing breath sampling systems upstream of the SIFT-MS sample inlet is unclear. Importantly, despite the difference in these figures, in each case the dynamic response is appropriate to on-line analysis of single exhalations of 5 seconds duration or more (Bates et al., 1983).

**Specificity**

The SIFT-MS technique is not always specific in its identification of an analyte. For example, in their reactions with the $O_2^+$ reagent ion, chlorofluorocarbons (found in some metered-dose inhalers) and ammonia dichloramine (a potential biomarker of airway inflammation) both generate product ions at $m/z$ 85 and 87, and this hampers efforts to detect ammonia dichloramine in the breath of patients taking chlorofluorocarbon-containing inhalers (Epton et al., 2009). However, the problem of isobaric product ions can usually be overcome by using multiple reagent ions to confirm the identity of a trace gas. For example, propanal and acetone both react with the $H_3O^+$ ion to give a product ion at $m/z$ 59, but can be distinguished by using the $NO^+$ reagent ion, which reacts to give product ions with different masses for each of these compounds (Smith and Spanel, 2007). This flexibility gives the SIFT-MS technique a significant advantage over proton transfer reaction – mass spectrometry, which is a similar soft ionization technique restricted to the use of $H_3O^+$ reagent ions (see Section 1.1.2, Page 4) (Lagg et al., 1994).

The SIFT-MS technique also has some important advantages over the competing technology of GC-MS, which can be used for trace gas analysis in samples of air and breath at the ppb and ppt level. Using this latter technique, collection of the trace gases from relatively large volumes of air and breath samples is usually required onto adsorption traps (Phillips and Greenberg, 1992), and on-line monitoring is not possible. On-line SIFT-MS analysis of trace gases in exhaled breath obviates the need for sample collection into bags or onto traps, which can compromise the sample (Neilsen, 2006), and allows closer study of the physiology of
trace gas exhalation (Dummer et al, 2007). Additionally, SIFT-MS can be used for the analysis of small molecules that are difficult to identify using GC-MS, for example, ammonia and formaldehyde (Phillips and Greenberg, 1992; Sanchez and Sacks, 2003).

1.5.2. Breath analysis using SIFT-MS

The SIFT-MS analytical technique has shown promise in the on-line analysis of several volatile compounds in exhaled breath (Turner et al, 2006a; Turner et al, 2006d; Turner et al, 2006c; Turner et al, 2006b; Enderby et al, 2009b). It has also been used for off-line analysis (Lad, 2006; Hryniuk and Ross, 2009; Boshier et al, 2010), and for analysis of the headspace over exhaled breath condensate (Cap et al, 2008). Attempts have been made to establish a normal range for trace gases that are readily detected by SIFT-MS (see Table 1-1), there has been some exploration of the effects of variables such as sex and diet (Turner et al, 2006a; Turner et al, 2006d; Turner et al, 2006c; Turner et al, 2006b), and some studies have investigated potential clinical applications (Davies et al, 1997; Enderby et al, 2009b; Ross et al, 2009). For example, SIFT-MS has been used to show that ammonia is elevated in the exhaled breath of patients with end-stage renal failure (Davies et al, 1997), and to define the chemical nature of malodorous breath (Ross et al, 2009). In addition, a recent SIFT-MS study examined the potential of hydrogen cyanide as a biomarker for P aeruginosa, (Enderby et al, 2009b).

Table 1-1 Concentrations of volatile compounds in the exhaled breath of 30 healthy volunteers quantified by SIFT-MS (Turner et al, 2006a; Turner et al, 2006d; Turner et al, 2006c; Turner et al, 2006b). * Geometric mean (multiplicative SD). † Median.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean (SD) (ppb)</th>
<th>Range (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>833 (1.2)*</td>
<td>248-2935</td>
</tr>
<tr>
<td>Acetone</td>
<td>477 (1.58)*</td>
<td>148-2744</td>
</tr>
<tr>
<td>Propanol</td>
<td>18†</td>
<td>0-135</td>
</tr>
<tr>
<td>Methanol</td>
<td>450 (1.62)*</td>
<td>32-1684</td>
</tr>
<tr>
<td>Ethanol</td>
<td>196 (244)</td>
<td>0-1663</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>24 (17)</td>
<td>0-104</td>
</tr>
<tr>
<td>Isoprene</td>
<td>118 (68)</td>
<td>0-474</td>
</tr>
</tbody>
</table>
To date, most breath analysis studies using SIFT-MS have required volunteers to perform a number of exhalations via the mouth into the sample inlet of a SIFT-MS instrument without accompanying measurements of expiratory flow and volume (Turner et al, 2006a; Turner et al, 2006d; Turner et al, 2006c; Turner et al, 2006b; Enderby et al, 2009b). Simultaneous on-line analysis of the analyte and the water vapour in the exhaled gas is performed by SIFT-MS. A plateau in water vapour concentration of greater than 5% by volume is identified, and the mean concentration of the analyte over the duration of this plateau is measured. This approach to analysis is based on two suppositions, firstly, that an exhaled water vapour concentration of greater than 5% by volume identifies the alveolar fraction of breath and, secondly, that the concentrations of trace gases in the alveolar fraction are in equilibrium with the blood (Spanel and Smith, 2001).

Recent studies, using SIFT-MS and PTR-MS, have demonstrated flaws in the approach described above. For example, ammonia and HCN levels were found to be markedly higher in oral, compared with nasal, exhalations (Wang et al, 2008). These results rendered irrelevant much of the previous SIFT-MS literature on ammonia and HCN in uncontrolled oral exhalations, and might have been anticipated, given that the oral cavity was already a known source of both of these volatiles (Lundquist et al, 1988; Kleinberg and Westbay, 1990).

Another problem related to the physiology of exhalation of a volatile has recently emerged in the analysis of isoprene in breath: studies of SIFT-MS analysis of isoprene in exhaled breath (Spanel et al, 1999) have been criticised because the plateau in water vapour concentration over which the measurement was taken, was not accompanied by a plateau in isoprene concentration (O'Hara et al, 2008). Measurements from on-line exhalations, using PTR-MS, have since shown that the end-exhaled concentration of isoprene depends on the duration of the exhalation, and that an uncontrolled single on-line exhalation, such as that used in SIFT-MS studies to date, is inappropriate for the analysis of isoprene in exhaled breath (O'Hara et al, 2008). The assumption that a plateau in water vapour concentration indicates the alveolar fraction of breath may be challenged because it does not reflect the physiology of water exchange in the respiratory tract. Transfer of water and heat from the airways to the inhaled air occurs on inspiration; on exhalation, some of that water and heat is returned back to the airways, and some is exhaled in the breath (Hlastala, 2003). A plateau in water vapour may simply mean that all water and heat that can be returned to the airways has been returned, and the point at which this occurs may have little to do with whether the breath originated from
the alveoli. Furthermore, some trace gases in the alveolar fraction of exhaled breath are not in equilibrium with the alveolar blood, as in the case of isoprene (O’Hara et al., 2008), for reasons of varying diffusion and perfusion limitation, gas solubility and site of production (Risby and Sehnert, 1999; Hlastala, 2003).

The above findings highlight the need to establish an appropriate exhalation manoeuvre for the analysis of each separate volatile compound in exhaled breath. The exhalation manoeuvre at a fixed flow against a fixed resistance, such as is performed in the analysis of FE\textsubscript{NO} (ATS/ERS, 2005), has been recommended as a standardised manoeuvre for the analysis of all trace volatile compounds in exhaled breath (Risby and Solga, 2006). However, a single standardised manoeuvre may not be suitable for the analysis of all volatiles, such as in the case of exhaled HCN, ammonia and isoprene (O'Hara et al., 2008; Smith et al., 2008; Wang et al., 2008), and closer examination of the effects of different breathing manoeuvres on the concentration of individual volatiles in exhaled breath may be required, as was the case in the development of FE\textsubscript{NO} measurement (Silkoff et al., 1997). In order to do this, the on-line analysis of a trace gas must be synchronised with measurements of expiratory flow and volume. The synchronisation of a pneumotachometer and a single quadrupole mass spectrometer has previously been achieved (Anderson et al., 2006), but this has not been attempted, to date, using SIFT-MS.

1.6. Summary and overall objectives of the thesis

This chapter has described the potential role of volatile compounds in exhaled breath as biomarkers of airway inflammation, and the technical and methodological issues that must be considered when analysing them. The role that FE\textsubscript{NO} plays in assessment of eosinophilic airway inflammation has been described, with emphasis on its potential application as a biomarker of corticosteroid response in COPD. The need for additional biomarkers of airway inflammation has been highlighted, and two potential candidates, hydrogen sulphide and hydrogen cyanide in exhaled breath, have been described. The SIFT-MS analytical technique has been described, as has the current state of SIFT-MS breath analysis and its potential in this field.

The work undertaken for this thesis will investigate the measurement of the concentration of volatile compounds in exhaled breath as biomarkers of airway inflammation. Firstly, an
extension of the role of FE\textsubscript{NO} measurement will be explored, to address the question of whether FE\textsubscript{NO} levels can predict the clinical response to corticosteroid in COPD. A study will be undertaken to determine the utility of FE\textsubscript{NO} measurement as a predictor of changes in functional exercise capacity, lung function and health-related quality of life in response to a trial of treatment with oral prednisone.

Secondly, the potential of the SIFT-MS technique for the analysis of novel biomarkers of airway inflammation in exhaled breath will be investigated. In an attempt to advance the technique, the synchronisation of exhalation measurements from a SIFT-MS instrument and a pneumotachometer will be investigated. Following this, a set of experiments will be performed to characterise the accuracy and repeatability of the instrument for the measurement of several volatile compounds, and to determine the effects of expiratory flow, volume and oral or nasal route on the concentration of the volatile compound in exhaled breath. Initially, these experiments will be performed using acetone as a model volatile compound. Similar experiments will then be performed using the potential biomarkers of airway inflammation, hydrogen sulphide and hydrogen cyanide.

Finally, the concentrations of hydrogen sulphide and hydrogen cyanide in the exhaled breath of patients with airway inflammation will be investigated. The levels of these volatile compounds in patients with asthma and COPD will be compared to the levels in control groups, and any relationship between these volatile compounds and current biomarkers of neutrophilic and eosinophilic airway inflammation will be explored.
2.

Predicting Corticosteroid Response in Chronic Obstructive Pulmonary Disease using Exhaled Nitric Oxide

2.1. Introduction

Inhaled corticosteroids (ICS) are widely used in patients with chronic obstructive pulmonary disease (COPD) but their effectiveness remains controversial (Suissa et al., 2007). They are recommended for reducing exacerbation frequency in severe disease (GOLD, 2008), but do not reduce overall mortality (Drummond et al., 2008), and overall, have only borderline effects on lung function or quality of life (Suissa et al., 2007). One of the challenges in the treatment of COPD is to identify potential “steroid responders”. COPD is heterogeneous (Marsh et al., 2008), but there is a recognised subgroup of patients who may potentially benefit from inhaled corticosteroid treatment (Weir et al., 1990; Weir and Burge, 1993). Against a background of concerns that treatment with ICS may predispose to pneumonia in at-risk patients (Drummond et al., 2008; Singh et al., 2009), identifying and treating responders selectively is potentially important in improving the overall risk-benefit ratio for ICS therapy.

There is evidence that “steroid responders” are more likely to be characterised by the presence of eosinophilic airway inflammation. Studies have shown that sputum eosinophilia in patients with COPD is associated with a short-term response to corticosteroid, demonstrated by increased airway calibre and improved health-related quality of life (Pizzichini et al., 1998; Brightling et al., 2000; Brightling et al., 2005; Leigh et al., 2006). However, the clinical applicability of sputum induction is limited because it is technically demanding and results are not immediately available. In contrast, measurement of the fraction of nitric oxide in exhaled breath (FE\text{NO}) is simple and reliable (Pavord et al., 2008). \text{FE}\text{NO} correlates with
eosinophilic airway inflammation (Jataban et al., 1998; Berlyne et al., 2000; Payne et al., 2001), and has utility as a predictor of corticosteroid response in patients with non-specific chronic airways symptoms (Smith et al., 2005a). To date, its application among patients with COPD has not been systematically assessed.

We hypothesised that FE\textsubscript{NO} levels could be used to predict short-term response to corticosteroid in COPD. We performed a double-blind, placebo-controlled, cross-over trial to evaluate FE\textsubscript{NO} as a predictor of changes in functional exercise capacity, lung function and health-related quality of life in response to a trial of treatment with oral prednisone. Oral rather than inhaled steroid was chosen so as to minimise drug response variability associated with inadequate inhalation technique and drug deposition.

### 2.2. Methods

#### 2.2.1. Participants

We recruited patients with a diagnosis of COPD from our own research database and respiratory clinics in Christchurch and Dunedin Hospitals between April 2003 and October 2008. Patients were 45 years or older, had a smoking history of >10 pack years, persistent symptoms of chronic airflow obstruction, a post-bronchodilator FEV\textsubscript{1}/FVC of less than 70%, and FEV\textsubscript{1} of 30-80% predicted. Current smokers were excluded because of the effect of smoking on exhaled nitric oxide levels (McSharry et al., 2005). Other exclusions were: patients with a diagnosis of asthma, bronchiectasis, lung cancer, diabetes or any other co-morbidity likely to affect completion of the study. Patients taking regular oral corticosteroid or who had required oral corticosteroid for exacerbations more than twice during the previous six months were also excluded. Patients who developed an acute exacerbation during the study were reviewed, treated and, once clinically stable, considered for re-entry into the study. A second exacerbation resulted in withdrawal of the patient. The study was approved by the Canterbury and Otago Ethics Committees, and all patients gave written, informed consent.

#### 2.2.2. Study design and procedures

The study was a randomized, double-blind, placebo-controlled, cross-over trial of oral prednisone (30mg/day) for three weeks per treatment period (see Table 2-1). Any inhaled corticosteroid treatment was withdrawn four weeks before the first treatment period. The two
treatment periods were separated by a four-week washout period during which the patients did not receive any inhaled or oral corticosteroid. Patients attended the research clinic before and after each treatment period, and performed a fixed sequence of assessments at each visit: St. George’s Respiratory Questionnaire (Jones et al., 1992); \( \text{FE}_{\text{NO}} \) measured according to current recommendations (ATS/ERS, 2005); spirometry before and 15 minutes after 400μg of inhaled albuterol; six-minute walking test according to current guidelines (ATS, 2002); and lastly, sputum induction.

**Table 2-1** Schedule of study visits and procedures.

<table>
<thead>
<tr>
<th>Time interval</th>
<th>Visit 1</th>
<th>Visit 2</th>
<th>Visit 3</th>
<th>Visit 4</th>
<th>Visit 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 weeks</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>3 weeks</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

**2.2.3. Exhaled nitric oxide measurement**

\( \text{FE}_{\text{NO}} \) was measured using an on-line chemiluminescence analyser (Aerocrine AB, Solna, Sweden) according to current recommendations (ATS/ERS, 2005). Once seated comfortably, the patient inserted the mouthpiece, inhaled NO-free air (<5 ppb) through the mouth to total lung capacity over 2-3 seconds, and then exhaled against resistance to maintain mouthpiece pressure at 10-20 cm H\(_2\)O at a flow rate of 50 ml/s. A constant expiratory flow was achieved by biofeedback: the target and actual mouthpiece pressures were displayed on a screen during exhalation and the patient aimed to keep within the target parameters. Acceptable exhalations were of at least 6 seconds, with a plateau of at least 3 seconds in the NO versus time profile. Exhalations were repeated until three plateau values were obtained that agreed to within 10% of their mean value. \( \text{FE}_{\text{NO}} \) was then calculated as the mean of these three values.

**2.2.4. Lung function testing**

Spirometry was undertaken before and 15 minutes after 400 μg of inhaled salbutamol according to current standards (Miller et al., 2005). A minimum of three acceptable FVC
manoeuvres were performed. Acceptable repeatability was achieved when the difference between the largest and the next largest FVC was ≤150 ml and the difference between the largest and next largest FEV₁ was ≤150 ml. If these criteria were not met in three manoeuvres, up to five additional trials were attempted. At the Christchurch research centre, lung volume testing was performed using whole-body plethysmography. The predicted values for spirometry and lung volumes were calculated from appropriate reference ranges (Quanjer et al, 1993; Hankinson et al, 1999).

2.2.5. Six-minute walk test

Six-minute walk tests were performed according to current guidelines, and two practice tests were administered before commencing the study (ATS, 2002). Briefly, a 30 metre walking course was marked out on a flat, straight corridor, with turnaround points clearly marked by orange cones. Prior to the test, pulse oximetry was performed, and perceived dyspnoea was assessed using a Borg scale. Standardised instructions were then issued to the subject to walk as far as possible for six minutes. During the test, encouragement was given using standard phrases. At completion of the test, the distance walked by the subject was recorded, and pulse oximetry and rating of dyspnoea using the Borg scale were repeated. The longer distance of two tests, performed 15 minutes apart, was recorded at each visit.

2.2.6. St. George’s Respiratory Questionnaire

The St. George’s Respiratory Questionnaire comprised 76 items in three domains (symptoms, activity and impact on daily life), giving a measure of health in chronic airflow limitation (Jones et al, 1992). At each visit, subjects self-administered the questionnaire in a quiet room before undertaking any other tests. The scores for the 76 items were then entered on the St. George’s Respiratory Questionnaire Excel Template Sheet, from which the total score, and a score for each of the three domains, was then calculated.

2.2.7. Sputum induction and processing

Sputum induction was undertaken and processed as previously described (Aldridge et al, 2000). The procedure was performed 30 minutes after 400 µg of inhaled salbutamol. Nebulised 3% saline was generated using an ultrasonic nebuliser (Devilbiss Healthcare, Somerset, PA, USA) connected to a two-way non-rebreathing valve (Hans Rudolph, Shawnee, KS, USA) and rubber mouthpiece. Saline was inhaled for four minutes, after which patients rinsed their mouth out with water three times, and were encouraged to cough sputum
into a plastic container. If unable to expectorate a sample, saline was inhaled for a further two to four minutes. If no sample was produced after this, the procedure was stopped. The whole sample was homogenised by the addition of 10% dithiothreitol (Oxoid, Hants, England), the volume added equating to two times the volume of the sputum sample. The mixture was placed in a rocking water bath at 37°C for 30 minutes, and then filtered through a 60 μm mesh (Millipore, Billerica, MA, USA). Using a haemocytometer, total cell count, percentage squamous cells and percentage cell viability (trypan blue exclusion) were determined. An aliquot was diluted to give a concentration of approximately $1 \times 10^6$ cells/ml, from which cytospins were prepared. After May-Grünwald-Giemsa staining of the cytospins, a 400 differential cell count (excluding squamous cells) was determined in duplicate.

### 2.2.8. Statistical analysis

The primary outcome was change in six-minute walking distance (6MWD) after prednisone, with post-bronchodilator FEV$_1$, and SGRQ total score as co-primary outcomes. Pilot work demonstrated that a minimum of 48 completed patients would be required to determine a significant treatment-related difference in 6MWD of 35 metres (alpha=0.05, beta=0.2). Secondary outcomes were changes in individual SGRQ domains, F$_{ENO}$ and sputum eosinophil counts.

Randomized patients were excluded from analysis for non-adherence or because of adverse events other than deteriorating respiratory function. Patients who withdrew during the first treatment period were excluded from analysis. Patients who withdrew during the second treatment period or during the between-treatment washout were assigned a net change of zero for outcome variables for the second treatment period.

Since the distributions of F$_{ENO}$ measurements and sputum eosinophil counts were positively skewed, they were logarithmically transformed prior to parametric analyses. Comparisons of baseline characteristics of subjects completing either one or both of the treatment arms were performed by independent $t$-test. Comparisons of treatment-related outcomes (6MWD, FEV$_1$, SGRQ and secondary outcomes) before and after placebo and prednisone were performed using repeated-measures analysis of variance. The significance of the change in outcomes across tertiles was analysed by linear regression (see Figure 2-3 and Table 2-4). Correlations were determined using Spearman’s rank correlation. Receiver operator characteristic analyses were performed to determine the predictive utility of F$_{ENO}$ for improvement in primary outcomes with prednisone (Hanley and McNeil, 1983). ROC curves were constructed to
show the sensitivity and specificity of all cut-points of the continuous variables, $FE_{NO}$ and sputum eosinophil percentage, in predicting the dichotomous variables, achievement / non-achievement of minimum clinically important differences in primary outcomes. For each outcome, the area under the curve was calculated and the asymptotic significance determined. Minimum clinically important differences for each of the primary end-points were: 35m for the 6MWD (Puhan et al, 2008), 200 ml for $FEV_1$ (Celli and MacNee, 2004; Cazzola et al, 2008), 20% for $FEV_1$ (Callahan et al, 1991) and -4 units for the SGRQ (Jones, 2002; Cazzola et al, 2008). Analyses were performed using SPSS 16.

2.3. Results

The study profile is shown in Figure 2-1. 82 patients were recruited, of whom 65 proceeded to randomisation. Of the 17 patients not randomised, 13 were symptomatic after withdrawal of ICS, two were too busy to continue, one had inadequate $FE_{NO}$ technique, and one had an unrelated illness. Thus there was the potential for selection bias because patients unable to tolerate the absence of inhaled steroid treatment were excluded from the study.

Data from 62 patients were included in the analysis: two patients were excluded because of non-adherence and one was excluded because of a new diagnosis of angina. 55 patients completed all parts of the study. Of the seven who completed only the first treatment arm, four withdrew during the washout after receiving prednisone (three with an acute exacerbation of COPD (AECOPD), one restarted smoking); two withdrew while on placebo in the second treatment arm (both with AECOPD) and one withdrew in the washout after placebo (AECOPD). Adherence to treatment, assessed by pill count from retrieved medication containers, was 96%. Treatment order had no significant effect on the primary and co-primary outcomes.

2.3.1. Subject characteristics

The baseline (corticosteroid-naïve) characteristics of the 62 subjects included in the analysis are shown in Table 2-2. Subjects are stratified according to $FE_{NO}$ tertile. There were no significant differences in age, sex, BMI, smoking history or GOLD (Rabe et al, 2007) classification of disease severity across low, middle and high $FE_{NO}$ tertiles. Nor were there any differences in 6MWD, $FEV_1$ or SGRQ score across the tertiles at baseline. Of the seven subjects who completed only the first treatment arm, five subjects were in the high $FE_{NO}$
tertile, and two were in the low \( \text{FE}_{\text{NO}} \) tertile. Compared to the 55 subjects who completed the study, these seven were on a higher daily dose of ICS (1257\( \mu \)g vs. 376\( \mu \)g beclomethasone equivalents, \( p=0.02 \)), showed greater percentage reversibility after bronchodilator (31\% vs. 15\%, \( p<0.01 \)), and had a higher baseline geometric mean \( \text{FE}_{\text{NO}} \) (38.5ppb vs. 23.6ppb, \( p=0.02 \)).

**Figure 2-1** Study profile.
### Chapter Two

#### Table 2-2 Baseline subject characteristics after withdrawal of inhaled corticosteroid.

<table>
<thead>
<tr>
<th></th>
<th>All subjects</th>
<th>&lt; 19.0</th>
<th>19.0–30.2</th>
<th>&gt; 30.2</th>
<th>Subjects completing 1st treatment only</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of patients</strong></td>
<td>62</td>
<td>21</td>
<td>21</td>
<td>20</td>
<td>7</td>
</tr>
<tr>
<td><strong>Age (yr) (range)</strong></td>
<td>72 (59–86)</td>
<td>70</td>
<td>73</td>
<td>73</td>
<td>75</td>
</tr>
<tr>
<td><strong>Sex, female</strong></td>
<td>18 (29%)</td>
<td>7</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>27.1 (26.0–28.2)</td>
<td>26.7</td>
<td>28.6</td>
<td>26.0</td>
<td>25.4</td>
</tr>
<tr>
<td><strong>No. of pack years</strong></td>
<td>47 (41–53)</td>
<td>46</td>
<td>48</td>
<td>47</td>
<td>50</td>
</tr>
<tr>
<td><strong>ICS dose (mcg) (range)</strong></td>
<td>475 (0–2000)</td>
<td>498</td>
<td>229</td>
<td>710</td>
<td>1257</td>
</tr>
<tr>
<td><strong>Patients taking long-acting bronchodilator</strong></td>
<td>11 (18%)</td>
<td>5 (24%)</td>
<td>1 (5%)</td>
<td>5 (25%)</td>
<td>3 (43%)</td>
</tr>
<tr>
<td><strong>FEV₁/FVC (%)</strong></td>
<td>49 (47–51)</td>
<td>50</td>
<td>50</td>
<td>47</td>
<td>45</td>
</tr>
<tr>
<td><strong>FEV₁ (l) (post-bronchodilator)</strong></td>
<td>1.58 (1.46–1.71)</td>
<td>1.68</td>
<td>1.50</td>
<td>1.57</td>
<td>1.45</td>
</tr>
<tr>
<td><strong>GOLD classification</strong></td>
<td>42/20</td>
<td>14/7</td>
<td>15/6</td>
<td>13/7</td>
<td>3/4</td>
</tr>
<tr>
<td><strong>Patients with bronchodilator reversibility</strong></td>
<td>29 (47%)</td>
<td>9 (43%)</td>
<td>9 (43%)</td>
<td>11 (55%)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td><strong>6MWD (m)</strong></td>
<td>482 (460–503)</td>
<td>492</td>
<td>471</td>
<td>482</td>
<td>432</td>
</tr>
<tr>
<td><strong>SGRQ symptoms score</strong></td>
<td>53.3 (48.1–58.5)</td>
<td>47.7</td>
<td>49.7</td>
<td>62.8</td>
<td>60.8</td>
</tr>
<tr>
<td><strong>SGRQ activity score</strong></td>
<td>53.6 (47.9–59.3)</td>
<td>52.8</td>
<td>48.8</td>
<td>59.2</td>
<td>60.7</td>
</tr>
<tr>
<td><strong>SGRQ impact score</strong></td>
<td>24.9 (20.7–29.1)</td>
<td>25.2</td>
<td>20.8</td>
<td>28.5</td>
<td>30.4</td>
</tr>
<tr>
<td><strong>SGRQ total score</strong></td>
<td>38.1 (33.9–42.3)</td>
<td>37.2</td>
<td>33.9</td>
<td>43.3</td>
<td>44.5</td>
</tr>
<tr>
<td><strong>FE NO (ppb)</strong></td>
<td>2.05 (1.24–3.37)</td>
<td>0.56</td>
<td>2.78</td>
<td>4.78</td>
<td>7.29</td>
</tr>
<tr>
<td><strong>Eosinophils (%)</strong></td>
<td>69.4 (64.4–74.5)</td>
<td>79.7</td>
<td>68.7</td>
<td>60.9</td>
<td>55.4</td>
</tr>
<tr>
<td><strong>Neutrophils (%)</strong></td>
<td>15.1 (12.7–17.6)</td>
<td>13.8</td>
<td>16.6</td>
<td>14.7</td>
<td>16.4</td>
</tr>
<tr>
<td><strong>Macrophages (%)</strong></td>
<td>0.8 (0.6–1.0)</td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Lymphocytes (%)</strong></td>
<td>5.2 (3.3–7.2)</td>
<td>4.4</td>
<td>4.3</td>
<td>7.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean (95% confidence interval) unless otherwise stated. * Bronchodilator reversibility defined as increase in FEV₁ of >12% and >200 ml. † Data are expressed as geometric means (95% confidence interval).
2.3.2. Relationship between baseline $\text{FE}_{\text{NO}}$ measurements and sputum eosinophils

There was a significant correlation between baseline (corticosteroid-naïve) $\text{FE}_{\text{NO}}$ measurements and percentage sputum eosinophils at Visit 2 ($r=0.46$, $p<0.01$) (see Figure 2-2).

**Figure 2-2** Correlation between $\text{FE}_{\text{NO}}$ measurements and percentage sputum eosinophils.

2.3.3. Overall response to prednisone

Outcomes before and after treatment with oral prednisone and placebo are shown in Table 2-3. With prednisone, the 6MWD increased by 13 metres (95% C.I.: 3–22m, $p=0.02$) compared to placebo, and FEV$_1$ increased by 0.06 litres (95% C.I.: 0.02–0.11 litres, $p=0.02$). There was a non-significant decrease in SGRQ score of -2.4 (-5.3–0.6, $p=0.16$). The number of “responders” who demonstrated changes greater than or equal to the minimum clinically important difference (MCID) was: 8 for 6MWD (12.9%), 14 for FEV$_1$ (22.6%), and 21 for SGRQ (33.9%).

The geometric mean $\text{FE}_{\text{NO}}$ decreased from 26.1ppb to 19.8ppb after prednisone compared with an increase from 23.6ppb to 24.4ppb after placebo ($p<0.001$). There was a significant decrease in geometric mean sputum eosinophil count after prednisone from 1.8% to 0.4%, compared with an increase from 2.0% to 2.2% after placebo ($p<0.001$). There was a significant correlation between off-steroid $\text{FE}_{\text{NO}}$ and sputum eosinophil percentage ($r=0.46$, $p<0.01$).
2.3.4. Response to prednisone according to baseline $FE_{NO}$

The correlation coefficients for the relationship between baseline $FE_{NO}$ and the change in the primary end-points were: 6MWD, $r=0.10$, $p=0.45$; FEV$_1$, $r=0.32$, $p=0.01$; SGRQ, $r=0.12$, $p=0.36$). A significant improvement from the lowest to the highest $FE_{NO}$ tertile was observed for FEV$_1$ ($p=0.03$) but not for 6MWD or SGRQ (see Figure 2-3 and Table 2-4). Results for other outcomes are also shown in Table 2-4. The relationships between baseline sputum eosinophils and the primary end-points were all non-significant.

Figure 2-4 shows the changes in $FE_{NO}$ and percentage sputum eosinophils in response to placebo and prednisone for subjects stratified by baseline $FE_{NO}$ tertile. With ascending $FE_{NO}$ tertiles, there were greater reductions of $FE_{NO}$ ($p<0.01$) and percentage eosinophils ($p=0.01$) with prednisone compared to placebo. After prednisone treatment, geometric mean $FE_{NO}$ levels ranged from low-normal to high-normal across ascending tertiles, and percentage sputum eosinophils in the upper two tertiles fell into the normal range (Balbi et al, 2007) and were similar to the low tertile.
Table 2-3  Outcomes before and after treatment with oral prednisone and placebo in 62 patients with COPD.

<table>
<thead>
<tr>
<th></th>
<th>Before placebo</th>
<th>After placebo</th>
<th>Change after placebo</th>
<th>Before prednisone</th>
<th>After prednisone</th>
<th>Change after prednisone</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>6MWD (m)</td>
<td>483 (11)</td>
<td>481 (12)</td>
<td>-2 (4)</td>
<td>481 (11)</td>
<td>491 (11)</td>
<td>11 (3)</td>
<td>0.02</td>
</tr>
<tr>
<td>FEV₁ (l) (post-bronchodilator)</td>
<td>1.57 (0.06)</td>
<td>1.57 (0.06)</td>
<td>-0.0 (0.01)</td>
<td>1.56 (0.06)</td>
<td>1.62 (0.06)</td>
<td>0.06 (0.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>FVC (l) (post-bronchodilator)</td>
<td>3.22 (0.11)</td>
<td>3.24 (0.11)</td>
<td>0.02 (0.03)</td>
<td>3.24 (0.11)</td>
<td>3.29 (0.10)</td>
<td>0.05 (0.04)</td>
<td>0.57</td>
</tr>
<tr>
<td>SGRQ total</td>
<td>38.5 (2.1)</td>
<td>39.4 (2.3)</td>
<td>0.9 (0.9)</td>
<td>38.7 (2.1)</td>
<td>37.2 (2.0)</td>
<td>-1.5 (1.2)</td>
<td>0.16</td>
</tr>
<tr>
<td>SGRQ symptoms</td>
<td>54.6 (2.6)</td>
<td>56.8 (2.7)</td>
<td>2.1 (1.6)</td>
<td>54.2 (2.7)</td>
<td>52.0 (2.6)</td>
<td>-2.2 (1.7)</td>
<td>0.08</td>
</tr>
<tr>
<td>SGRQ activity</td>
<td>55.9 (2.8)</td>
<td>55.3 (3.1)</td>
<td>-0.7 (1.6)</td>
<td>54.6 (3.0)</td>
<td>54.3 (2.8)</td>
<td>-0.2 (1.8)</td>
<td>0.88</td>
</tr>
<tr>
<td>SGRQ impacts</td>
<td>23.9 (2.0)</td>
<td>25.4 (2.3)</td>
<td>1.4 (1.1)</td>
<td>25.2 (2.1)</td>
<td>23.3 (2.0)</td>
<td>-1.9 (1.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>FE_NO (ppb)*</td>
<td>23.6 (0.03)</td>
<td>24.4 (0.03)</td>
<td>0.97 (0.02)</td>
<td>26.1 (0.03)</td>
<td>19.8 (0.03)</td>
<td>1.32 (0.03)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Eosinophils (%)*</td>
<td>2.04 (0.10)</td>
<td>2.17 (0.11)</td>
<td>0.94 (0.06)</td>
<td>1.82 (0.11)</td>
<td>0.37 (0.09)</td>
<td>4.94 (0.11)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>72.0 (2.4)</td>
<td>67.1 (2.7)</td>
<td>-4.9 (2.2)</td>
<td>71.4 (2.6)</td>
<td>78.7 (2.0)</td>
<td>7.3 (2.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>15.9 (1.7)</td>
<td>17.5 (1.5)</td>
<td>1.6 (1.6)</td>
<td>14.5 (1.2)</td>
<td>14.8 (1.3)</td>
<td>0.3 (1.4)</td>
<td>0.47</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.92 (0.11)</td>
<td>1.03 (0.14)</td>
<td>0.1 (0.1)</td>
<td>0.78 (0.12)</td>
<td>0.46 (0.07)</td>
<td>-0.3 (0.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Epithelials (%)</td>
<td>4.19 (0.67)</td>
<td>6.58 (1.24)</td>
<td>2.4 (1.3)</td>
<td>5.81 (1.28)</td>
<td>5.51 (1.36)</td>
<td>-0.3 (1.2)</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Data are expressed as mean (SE) unless otherwise stated. * Data are expressed as geometric means (log SE). Changes after placebo and prednisone are expressed as fold decrease (log SE).
Table 2-4  Mean change in outcomes after prednisone compared to placebo for subjects stratified by FENO tertiles.

<table>
<thead>
<tr>
<th>Tertiles by FENO (ppb)</th>
<th>&lt; 19.0</th>
<th>19.0 – 30.2</th>
<th>&gt; 30.2</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>6MWD (m)</td>
<td>+15</td>
<td>+7</td>
<td>+15</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>(-11 to +41)</td>
<td>(-7 to +22)</td>
<td>(6 to +25)</td>
<td></td>
</tr>
<tr>
<td>FEV₁ (l) (post-bronchodilator)</td>
<td>-0.01</td>
<td>+0.07</td>
<td>+0.12</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>(-0.09 to +0.07)</td>
<td>(-0.01 to +0.15)</td>
<td>(+0.04 to +0.21)</td>
<td></td>
</tr>
<tr>
<td>FVC (l) (post-bronchodilator)</td>
<td>-0.07</td>
<td>-0.01</td>
<td>+0.18</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>(-0.22 to +0.09)</td>
<td>(-0.19 to +0.16)</td>
<td>(-0.04 to +0.40)</td>
<td></td>
</tr>
<tr>
<td>SGRQ total</td>
<td>-1.3</td>
<td>-1.7</td>
<td>-4.1</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>(-6.0 to +3.3)</td>
<td>(-8.0 to +4.5)</td>
<td>(-10.3 to +2.1)</td>
<td></td>
</tr>
<tr>
<td>SGRQ symptoms</td>
<td>+0.8</td>
<td>-7.4</td>
<td>-6.8</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>(-8.8 to +10.5)</td>
<td>(-14.8 to 0.0)</td>
<td>(-14.1 to +0.5)</td>
<td></td>
</tr>
<tr>
<td>SGRQ activity</td>
<td>-3.0</td>
<td>+2.2</td>
<td>+2.3</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>(-10.6 to +4.5)</td>
<td>(-9.2 to +13.6)</td>
<td>(-8.8 to +13.5)</td>
<td></td>
</tr>
<tr>
<td>SGRQ impacts</td>
<td>-1.1</td>
<td>-2.1</td>
<td>-6.9</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(-6.4 to +4.2)</td>
<td>(-7.7 to +3.5)</td>
<td>(-13.4 to -0.4)</td>
<td></td>
</tr>
<tr>
<td>FENO (ppb)*</td>
<td>+1.1</td>
<td>+1.4</td>
<td>+1.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>(+0.9 to +1.3)</td>
<td>(+1.1 to +1.7)</td>
<td>(+1.4 to +2.2)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)*</td>
<td>+1.5</td>
<td>+6.6</td>
<td>+12.0</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>(+0.7 to +3.6)</td>
<td>(+2.9 to +15.1)</td>
<td>(+3.2 to +45.0)</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>+6.5</td>
<td>+4.6</td>
<td>+19.2</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>(-3.2 to +16.3)</td>
<td>(-5.0 to +14.2)</td>
<td>(+10.2 to +28.3)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as mean increase (95% confidence interval) unless otherwise stated. * Data are expressed as fold decrease (95% confidence interval). p values are given for the changes in outcomes across tertiles analyzed by linear regression.
Figure 2-3  Mean (SE) changes in 6MWD, FEV₁, and SGRQ for each tertile after prednisone compared to placebo. * p<0.05 for the change in FEV₁ across tertiles analyzed by linear regression.
Figure 2-4 Geometric mean (95% C.I.) changes in (A) $\text{FE}_{\text{NO}}$ and (B) percentage sputum eosinophils in response to placebo and prednisone for subjects stratified by baseline (corticosteroid-naïve) $\text{FE}_{\text{NO}}$ tertile.

A.

B.
2.3.5. Predicting response to prednisone using $\text{FE}_{\text{NO}}$

Figure 2-5 shows ROC curves demonstrating the utility of $\text{FE}_{\text{NO}}$ and percentage sputum eosinophils for predicting a response to prednisone. The predictive value of baseline $\text{FE}_{\text{NO}}$ for an increase of 0.2 litres in $\text{FEV}_1$ with prednisone was borderline significant (AUC 0.69, $p=0.04$) with an optimum $\text{FE}_{\text{NO}}$ cut point of 50ppb (sensitivity 29%, specificity 96%, positive predictive value (PPV) 67%, negative predictive value (NPV) 82%; see Table 2-5 A). The predictive values of baseline $\text{FE}_{\text{NO}}$ for an increase in $\text{FEV}_1$ of 20% were of even greater significance, with an AUC of 0.80, $p<0.01$; see Table 2-5 B. Six patients had a baseline $\text{FE}_{\text{NO}} > 50$ppb, and the changes in outcomes for these patients were (mean (range)): $\text{FEV}_1$ 0.21 litres (range -0.19 to 0.39 litres); 6MWD 13.5 metres (range -8 to 32 metres); SGRQ -7.4 (range -28.7 to 9.7). The predictive values of baseline $\text{FE}_{\text{NO}}$ for either a 35 metre increase in 6MWD or a 4-point reduction in SGRQ total score were not significant (AUC for 6MWD: 0.467, $p=0.97$; AUC for SGRQ: 0.569, $p=0.38$). The baseline sputum eosinophil count (%) was a significant predictor of an increase in $\text{FEV}_1$ of 20% (AUC 0.77, $p=0.02$) but, somewhat surprisingly, did not predict any of the remaining clinical end points (AUC for 35 metre increase in 6MWD: 0.49, $p=0.921$; AUC for 0.2 litres increase in $\text{FEV}_1$: 0.63, $p=0.15$; AUC for 4-unit reduction in SGRQ total score: 0.65, $p=0.06$).

Table 2-5 Sensitivities, specificities, positive and negative predictive values (PPV and NPV respectively) and accuracy of cut-points for $\text{FE}_{\text{NO}}$ as a predictor for an increase in $\text{FEV}_1$ of (A) 0.2 litres or greater in response to prednisone and (B) for an increase in $\text{FEV}_1$ of 20% or greater.

<table>
<thead>
<tr>
<th>$\text{FE}_{\text{NO}}$ cut-point (ppb)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>71</td>
<td>56</td>
<td>32</td>
<td>87</td>
<td>60</td>
</tr>
<tr>
<td>35</td>
<td>36</td>
<td>79</td>
<td>33</td>
<td>81</td>
<td>69</td>
</tr>
<tr>
<td>50</td>
<td>29</td>
<td>96</td>
<td>67</td>
<td>82</td>
<td>81</td>
</tr>
<tr>
<td>70</td>
<td>14</td>
<td>98</td>
<td>67</td>
<td>80</td>
<td>79</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>86</td>
<td>55</td>
<td>19</td>
<td>97</td>
<td>58</td>
</tr>
<tr>
<td>35</td>
<td>57</td>
<td>78</td>
<td>25</td>
<td>93</td>
<td>76</td>
</tr>
<tr>
<td>50</td>
<td>43</td>
<td>95</td>
<td>50</td>
<td>93</td>
<td>89</td>
</tr>
<tr>
<td>70</td>
<td>14</td>
<td>96</td>
<td>33</td>
<td>90</td>
<td>87</td>
</tr>
</tbody>
</table>
Figure 2-5  Receiver operator characteristic curves demonstrating the utility of FENO and % sputum eosinophils for predicting response to prednisone, defined as a change in outcome, with prednisone compared to placebo, equal to or greater than the MCID. MCIDs: 6MWD 35m; FEV₁ 0.2 litres (black line), 20% (red line); SGRQ -4 units.
2.4. Discussion

The results of the present study demonstrate that FE\textsubscript{NO} is a weak predictor of short-term response to oral corticosteroid in patients with stable, moderately severe COPD. Using receiver operator characteristic analyses, the AUC was of borderline significance for FE\textsubscript{V1} (0.69, p=0.04) but not for 6MWD or SGRQ. Baseline FE\textsubscript{NO} measurements correlated with percentage sputum eosinophils (r=0.46, p<0.01), and both were reduced by the administration of oral corticosteroid (p<0.001), with increasing magnitude of effect across ascending FE\textsubscript{NO} tertiles (p<0.01 and p=0.01 respectively).

The weak predictive utility of FE\textsubscript{NO} was reflected in an AUC of 0.69 (a value above 0.8 denoting a strong predictor (Hanley and McNeil, 1982)), and a modest positive predictive value of 67% at the optimum cut-point for predicting an increase in FE\textsubscript{V1} (>50ppb). However, a low FE\textsubscript{NO} (<25ppb) helpfully predicted the absence of a response to corticosteroid, with a high negative predictive value of 87%. This was consistent with the results of another recent study of patients with COPD, in which a post-hoc analysis demonstrated that FE\textsubscript{NO} was a significant predictor of increase in FE\textsubscript{V1} in response to ICS: at a low FE\textsubscript{NO} cut-point of 19ppb, the negative predictive value was 100% (Kunisaki \textit{et al}, 2008). In the context of treating COPD, in which at best only 20% of patients will demonstrate steroid responsiveness (Weir \textit{et al}, 1990; Weir and Burge, 1993), this information would help the clinician to avoid prescribing unnecessary ICS treatment.

The optimum FE\textsubscript{NO} cut-point of 50ppb closely corresponded with the suggested upper limit of the normal range (47ppb) (Olin \textit{et al}, 2007), and with results from studies of asthma and non-specific respiratory symptoms, in which the optimum cut-points for FE\textsubscript{NO} to predict corticosteroid response were 49ppb and 47ppb respectively (Pijnenburg \textit{et al}, 2005b; Smith \textit{et al}, 2005a). Positive and negative predictive values are, in part, determined by the prevalence of a condition in the test population, so a direct comparison with predictive values observed in those studies is uninformative about the properties of the test. However, a useful comparison of sensitivities and specificities can be made: in the studies of asthma and non-specific respiratory symptoms, specificities of 74-93% and sensitivities of 43-82% were noted at the optimum FE\textsubscript{NO} cut-point. The specificity of the optimum FE\textsubscript{NO} cut-point in the present study (96%) compared favourably, while the sensitivity (29%) did not. The reasons for the lower sensitivity are not clear, and it may be that other factors compete against eosinophilic inflammation to lower FE\textsubscript{NO} in corticosteroid-responsive patients with COPD, but such a
finding highlights the need for separate studies of the predictive utility of FE\textsubscript{NO} in different airway diseases so that the clinical application of the test can be refined.

The average and distribution of baseline FE\textsubscript{NO} measurements (geometric mean, 25ppb; 95% RI, 9 to 72ppb) showed considerable overlap with FE\textsubscript{NO} measurements in healthy subjects without airway disease (geometric mean, 17ppb; 95% RI, 6 to 47ppb) (Olin\textit{ et al}, 2007). This finding is consistent with the results of previous studies suggesting that FE\textsubscript{NO} in COPD shows little or no difference compared to controls (Delen\textit{ et al}, 2000; Fabbri\textit{ et al}, 2003). It is therefore unsurprising that FE\textsubscript{NO} is an imperfect predictor of steroid response in COPD, given that FE\textsubscript{NO} measurements within the “normal” range may be influenced by factors other than eosinophilic inflammation. It has been suggested, however, that FE\textsubscript{NO} measurements in COPD might still be of use, given that, even though levels may be within the normal range, there is still a significant relationship between baseline FE\textsubscript{NO} and the subsequent increase in FEV\textsubscript{1} after bronchodilator (Papi\textit{ et al}, 2000) or inhaled corticosteroid (Ferreira\textit{ et al}, 2001; Zietkowsk\textit{ et al}, 2005). The present study provides further confirmatory evidence of this, demonstrating a significant relationship between baseline FE\textsubscript{NO} and a subsequent increase in FEV\textsubscript{1} after oral corticosteroid. Reference equations, accounting for factors such as height and age, currently explain only between 10% and 26% of variance in FE\textsubscript{NO} measurements in normal subjects (Olin\textit{ et al}, 2007; Dressel\textit{ et al}, 2008). Refinement of these equations may result in higher explanatory values and narrower reference intervals, and may enhance the predictive utility of FE\textsubscript{NO} measurements.

FE\textsubscript{NO} and sputum eosinophil percentage, at baseline, were positively correlated ($r=0.46$, $p<0.01$), supporting previous findings of a correlation between corticosteroid-naïve FE\textsubscript{NO} measurements and percentage sputum eosinophils in COPD ($r=0.65$) (Rutgers\textit{ et al}, 1999). The strength of the correlation was comparable to the correlations observed in studies of asthma ($r=0.44$ to 0.78) (Jatakanon\textit{ et al}, 1998; Piacentini\textit{ et al}, 1999; Berlyne\textit{ et al}, 2000; Warke\textit{ et al}, 2002). As expected, FE\textsubscript{NO} and sputum eosinophil percentage were reduced by prednisone treatment (see Figure 2-4), with the greatest reductions occurring in patients with the highest baseline FE\textsubscript{NO} levels – for eosinophils, there was a 12-fold reduction in patients in the highest FE\textsubscript{NO} tertile. After prednisone treatment, FE\textsubscript{NO} levels ranged from low-normal to high-normal across ascending baseline FE\textsubscript{NO} tertiles, while percentage sputum eosinophils in the upper two tertiles fell into the normal range (Balbi\textit{ et al}, 2007) and were similar to the low FE\textsubscript{NO} tertile. These outcomes and their relationship to baseline levels of airway inflammation are consistent with those of a previous study (Brightling\textit{ et al}, 2000), and indicate that in
COPD, just as in asthma, the eosinophilic component of a disease with mixed airway inflammation is responsive to corticosteroid. $\text{FE}_{\text{NO}}$ measurements provide a similar but easier-to-obtain perspective.

While $\text{FE}_{\text{NO}}$ measurements were predictive of a clinically significant increase in $\text{FEV}_1$ in response to corticosteroid, no such relationship was demonstrated for 6MWD or SGRQ. As a whole, the study group achieved only a slight increase in 6MWD after prednisone compared with placebo. The 13m increase was well below the MCID of 35m and consistent with the findings of Brightling and colleagues (Brightling et al, 2000) who, when testing functional exercise capacity using a similar study design, observed an increase of 12m in incremental shuttle walk, well below the recently-established MCID of 47.5m (Singh et al, 2008). There was no difference amongst the tertiles stratified by $\text{FE}_{\text{NO}}$, and neither $\text{FE}_{\text{NO}}$ nor sputum eosinophils predicted clinically significant improvement. The cause of exercise limitation in COPD is disputed and may comprise elements of dynamic compression of the airways, inadequate metabolic energy supply to the respiratory and locomotor muscles, and lower limb muscle dysfunction (Aliverti and Macklem, 2008; Debigare and Maltais, 2008; O'Donnell and Webb, 2008). The mechanism by which prednisone induced a small increase in exercise capacity in the present study is therefore uncertain: it may be related to the modification of eosinophilic airway inflammation but with a poor relationship over the short-term, or it may be due to another mechanism.

SGRQ total score did not change after prednisone compared with placebo, although changes in the symptoms and impacts domains of the questionnaire approached significance. This finding contrasts with other studies using the chronic respiratory questionnaire to measure health-related quality of life (Pizzichini et al, 1998; Brightling et al, 2000) in which significant changes were observed. In these studies, increasing response to corticosteroid was observed with increasing baseline sputum eosinophilia. In the present study, we were unable to demonstrate a similar trend with increasing sputum eosinophilia or $\text{FE}_{\text{NO}}$ and, while ROC analysis showed that although the utility of sputum eosinophil percentage as a predictor of change in SGRQ total score approached significance, the area under the curve was small, indicating that its accuracy was poor.

A potential criticism of our study design is that oral rather than rather than inhaled corticosteroid was used. The relationship between outcomes following a short term trial of oral steroid and outcomes with long term inhaled steroid in patients with COPD is not a
consistent one (Burge et al, 2003). Thus, arguably, it would have been more clinically relevant to determine whether \( \text{FeNO} \) measurements predict the response to long term ICS treatment. This issue was keenly debated when the study was designed. The conclusion was reached that it was important at this stage to answer the question: “Does \( \text{FeNO} \) predict response to steroid in COPD if this is at all possible?” By choosing to administer oral prednisone, the confounding effects of variable inhaler technique and airway drug deposition on treatment responses were minimised. Ideally, the study would have involved a sequential trial of oral followed by inhaled steroid in each patient. However, it has been highlighted that despite the overall lack of effect of ICS on COPD outcomes, the results of a short term trial of oral steroid do indeed have predictive significance (Pavord et al, 2004). In the ISOLDE study, subjects with the greatest increase in \( \text{FEV}_1 \) after prednisolone had the largest reduction in exacerbations during subsequent treatment with inhaled fluticasone (Burge et al, 2003). This observation provides indirect support for using \( \text{FeNO} \) as a predictive biomarker, given its utility as a predictor of change in \( \text{FEV}_1 \) with prednisone. Further, it has been shown that long-term ICS treatment is more likely to be of benefit in patients whose pre-treatment airway inflammation includes a significant eosinophilic component (Siva et al, 2007). Given that \( \text{FeNO} \) measurements are a surrogate marker for sputum eosinophil counts, one would therefore speculate that the predictive values for \( \text{FeNO} \) in relation to long-term outcomes might be equally or even more significant than in the present report. A further study to test this hypothesis would be justified.

The results of our study might have been more definitive had it been possible for all enrolled subjects to enter the randomised phase of the study. Unfortunately, 13 patients were unable to tolerate cessation of ICS during the run-in, resulting in a potential selection bias and underestimation of the beneficial effects of prednisone. We can only speculate that these 13 patients might have had eosinophilic airway inflammation with correspondingly elevated \( \text{FeNO} \) levels. In other studies of steroid withdrawal in COPD, a similar proportion of patients who are clearly “steroid-requiring” has been identified when treatment is discontinued (O’Brien et al, 2001; van der Valk et al, 2002). It is worth noting that the seven patients who withdrew after randomisation (of whom six suffered an AECOPD) had marked sputum eosinophilia (7.3%) and elevated \( \text{FeNO} \) (38.5ppb) at randomisation (see Table 2-2).

\( \text{FeNO} \) measurements are affected by a number of factors including current cigarette smoking and, obviously, inhaled corticosteroid use (Taylor et al, 2006). \( \text{FeNO} \) levels are approximately 30-40% lower in current smokers although adjustments may be applied (McSharry et al,
The effect of exposure to corticosteroid may be 4-6 weeks in duration. Thus our results are only applicable in patients who are ex-smokers and are currently steroid-free.

For many years, identifying markers of corticosteroid responsiveness in COPD has been a “holy grail”. Earlier studies explored the value of both bronchodilator reversibility and airway hyper-responsiveness as predictors of corticosteroid responsiveness. Unfortunately, the relationships between these measurements and treatment outcomes are weak, and these tests are unreliable in this setting (Yang et al, 2007). Clinicians often resort to a “trial of steroid” in COPD, but here again evidence to justify this strategy is poor (Yang et al, 2007). In practice, n of 1 trials are difficult, involving repeat consultations and spirometric measurements, and they are now no longer recommended (GOLD, 2008). Empiric therapy is often undertaken and patients may remain on long-term treatment in the absence of confirmatory evidence regarding their efficacy. To date, using a biomarker to identify potential therapeutic responses has not been carefully investigated in COPD. It is theoretically desirable to use a biomarker in any disease state if the biomarker in question (in this case a marker of airway inflammation) reflects the underlying pathology and is responsive to a disease-modifying treatment intervention. Given that this is the case for the relationship between FeNO and eosinophilic inflammation, this is a potentially important advance in the management of airways disease (Pavord et al, 2008). In COPD, only one previous study with FeNO has shown a correlation between baseline FeNO and change in FEV1 after ICS (Zietkowski et al, 2005), although there is also evidence that an alternative biomarker of airway inflammation, sputum eosinophils, for which FeNO is a surrogate, is associated with steroid responsiveness (Pizzichini et al, 1998; Brightling et al, 2000; Brightling et al, 2005; Leigh et al, 2006). In these studies, however, predictive values, ideally the issue of interest, were not calculated for FeNO or sputum eosinophils.

2.5. Summary

The results of this study have demonstrated that FeNO measurements in patients with COPD are a predictor for changes in airflow obstruction, but not improvements in functional exercise capacity or health-related quality of life, with corticosteroid therapy. Low FeNO values are highly predictive that improvements in FEV1 are unlikely. Despite the fact that the indications for using ICS in COPD are limited and there are risks of adverse effects (GOLD, 2008), they are widely and empirically prescribed, largely because objective data upon which
rational therapeutic choices may be based are not easily obtainable. Using an appropriate biomarker such as FE_{NO} has the potential to improve this situation.
3.

Accurate, Reproducible Measurement of Acetone Concentration in Breath using Selected Ion Flow Tube – Mass Spectrometry

3.1. Introduction and aims

Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS) is an analytical technique with the capacity for on-line measurement of volatile compounds in exhaled breath at the low parts per billion (ppb) level. SIFT-MS shows promise in the analysis of several compounds in breath (Turner et al, 2006a), and has recently been proposed as a technique to measure the concentration of hydrogen cyanide in the exhaled breath of patients with cystic fibrosis as a possible means of detecting infection with *P aeruginosa* (Enderby et al, 2009b).

In order to realize its potential clinical applications, further steps must be taken in the development of SIFT-MS. Its validation for an individual volatile compound requires that the instrument gives accurate and repeatable measurements, with an appropriate dynamic response time for on-line analysis of exhalations. Measurements of the exhaled volatile compound concentration must then be synchronised with measurements of expiratory flow and volume, to explore the effects of these variables. An optimum exhalation for analysis can then be defined, taking into account the expired flow and volume, to ensure an accurate and reproducible measure of the volatile concentration from within that exhalation. Within-session, intra-day and inter-day coefficients of variation for measurement of the concentration of the volatile compound can then be determined.
In this study, exhaled acetone, one of the most abundant volatile compounds in breath, was investigated. Analysis of acetone in breath has been examined as a tool to approximate the blood glucose level (Galassetti et al., 2005), to monitor metabolic stress during cardiac surgery (Pabst et al., 2007), to monitor the effectiveness of ketogenic diets in some forms of epilepsy (Musa-Veloso et al., 2002), and as a motivational tool in some weight-loss programs (Kundu et al., 1993). The SIFT-MS technique was used, for the first time, to explore the effects of expiratory flow and volume on the concentration of acetone in exhaled breath, to measure the phase III slope of exhaled acetone concentration versus total exhaled volume (see Figure 3-3, Page 63 for a description of the phases of exhalation), and to measure the repeatability of exhaled acetone concentration while using a controlled breathing manoeuvre. These data were complemented by measurements of instrument accuracy and repeatability.

As with other highly soluble gases, experimental data suggest that acetone in exhaled breath originates from the airway rather than from alveolar gas exchange (Anderson et al., 2006). Therefore, the acetone concentration in end-exhaled breath may not be in equilibrium with the systemic blood. To be clinically useful, a measurement of acetone concentration taken from an exhalation must reflect the systemic acetone level. Other investigators have shown that the concentration of acetone in the systemic blood can be estimated from breath using a re-breathing sampling technique, in which a sample is taken from air that has been re-breathed multiple times by a subject (O'Hara et al., 2009). In an earlier study, the same group showed that the concentration of end-exhaled acetone in a single breath was within the uncertainty of the value obtained from a re-breathed sample, suggesting that sampling of acetone in end-exhaled breath may be an acceptable alternative (O'Hara et al., 2008). Previous work has shown no difference between the concentrations of acetone in orally and nasally exhaled breath (Dummer et al., 2007; Wang et al., 2008), therefore oral exhalations were performed in this experiment.

In this study, the aim was to establish the accuracy, repeatability and dynamic response for measurement of acetone concentration using a Voice 100™ SIFT-MS instrument. Secondly, a SIFT-MS instrument was synchronised with a pneumotachometer to determine the effects of expiratory flow and volume on the acetone concentration in breath, and to determine an appropriate single-exhalation breathing manoeuvre from which a measure of acetone concentration could be obtained. Within-session, intra-day and inter-day coefficients of variation for these measurements were calculated.
3.2. Methods

3.2.1. Voice100™ SIFT-MS instrument

The Voice100™ SIFT-MS instrument (Syft Technologies Ltd, New Zealand) used for this work has been described in detail previously (Francis et al, 2007). A detailed description of the SIFT-MS technique is given in Section 1.5.1, Page 25. The reagent ions were generated by a microwave discharge that ionised a saturated mixture of air and water at ~0.3 Torr. The reagent ions were individually mass selected in the upstream chamber (at ~1 × 10^{-5} Torr) by a quadrupole mass spectrometer and injected into the flow tube, through a Venturi orifice. The Venturi effect was created by using a dual-inlet method: helium was used to create the Venturi effect on an inner ring, and argon was added through an outer ring on the Venturi plate. All experiments were performed with a flow tube pressure of 0.5 Torr, and a carrier gas mixture of 40% helium and 60% argon. The flow tube was 30 cm in length and 5 cm in diameter. Samples were introduced into the flow tube at approximately 3 ml atmosphere s\textsuperscript{-1} at a distance of 6 cm downstream from the Venturi orifice via a custom-made sample inlet. Reactions between reagent ions and sample molecules occurred in the remaining 24 cm of the flow tube, with a measured ion transit time of 4 ms. Ions were then sampled through an electrostatic orifice at the end of the flow tube, into the downstream chamber. On entering the downstream chamber (at <1 × 10^{-5} Torr), ions resulting from the ion/molecule reaction of interest were mass selected by a second quadrupole mass spectrometer, and detected on a continuous dynode particle multiplier.

The instrument was fitted with a custom-made sample inlet protruding 40 cm from the side of the instrument and 120 cm from the ground. The inlet arm was constructed from ¼ inch stainless steel tubing wrapped in heating wire and heated to 105°C by a cal3300 temperature controller (33Volt) (CAL controls, Brighton, UK), and insulated with aluminium foil and fibreglass insulation. At the end of the inlet arm a tapered stainless steel adaptor, 1.5 cm in length, was heated by the same temperature controller, and allowed the inlet arm to be connected to the breath analysis system.

The existing data acquisition application (Syft Technologies Ltd, New Zealand) for the SIFT-MS instrument was modified to communicate with a pneumotachometer (RSS 100, Hans Rudolph Inc, USA) via its serial port. This was achieved with a publicly available program (uCon, Microcross, USA) to control the serial communications with the pneumotachometer.
hardware. The data file format used by the data acquisition program was extended to permit saving of the pneumotachometer data along with the analyte concentration data.

3.2.2. SIFT-MS analysis of acetone

The NO$^+$ reagent ion was used to analyse breath acetone. The reaction is an ion-molecule collisional association with He atom stabilization as follows (Spanel et al, 1997b):

\[
\text{NO}^+ + \text{CH}_3\text{COCH}_3 + \text{He} \rightarrow \text{NO}^+.\text{CH}_3\text{COCH}_3
\]

Monitoring was performed using Selected Ion Monitoring mode (see Section 1.5.1, Page 25). The NO$^+$ reagent ion was monitored at a mass-to-charge ratio (m/z) of 30, and the NO$^+.\text{H}_2\text{O}$ hydrated reagent ion at m/z 48 as this ion is formed in moist air mixtures. The NO$^+.\text{CH}_3\text{COCH}_3$ product ion was monitored at m/z 88. The H$_3\text{O}^+$ reagent ion at m/z 19 was also monitored for the purpose of synchronising the SIFT-MS instrument and the pneumotachometer (see below). Monitoring cycles for breath analysis took 400 ms, and resulted in the acquisition of one data point, giving a sampling rate of 2.5 Hz. Monitoring cycles for determining transit time and dynamic response were performed at 5 Hz.

3.2.3. Instrument accuracy, repeatability and dynamic response

The accuracy and repeatability of the instrument, for the measurement of acetone, were determined using a custom permeation system consisting of a dilution apparatus (Syft Technologies Ltd., New Zealand) and permeation chamber (Dynacalibrator Model 150, VICI Metronics, USA), and acetone permeation tube (Kin-tek, USA) with a known emission rate (1269 ng/min) at 40ºC. The system delivered a flow of a known concentration of acetone in air at 100% relative humidity. Five known acetone concentrations between 600 and 3000 ppb were then measured by the instrument on three weekdays of every week for ten weeks. In addition, on five of those days (17% of the days), morning and afternoon measurements were made.

The dynamic response of the instrument was determined by measuring the time taken for the instrument to respond to a step change in acetone concentration from the background level in the ambient air to a physiological concentration in humid air (see Figure 1-1A, Page 7). The time taken between achieving a 10% and 90% response to the step change was measured when performing the same experimental method as described in Section 3.2.5 (Page 60).
3.2.4. Breath analysis system

The SIFT-MS instrument and pneumotachometer were configured to make simultaneous expiratory measurements (see Figure 3-1): a disposable respiratory filter (SureGuard, BIRD Healthcare, Australia), a disposable cardboard roll (A-M Systems, USA) and the pneumotachometer were attached in series. A side port was created in the disposable cardboard roll, 2 cm distal to the disposable respiratory filter and, using a purpose-built adaptor, we introduced the SIFT-MS sample inlet arm into this via the purpose-built adaptor. The flow measured by the pneumotachometer was displayed on a screen during exhalation so that a subject could exhale at a target flow.

**Figure 3-1** Schematic diagram of the breath analysis system. The data for acetone concentration and expiratory flow and volume were gathered simultaneously. The SIFT-MS instrument sampled at 3 ml/s.

3.2.5. Synchronisation of the SIFT-MS instrument and the pneumotachometer

The SIFT-MS instrument and the pneumotachometer recorded measurements independently, each on their own internal timer. They were synchronised using the flow of a volume of humid air through the breath analysis system as an input reference signal.

Firstly, it was necessary to determine any difference in transit time between water vapour and acetone through the breath analysis system and SIFT-MS instrument, so that SIFT-MS measurements of exhaled acetone concentration and pneumotachometer measurements of
expiratory flow and volume could be aligned using the flow of a volume of humid air as an input reference signal. One port of a respiratory humidifier (HC150, Fisher and Paykel Healthcare, New Zealand) was connected to the disposable respiratory filter on the breath analysis system, and the second port was attached to a 1 litre syringe (Vitalograph, UK). Acetone was added to the humidifier to give a headspace acetone concentration of between 500 and 2000 ppb. The syringe was emptied within 3 seconds, causing the humid air and acetone to flow past the SIFT-MS inlet and through the pneumotachometer, displacing the ambient air. A flow signal was detected by the pneumotachometer, while the introduction of a sample of humid air into the SIFT-MS flow tube caused the formation of the hydrated hydronium ions $\text{H}_3\text{O}^+.(\text{H}_2\text{O})_{1,2,3}$ (Smith and Spanel, 1996).

$$\text{H}_3\text{O}^+ + n\text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+.\text{(H}_2\text{O})_n$$

This association reaction caused a detectable drop in the count rate of $\text{H}_3\text{O}^+$ reagent ions at $m/z$ 19. After emptying the syringe, the time-points were measured at which there was a decrease in $m/z$ 19 ion count and an increase in acetone concentration of >2 SDs. The difference between the two time-points was then calculated.

In order to prevent contamination of the system with exogenous acetone during analysis of exhalations, synchronisation of the SIFT-MS instrument and the pneumotachometer was performed using water vapour alone. To characterise the signal at $m/z$ 19, we performed 25 syringe discharges. Figure 3-2 shows a scheme of the typical response to syringe discharge at $m/z$ 19. The mean and standard deviation (SD) in the $\text{H}_3\text{O}^+$ ions counted per second at $m/z$ 19 was calculated for 10 s prior to each syringe discharge. We defined the time at which the signal from the interface between dry and humid air was first detected (Figure 3-2, A) as the time at which the $\text{H}_3\text{O}^+$ ion count at $m/z$ 19 first dropped below the mean by greater than 2 SD as it fell to its nadir (Figure 3-2, B). We defined the time at which signal detection ended (Figure 3-2, C) as the first point after the nadir in the $\text{H}_3\text{O}^+$ ion count at which the signal returned to within 2 SD of the original mean. The time from signal detection to signal nadir, and the total duration of detection of the signal were measured. The signal nadir was recorded and area under the curve during signal detection (Figure 3-2, shaded area) was calculated. Signal-to-noise ratio was also calculated [(mean plateau – mean signal nadir)/ mean intra-plateau SD]. For the pneumotachometer, we defined the time at which the signal was first detected as the time of the first data point at which flow was greater than zero. We defined the time at which signal detection ended as the time of the first data point after peak flow at
which the flow returned to zero. The duration, peak flow and volume of each signal were recorded and the mean flow calculated.

**Figure 3-2** Scheme of the SIFT-MS signal, at $m/z$ 19, to an input of humidified air. A = start of signal detection. B = time of signal nadir. C = end of signal detection. Shaded area is area under the curve during signal detection.

Twelve pairs of syringe discharges were then performed. There was an interval of approximately 90 s between the syringe discharges within a pair. The time between them was measured by detection of flow by the pneumotachometer and by change in $\mathrm{H}_3\mathrm{O}^+$ ion count at $m/z$ 19 by the SIFT-MS instrument. Any discrepancy in the times between the signals for the two instruments was then calculated and the statistical significance of any difference analyzed by paired $t$-test.

### 3.2.6. Study design for testing of participants

The experimental procedures were approved by the Upper South Regional Ethics Committee. Twelve consenting, healthy, non-smoking volunteers with no history of respiratory disease were studied. Volunteers attended two visits within a two week period, and all visits took place over the same two month period during which instrument accuracy and repeatability were studied.

At the first visit, spirometry was measured, using an EasyOne™ spirometer (NDD, USA), according to current international standards (see Section 2.2.4, Page 36 for further details of the procedure) (Miller *et al*, 2005). A single-breath nitrogen washout test ($\mathrm{SBN}_2$) (Viasys,
USA) was also performed (Ruppel, 2009). The subject exhaled to residual volume, inhaled a vital capacity breath of 100% oxygen from a demand valve, and then immediately exhaled to residual volume at an expiratory flow of 300-500 ml/s. Nitrogen concentration and exhaled volume were measured, and the nitrogen concentration was plotted against exhaled volume on a graph (see Figure 3-3). Acceptable repeatability was achieved when expiratory flow was 300-500 ml/s throughout exhalation, and when vital capacity achieved during the test was within 200 ml of the volunteer’s vital capacity as measured by spirometry.

**Figure 3-3** Single-Breath Nitrogen Washout (SBN2). After taking a single inhalation of 100% O₂ to total lung capacity, the subject exhales to residual volume. The graph shows a plot of nitrogen concentration vs. exhaled volume for that exhalation. At the beginning of exhalation, only O₂ is exhaled (Phase I). As mixed bronchial and alveolar air is exhaled, there is a rapid increase in N₂ concentration (Phase II). Phase III is the alveolar gas plateau, in which N₂ concentration rises slowly as long as ventilation is uniformly distributed. As the end of exhalation is approached, there is a more rapid increase in N₂ concentration because basal airways close and a greater proportion of gas comes from the apices, where the N₂ concentration is higher.

![Exhaled volume vs. N₂ concentration](image)

At the second visit, analysis of exhaled acetone was performed using SIFT-MS. Analysis of exhaled acetone was performed between 10 a.m. and 12 p.m. for all 12 volunteers. In addition, three of the volunteers also attended in the afternoon (between 2 p.m. and 4 p.m.) on the day of the second visit for breath analysis of acetone, and repeated the morning and afternoon visits ten days later. For the analysis of exhaled acetone, the sampling apparatus was set up and the instruments synchronised as described above. Subjects refrained from eating or drinking anything other than water, and exercising for at least an hour before being tested. After five minutes of rest, the subject performed three exhalations of vital capacity at
a low target expiratory flow of 170 ml/min and three exhalations at a high target expiratory flow of 330 ml/min in random order and at two minute intervals.

3.2.7. Statistical analysis

The analyte concentration data from the SIFT-MS instrument and the data from the pneumotachometer were saved and then processed as Excel 2003© files (Microsoft, USA). Using the input reference signal described above, the data from the two instruments were manually synchronised. A worksheet was then constructed to automate the reduction of the pneumotachometer data rate from 50 Hz to the same data rate as the SIFT-MS instrument (2.5-5 Hz). This enabled the plotting of analyte concentration against exhaled volume, from which the values below could be calculated.

Exhalations of a volume less than 90% of an individual’s forced vital capacity were excluded, and the volumes of the remaining exhalations were curtailed to that of the smallest exhaled volume for analysis. For each individual, the arithmetic mean and coefficient of variation of acetone concentration at two breath fractions, 70-85% and 85-100% by exhaled volume (V₇₀₋₈₅% and V₈₅₋₁₀₀% respectively), at expiratory flows of 170 and 330 ml/s was calculated. The phase III slope in acetone concentration (normalized to end-exhaled acetone concentration as given by the mean acetone concentration in V₈₅₋₁₀₀%) was then determined by line-of-best-fit through the interval of 50-90% of exhaled volume (Anderson et al, 2006). Multiple measurements of breath acetone concentration were obtained for three individuals, and intra-individual within-day and between-day coefficients of variation were calculated. For the single-breath nitrogen washout test, the phase III slope was determined by line-of-best-fit from the point where 30% of the vital capacity remained above residual volume to the onset of phase IV (Ruppel, 2009). Because the distribution of exhaled acetone concentrations across the group of 12 participants was positively skewed, consistent with the log-normal distribution previously described (Turner et al, 2006a; Schwartz et al, 2009), the mean exhaled acetone concentration from each participant was log-transformed before statistical analysis. Comparisons were performed by paired t-test and analysis of variation with the exception of coefficients of variation, which were performed by Wilcoxon signed-rank test. Correlations were determined using Spearman rank correlation. Analyses were performed using SPSS 16.
3.3. Results

3.3.1. SIFT-MS instrument characteristics

Measurements were taken of acetone in humid air at concentrations of 600-3000 ppb on 30 days over two months. On five of those days, morning and afternoon measurements were made. Across all concentrations there was an instrument measurement bias of 8% (see Figure 3-4); the measured concentration was lower than the expected concentration (the known concentration delivered by the custom permeation system). The bias did not change with time over the two months of testing. The inter-day and intra-day coefficients of variation of measurement of acetone concentration were 5.6% and 0.0% respectively.

The 10-90% dynamic response time for the measurement of acetone in humid air was repeatedly measured 18 times, over a range of acetone concentrations (350-1500 ppb) similar to those seen in exhaled breath (see Figure 3-5). For a step change from a mean acetone concentration of 54 ppb in ambient air to a mean acetone concentration of 780 ppb in humid air, the dynamic response time was 500±50 ms (mean±SE). There was no correlation between the acetone concentration and the dynamic response time.

3.3.2. Synchronisation of the SIFT-MS instrument and the pneumotachometer

In order to determine any difference between the transit times of acetone and water vapour through the breath analysis system and SIFT-MS instrument, twenty syringe discharges of a gas mixture of humid air and acetone were performed. Figure 3-6 shows a typical SIFT-MS trace for m/z 19 and acetone concentration in response to the emptying of the syringe. The SIFT-MS transit time of acetone in humid air was 400±50 ms (mean±SE) faster than that of the water vapour as measured by the drop in H$_3$O$^+$ ions counted per second at m/z 19.

In order to characterise the signal to the two instruments, a total of 25 syringe discharges were performed. One was excluded because of incomplete data collection, leaving 24 for analysis. Over the 10 s prior to syringe discharge (see Figure 3-2 (Page 62), plateau before bolus delivery), the count rate at m/z 19 for the 24 plateaus was 160±3 x 10$^3$ cps (mean ± SD) with a mean intra-plateau SD of 2 x 10$^3$ cps. The mean time from the start of signal detection (Figure 3-2, A) to signal nadir (Figure 3-2, B) was 1.2 ± 0.3 s. The mean signal nadir was 105±3 x 10$^3$ cps. The mean duration of signal detection (Figure 3-2, time from A to C) was 23.9 ± 5.2 s. During signal detection, the mean area under the curve (Figure 3-2, shaded area)
was calculated to be $3.37 \times 10^6$ count seconds, i.e. the ion count at $m/z$ 19 was reduced by a mean of $0.47 \times 10^6$ counts over the duration of signal detection. The signal-to-noise ratio was 24. For the pneumotachometer, a volume of $0.90 \pm 0.04$ l was detected at a mean flow of $31.4 \pm 1.8$ l/min, giving a signal duration of $1.7 \pm 0.1$ s. Twelve pairs of syringe discharges were then performed. Mean time between the 12 pairs was $93.1 \pm 2.7$ s measured by detection of flow by the pneumotachometer and $93.1 \pm 2.7$ s measured by SIFT-MS. There was no significant difference in time between paired syringe discharges measured by the pneumotachometer and SIFT-MS ($0.01 \pm 0.13$ s; SED 0.04 s; $P = 0.76$).

**Figure 3-4** Bland-Altman plots showing that (A) the acetone concentration from the custom permeation system measured by the SIFT-MS instrument was less than that expected, and (B) the percentage bias was the same across all acetone concentrations. Mean percentage bias (black line) ± 2 SDs (red lines) is shown.
Figure 3-5  SIFT-MS instrument dynamic response times for a step change from sampling ambient air to sampling a range of acetone in humid air at concentrations found in exhaled breath. There was no correlation between 10-90% response time and the acetone concentration in humid air.

Figure 3-6  Example of SIFT-MS trace for m/z 19 and acetone concentration in response to the discharge of a syringe containing a mixture of humid air and acetone. Following the emptying of the syringe, acetone (orange line) was detected at A, and water vapour (purple line) at B.
3.3.3. Breath analysis

Seven women and five men successfully completed all parts of the study. Their characteristics are shown in Table 3-1. The volunteers had normal spirometry, and phase III slope gradients for the single-breath nitrogen washout test ranged from 0.58 to 1.13% N₂/l (normal values in healthy young adults are 0.5-1.0% with wide variability (Ruppel, 2009)).

Table 3-2 shows the exhaled volumes and flows of the exhalations used for the breath analysis of acetone for the 12 volunteers. Six out of a total of 72 exhalations were excluded from the analysis because the exhaled volume was less than 90% of the forced vital capacity recorded at spirometry. Four individuals had one exhalation excluded, and one individual had two exhalations excluded. The mean actual flows recorded for the low and high target expiratory flows, at 193±18 (mean±SD) and 313±32 ml/s respectively, were significantly different from one another (p<0.001). The mean actual exhaled volumes for the low and high target expiratory flows, at 4570±1170 and 4660±1140 ml respectively, were also significantly different from one another (p<0.01).

An example of acetone concentration plotted against exhaled volume in six exhalations from one volunteer is shown in Figure 3-7. Measurements of acetone concentration were corrected for the instrument bias described in Section 3.3.1 (Page 65). The concentration of acetone in the ambient air was 30±13 ppb (arithmetic mean ± SD). Concentrations of acetone in breath according to expiratory flow and fraction of exhaled vital capacity are shown in Table 3-3. Acetone concentrations at expiratory flows of 170 and 330ml/s were 619±1.83 and 618±1.82 ppb (geometric mean ± logSD) in the breath fraction V₇₀-₈₅%, and 636±1.82 and 631±1.83 ppb (geometric mean ± logSD) in the breath fraction V₈₅-₁₀₀%. A difference was observed between acetone concentrations in the V₇₀-₈₅% and V₈₅-₁₀₀% fractions (p<0.01), but no difference was observed between acetone concentrations at target flows of 170 and 330 ml/s (p=0.28). The phase III slope was positive, and there was no difference between low and high expiratory flows (0.062±0.005 vs. 0.071±0.006 l⁻¹ (arithmetic mean ± SE), p=0.13). No difference was observed between median coefficients of variation at the two expiratory flows or fractions of exhaled vital capacity, which were all between 1.6 and 2.6%. For the three subjects tested in the morning and afternoon of two days, ten days apart, the intra-individual within-day and between-day coefficients of variation were 36% and 15% respectively (see Figure 3-8).

End-exhaled acetone concentration, as given by an individual’s arithmetic mean acetone concentration in V₈₅-₁₀₀, did not correlate with FVC or SBN₂ phase III slope. The phase III
slope in exhaled acetone concentration showed a negative correlation with FVC ($= -0.8$, $p=0.001$), but no correlation with SBN$_2$ phase III slope.

Table 3-1  Subject characteristics.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>FEV$_1$ (l)</th>
<th>% of predicted FEV$_1$ (l)</th>
<th>FVC (l)</th>
<th>% of predicted FVC (l)</th>
<th>SBN$<em>2$ S$</em>{III}$ (% N$_2$/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>f</td>
<td>29</td>
<td>166</td>
<td>62</td>
<td>3.31</td>
<td>106</td>
<td>3.95</td>
<td>107</td>
<td>1.08</td>
</tr>
<tr>
<td>2</td>
<td>f</td>
<td>29</td>
<td>173</td>
<td>69</td>
<td>3.38</td>
<td>100</td>
<td>3.87</td>
<td>97</td>
<td>0.63</td>
</tr>
<tr>
<td>3</td>
<td>f</td>
<td>29</td>
<td>155</td>
<td>49</td>
<td>2.91</td>
<td>105</td>
<td>3.49</td>
<td>109</td>
<td>0.79</td>
</tr>
<tr>
<td>4</td>
<td>f</td>
<td>34</td>
<td>176</td>
<td>72</td>
<td>3.16</td>
<td>94</td>
<td>3.78</td>
<td>93</td>
<td>0.89</td>
</tr>
<tr>
<td>5</td>
<td>f</td>
<td>35</td>
<td>161</td>
<td>61</td>
<td>2.99</td>
<td>105</td>
<td>3.74</td>
<td>111</td>
<td>0.82</td>
</tr>
<tr>
<td>6</td>
<td>f</td>
<td>36</td>
<td>174</td>
<td>76</td>
<td>3.55</td>
<td>109</td>
<td>4.84</td>
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<td>7</td>
<td>f</td>
<td>42</td>
<td>165</td>
<td>67</td>
<td>3.09</td>
<td>108</td>
<td>3.88</td>
<td>113</td>
<td>0.99</td>
</tr>
<tr>
<td>8</td>
<td>m</td>
<td>32</td>
<td>185</td>
<td>80</td>
<td>5.53</td>
<td>114</td>
<td>7.08</td>
<td>121</td>
<td>0.82</td>
</tr>
<tr>
<td>9</td>
<td>m</td>
<td>33</td>
<td>181</td>
<td>81</td>
<td>4.5</td>
<td>99</td>
<td>5.64</td>
<td>102</td>
<td>0.99</td>
</tr>
<tr>
<td>10</td>
<td>m</td>
<td>34</td>
<td>180</td>
<td>78</td>
<td>4.96</td>
<td>111</td>
<td>6.04</td>
<td>109</td>
<td>0.85</td>
</tr>
<tr>
<td>11</td>
<td>m</td>
<td>36</td>
<td>178</td>
<td>82</td>
<td>5.48</td>
<td>128</td>
<td>6.37</td>
<td>123</td>
<td>0.85</td>
</tr>
<tr>
<td>12</td>
<td>m</td>
<td>44</td>
<td>180</td>
<td>90</td>
<td>4.34</td>
<td>104</td>
<td>5.42</td>
<td>106</td>
<td>0.58</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>34</td>
<td>173</td>
<td>72</td>
<td>3.93 (0.98)</td>
<td>107 (9)</td>
<td>4.84 (1.23)</td>
<td>110</td>
<td>0.87 (0.16)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: FEV$_1$ = forced expiratory volume in one second; FVC = forced vital capacity; SBN$_2$ S$_{III}$ = single-breath nitrogen washout test phase III slope.

Figure 3-7  Example of the acetone concentration plotted against exhaled volume in six exhalations from one volunteer (case 7). Red and blue lines are exhalations at target expiratory flows of 170 and 330 ml/s respectively. Percentages of exhaled volume are shown for the smallest exhaled volume greater than 90% of forced vital capacity, to which all others were curtailed for analysis.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Target flow (ml/s)</th>
<th>Actual flow (ml/s)</th>
<th>Exhaled volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170</td>
<td>216 (3)</td>
<td>3620 (50)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>320 (8)</td>
<td>3780 (80)</td>
</tr>
<tr>
<td>2</td>
<td>170</td>
<td>183 (1)</td>
<td>3610 (20)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>298 (1)</td>
<td>3820 (40)</td>
</tr>
<tr>
<td>3</td>
<td>170</td>
<td>188 (2)</td>
<td>3270 (80)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>310 (14)</td>
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</tr>
<tr>
<td>4</td>
<td>170</td>
<td>181 (4)</td>
<td>3090 (20)</td>
</tr>
<tr>
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<td>330</td>
<td>267 (12)</td>
<td>3640 (200)</td>
</tr>
<tr>
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<td>170</td>
<td>177 (2)</td>
<td>3580 (10)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>277 (16)</td>
<td>3700 (50)</td>
</tr>
<tr>
<td>6</td>
<td>170</td>
<td>223 (3)</td>
<td>4480 (110)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>357 (23)</td>
<td>4590 (120)</td>
</tr>
<tr>
<td>7</td>
<td>170</td>
<td>192 (6)</td>
<td>3690 (30)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>286 (4)</td>
<td>3660 (130)</td>
</tr>
<tr>
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<td>170</td>
<td>209 (4)</td>
<td>6630 (210)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>359 (4)</td>
<td>6690 (310)</td>
</tr>
<tr>
<td>9</td>
<td>170</td>
<td>194 (11)</td>
<td>5290 (250)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>327 (16)</td>
<td>5320 (170)</td>
</tr>
<tr>
<td>10</td>
<td>170</td>
<td>198 (5)</td>
<td>5700 (150)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>293 (10)</td>
<td>5920 (70)</td>
</tr>
<tr>
<td>11</td>
<td>170</td>
<td>198 (6)</td>
<td>6140 (20)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>357 (18)</td>
<td>6100 (280)</td>
</tr>
<tr>
<td>12</td>
<td>170</td>
<td>158 (10)</td>
<td>5160 (130)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>304 (22)</td>
<td>5200 (160)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Mean (SD)</th>
<th>170</th>
<th>193 (18)*</th>
<th>4570 (1170)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>330</td>
<td>313 (32)</td>
<td>4660 (1140)</td>
<td></td>
</tr>
</tbody>
</table>

The data are expressed as mean (SD). *Significant difference in flow and volume between the manoeuvres at target flows of 170 and 330 ml/s (p<0.01).
### Table 3-3

Mean acetone concentrations in exhaled breath at breath fractions of 70-85% and 85-100% by volume, at target expiratory flows of 170 and 330 ml/s.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Target flow (ml/s)</th>
<th>( V_{70-85%} )</th>
<th>( V_{85-100%} )</th>
<th>Acetone phase III slope (l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetone concentration (ppb)</td>
<td>CV (%)</td>
<td>Acetone concentration (ppb)</td>
</tr>
<tr>
<td>1</td>
<td>170</td>
<td>1891</td>
<td>5.6</td>
<td>1935</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>1929</td>
<td>2.5</td>
<td>1943</td>
</tr>
<tr>
<td>2</td>
<td>170</td>
<td>1505*</td>
<td>1.8</td>
<td>1517*</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>1456</td>
<td>2.8</td>
<td>1538</td>
</tr>
<tr>
<td>3</td>
<td>170</td>
<td>463</td>
<td>6.3</td>
<td>480</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>466</td>
<td>5.4</td>
<td>468</td>
</tr>
<tr>
<td>4</td>
<td>170</td>
<td>432*</td>
<td>2.1</td>
<td>456*</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>453*</td>
<td>3.2</td>
<td>458*</td>
</tr>
<tr>
<td>5</td>
<td>170</td>
<td>280*</td>
<td>0.8</td>
<td>284*</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>285</td>
<td>2.3</td>
<td>284</td>
</tr>
<tr>
<td>6</td>
<td>170</td>
<td>718</td>
<td>0.6</td>
<td>751</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>735</td>
<td>3.5</td>
<td>749</td>
</tr>
<tr>
<td>7</td>
<td>170</td>
<td>535</td>
<td>3.2</td>
<td>543</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>526</td>
<td>1.5</td>
<td>534</td>
</tr>
<tr>
<td>8</td>
<td>170</td>
<td>814</td>
<td>2.1</td>
<td>839</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>816</td>
<td>1.0</td>
<td>843</td>
</tr>
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<td>9</td>
<td>170</td>
<td>253*</td>
<td>3.0</td>
<td>264*</td>
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</tr>
<tr>
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<td>170</td>
<td>903</td>
<td>4.1</td>
<td>895</td>
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<td>885</td>
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<td>0.3</td>
<td>598*</td>
</tr>
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<td></td>
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<td>1.8</td>
<td>577</td>
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<td>0.2</td>
<td>497</td>
</tr>
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<td>499</td>
</tr>
<tr>
<td>All</td>
<td>170</td>
<td>(619) (1.83)</td>
<td>(2.1) (0.7 to 3.7)</td>
<td>(636) (1.82)</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>(618) (1.82)</td>
<td>(2.6) (1.8 to 3.5)</td>
<td>(631) (1.83)</td>
</tr>
</tbody>
</table>

Abbreviations: \( V_{70-85\%} \) = fraction 70-85% by volume of an exhalation of vital capacity; \( V_{85-100\%} \) = fraction 85-100% by volume of an exhalation of vital capacity; ppb = parts per billion; CV = coefficient of variation. Acetone concentrations for individuals are expressed as arithmetic mean of 3 exhalations unless otherwise shown. *Acetone concentrations for individuals are expressed as arithmetic mean of 2 exhalations. †Acetone concentrations for the group are expressed as geometric mean (log SD): a significant difference in acetone concentration was observed between the two breath fractions (p<0.01), but none was observed between the two target expiratory flows. ‡CVs are expressed as median (IQR): there was no significant difference in CV between the two breath fractions or the two target expiratory flows. #Acetone phase III slopes for the group are expressed as arithmetic mean (SD).
**Figure 3-8** Intra-day and inter-day variation in acetone concentration in the exhaled breath of three volunteers, at breath fraction 85-100% by volume, of an exhalation of vital capacity, at a target expiratory flow of 170-330 ml/s.

3.4. **Discussion**

The aim of this study was to establish the accuracy, repeatability and dynamic response for measurement of acetone concentration using a Voice 100™ SIFT-MS instrument. Secondly, using a synchronised SIFT-MS instrument and pneumotachometer, the aim was to determine the effects of expiratory flow and volume on the acetone concentration in breath, and to determine an appropriate single-exhalation breathing manoeuvre from which a measure of acetone concentration could be obtained. Within-session, intra-day and inter-day coefficients of variation for these measurements were calculated.

3.4.1. **SIFT-MS instrument characteristics**

The SIFT-MS instrument was appropriate for the measurement of acetone concentration near the end of a vital capacity manoeuvre. The bias of 8% in the measurement of acetone concentration in humid air was consistent across the range of acetone concentrations found in the breath of the volunteers, and did not alter with time over the two months of testing. It may be that the bias was due to differences between the instrument used in this study, and the instruments used to calculate rate constants for the SIFT-MS reactions of acetone in humid air (Spanel et al, 1997b; Spanel and Smith, 2000a). The intra-day and inter-day coefficients of
variation of measurement of acetone in humid air were low. The dynamic response of the instrument was acceptable, given that it was less than 10% of the duration of any of the exhalations analysed (Bates et al, 1983).

3.4.2. Synchronisation of the SIFT-MS instrument and the pneumotachometer

The practical method and the subsequent calculations to synchronise the two instruments were simple to perform. The signal was clearly detected by both instruments. The duration of signal detection by SIFT-MS was not prolonged (less than 24 s, after which the ion count at m/z 19 returned to within 2 SDs of the preceding plateau). Rapid return of the signal to baseline was of practical significance because a persistent reduction in ion count at m/z 19 might have reduced the instrument’s sensitivity for subsequent breath analysis. It is likely that much of the time of decreased ion count at m/z 19 following signal nadir represented the time taken for SIFT-MS sampling to remove the remainder of humid air from the breath analysis system. It may be possible to reduce this time by flushing the breath analysis system with ambient air between syringe discharge and breath analysis, but this has not been tested.

Using humid air as an input reference signal accounted for any acquisition and processing delays in either the pneumotachometer or the SIFT-MS instrument. However, it should be noted that, using this method, the delay in signal acquisition by the SIFT-MS instrument may vary according to the flow rate of the humid air (i.e. the speed of syringe discharge), and the volume between the inlet to the breath analysis system and the SIFT-MS sample inlet. The time taken, by the front of humid air, to traverse this volume may affect synchronisation of subsequent exhalations if they are performed at flows much higher or lower than that of the synchronising syringe discharge.

There was no significant difference in time between paired syringe discharges as measured by the two instruments (0.01 ± 0.13 s; SED 0.04 s; p=0.76) demonstrating that the instruments remained synchronised after 90 s. This would have been an adequate length of time in which to perform an exhalation.

The SIFT-MS transit time of acetone in humid air was 400±50 ms (mean±SE) faster than that of the water vapour itself. The reason for this difference is unclear, but may be due to relative differences in the adsorption of the two compounds on to the surfaces of the breath analysis system upstream of the sample inlet. This difference must be accounted for in order to align measurements of exhaled acetone concentration and expiratory flow and volume. The relative
difference in transit time of acetone did not change across a range of acetone concentrations similar to the concentrations seen in exhaled breath (Anderson et al, 2006).

If using other reagent ions (NO$^+$ or O$_2^+$) for breath analysis, the additional monitoring of H$_3$O$^+$ will enable synchronisation. This method can be used for SIFT-MS breath analysis of any volatile compound when using H$_3$O$^+$ as a reagent ion. However, the technique for synchronising a SIFT-MS instrument and the pneumotachometer could be greatly simplified if the measurements of the two instruments were taken on the same internal timer.

### 3.4.3. Breath analysis

The aim of the study was to determine a single-exhalation breathing manoeuvre that resulted in accurate and reproducible analysis of acetone in breath using SIFT-MS. The twelve volunteers studied had normal lung function confirmed with spirometry and SBN$_2$. A normal SBN$_2$ phase III slope suggested a normal distribution of ventilation throughout the lung. The acetone concentrations measured in breath were similar to end-exhaled acetone concentrations recorded previously, and the profile of acetone concentration over exhaled volume was consistent with the airway exchange of a highly soluble gas, as has been previously observed (Anderson et al, 2006): acetone concentration rose immediately after the start of exhalation, and increased with increasing exhaled volume with no observed plateau in concentration.

These results suggest that acetone concentration in exhaled breath is independent of expiratory flow; therefore the breathing manoeuvre does not require the control of flow during an exhalation. This is in contrast to the findings of Anderson et al, who showed a difference in end-exhaled acetone normalised partial pressures of 0.79 and 0.85 at target flows of 200 and 350 ml/s respectively (Anderson et al, 2006). It is difficult to speculate on why there is a difference in the results between the two studies. A recent study showed that, on exhaling from total lung capacity to residual volume, the end-expired acetone concentration was independent of exhalation duration, suggesting that it was also independent of flow (O’Hara et al, 2008). The results of O’Hara et al are consistent with our findings in this study.

The acetone concentration increased with increasing exhaled volume: there was a statistically significant difference of 13 to 17 ppb in the concentration of exhaled acetone between the V$_{70-85\%}$ and V$_{85-100\%}$ fractions of exhaled vital capacity, and the phase III slope was positive and independent of flow. Given these findings, when analysing acetone in breath, it would appear reasonable to sample a fraction of end-exhaled breath from an exhalation of vital capacity,
such as the $V_{85-100\%}$ fraction used here. It might be argued that such a small difference in acetone concentration, whilst statistically significant, is unlikely to be clinically significant and, therefore, a strict requirement for exhalation from total lung capacity to residual volume is unnecessary. Nevertheless, an exhalation from total lung capacity to residual volume will maintain a low coefficient of variation in the measurement of acetone concentration. Furthermore, recent evidence from isothermal rebreathing experiments suggests that the partial pressure of acetone in end-exhaled breath, from an exhalation from total lung capacity to residual volume, is close to the partial pressure in blood (O’Hara et al, 2008; O’Hara et al, 2009). The maximum attainable end-exhaled acetone concentration is therefore desirable as the value most closely reflecting the concentration in blood.

The phase III slope in exhaled acetone concentration showed a strong negative correlation with FVC ($r=-0.8$, $p=0.001$). This finding has been predicted by modelling work (Hlastala and Anderson, 2007), but has not previously been experimentally confirmed. End-exhaled acetone concentration is dependent on exhaled volume and does not reach a plateau, as seen within individual exhalations. Therefore, in two subjects with the same systemic acetone concentration, it may be that the subject with the larger vital capacity achieves a higher end-exhaled acetone concentration simply because of a greater exhaled volume. Recently, larger studies have shown that exhaled acetone concentration is higher in men compared with women (Turner et al, 2006a), and adults compared with children (Schwartz et al, 2009). The possible confounding of results by vital capacity should be considered in future work.

The overall phase III slope in exhaled acetone concentration for the combined exhalations at target flows of 170 and 330 ml/s was $0.066\pm0.019 \ l^{-1}$ (arithmetic mean ± SD). In a previous study, the phase III slope ($0.054\pm0.016 \ l^{-1}$) was normalised by re-breathed acetone partial pressure (1.22 times greater than end-exhaled partial pressure in that study) (Anderson et al, 2006). After multiplying the phase III slope in that study by a factor of 1.22, the slopes from the two studies are similar.

In this study, it was necessary to curtail the volumes of exhalations so that differences in volume between an individual’s manoeuvres did not confound the analysis. However, this meant that the mean volume of the exhalations used for analysis of acetone in an individual was only 95% of that individual’s forced vital capacity. For clinical testing, it would be preferable to use exhalations as close to the maximum exhaled volume as possible, in order to reflect the concentration in blood as closely as possible. A similar protocol to that used for
spirometry testing might be appropriate, whereby a maximum but reproducible exhalation is
used to determine the end-exhaled acetone concentration (Miller et al, 2005).

The median within-session intra-individual coefficients of variation for the measurement of
exhaled acetone concentration were low (1.6 and 2.1% for the $V_{85-100\%}$ fraction of exhaled
vital capacity at the low and high expiratory flows respectively). The lower CVs at the lower
expiratory flows may not be random, the increased exhalation time allowing the instrument to
acquire a greater number of data points, hence reducing the standard error of measurement
over a given fraction of exhaled volume. The low variability of within-session measurements
contrasted with the high intra-day and inter-day variability (coefficients of variation of 36 and
15% respectively). While we only studied intra-day and inter-day variability in three
volunteers, our findings were not dissimilar to those of a previous longitudinal study using
SIFT-MS, over a six month period, that found the intra-individual coefficient of variation for
measurements of acetone concentration in breath to be 33% (Turner et al, 2006a). The
implication of this finding is that it may be important, depending on the clinical application,
not only to determine a normal range for the end-exhaled concentration of acetone, but also to
establish an intra-individual day-to-day change in acetone concentration that is clinically
important. Some of the variation in breath acetone concentration can be attributed to recent
dietary intake (Smith et al, 1999), and might be reduced by pre-test control of diet. However,
this may reduce the practical application of the test in a clinical setting.

3.5. Summary

The SIFT-MS instrument was appropriate for the measurement of acetone concentration near
the end of a vital capacity manoeuvre, and was successfully synchronised with a
pneumotachometer. For the analysis of acetone in breath using SIFT-MS, an exhalation from
total lung capacity to residual volume, at an expiratory flow of between 170 and 330 ml/s was
appropriate. Sampling the acetone concentration at the end of an exhalation gave low intra-
individual coefficients of variation. This breathing manoeuvre can now be used to explore,
more closely, the relationship between breath and blood acetone concentrations, and factors
affecting breath acetone concentrations in both normal subjects and those with pathologically
elevated levels.
4. The Analysis of Hydrogen Sulphide and Hydrogen Cyanide in Exhaled Breath

4.1. Introduction

Hydrogen sulphide (H\textsubscript{2}S) and hydrogen cyanide (HCN) are involved in inflammatory processes, although their roles have not been fully defined. H\textsubscript{2}S regulates the activation of leukocytes in inflammatory diseases, and the production of HCN is associated with neutrophil activation (Stelmaszynska, 1986; Zhang and Bhatia, 2008). Because they are volatile gases, the concentrations of H\textsubscript{2}S and HCN in exhaled breath might reflect levels of airway and/or systemic inflammation, and they therefore have potential to be inflammatory biomarkers.

H\textsubscript{2}S is an inflammatory mediator involved in the regulation of leukocyte function and the release of inflammatory mediators (Zhang and Bhatia, 2008), but has also been shown to have anti-inflammatory effects, such as protecting gastric mucosa against aspirin-induced injury (Fiorucci et al, 2005), and reducing airway inflammation and remodelling in a rat model of asthma (Chen et al, 2009a). Serum H\textsubscript{2}S levels in patients with stable COPD are higher than in healthy subjects, and higher in stable COPD than during acute exacerbations (Chen et al, 2005). Furthermore, serum H\textsubscript{2}S levels are positively correlated with serum NO levels and with the percentage of predicted FEV\textsubscript{1}, and serum H\textsubscript{2}S levels are negatively correlated with the proportion of sputum neutrophils (Chen et al, 2005). These findings suggest that H\textsubscript{2}S levels may be associated with disease activity and severity.

Increased production of HCN is associated with neutrophil activation. The action of myeloperoxidase on peptides and phagocytosed bacteria in neutrophils results in the formation of HCN (Stelmaszynska and Zgliczynski, 1978; Stelmaszynska, 1985). The production of HCN from thiocyanate by leukocytes challenged with bacteria has also been
demonstrated (Stelmaszynska, 1986). The exact biological role of hydrogen cyanide remains unclear, but one possible function is the stimulation of the respiratory burst that accompanies phagocytosis in order to degrade internalised particles and bacteria (DeChatelet et al, 1977).

Because H$_2$S and HCN are volatile gases, it is possible that any alteration in their levels in airway inflammation might be reflected by a change in their concentrations in exhaled breath, and thus they have the potential to be inflammatory biomarkers, as is the case for nitric oxide (Taylor et al, 2006). However, the mouth is a known source of both H$_2$S and HCN. H$_2$S produced by mouth bacteria contaminates orally exhaled breath, sometimes causing malodorous breath (Rosenberg and McCulloch, 1992; Pysanenko et al, 2008). HCN produced by salivary lactoperoxidase also contaminates orally exhaled breath (Lundquist et al, 1988; Wang et al, 2008). In order to gain measurements of the concentrations of these compounds in exhaled breath that reflect the concentrations in the lower respiratory tract, contamination from the mouth must be minimised or excluded. This might be achieved by sampling exhaled breath via the nose, which has been shown to reduce the concentrations of these volatile compounds in breath (Pysanenko et al, 2008; Wang et al, 2008). It might also be achieved by performing procedures to reduce the levels in the mouth prior to testing. For example, in the case of H$_2$S, the concentration in the mouth is greatly reduced after using hydrogen peroxide mouthwash (Suarez et al, 2000).

Expiratory flow may have an effect on the concentration of a volatile compound associated with airway inflammation, as has been shown in the case of nitric oxide (Silkoff et al, 1997) (see Section 1.2.3, Page 11). Given that the concentrations of H$_2$S and HCN in exhaled breath are to be investigated as biomarkers of airway inflammation, it is important to consider the effect of expiratory flow on the concentration of each volatile. Exhaled volume may have an effect on the concentration of a soluble gas, as in the case of acetone (Anderson et al, 2006) (see Section 3.3.3, Page 68). Because H$_2$S and HCN are both water-soluble, it is important that the effect of exhaled volume on H$_2$S and HCN concentrations in breath is also considered. The effects of expiratory flow and volume on the concentrations of H$_2$S and HCN in exhaled breath have not previously been studied.

In this study, we aimed to establish the accuracy, repeatability and dynamic response for measurements of H$_2$S and HCN concentrations using a Voice200™ SIFT-MS instrument. Secondly, using a synchronised SIFT-MS instrument and pneumotachometer, we aimed to determine the effects of oral and nasal exhalation, and expiratory flow and volume on the
concentrations of H₂S and HCN in the breath of healthy volunteers. We then aimed to
determine appropriate single-exhalation breathing manoeuvres from which measures of H₂S
and HCN concentration could be obtained.

4.2. Methods

4.2.1. Voice200™ SIFT-MS instrument

The Voice200™ SIFT-MS instrument (Syft Technologies Ltd, New Zealand) used for this
work has been described in detail previously (Prince et al, 2010); it is a smaller, lighter and
more sensitive version of the Voice100™ (Francis et al, 2009). As described previously for
the Voice100™ (see Section 3.2.1, Page 58), reagent ions were generated from a saturated
mixture of air and water by a microwave discharge. Ions from this mixture passed into an
upstream chamber, where the desired reagent ions were selected by passing the ion mixture
through an array of electrostatic lenses and the upstream quadrupole mass filter. The selected
reagent ions were then injected into the flow tube through a Venturi orifice. The operation of
the flow tube was also similar to that described for the Voice100™, the notable difference
being the use of helium as the sole carrier gas. In the downstream detection chamber, signal
levels for each of the reagent ions were typically 10⁶-10⁷ counts per second. These signal
levels were an order of magnitude greater than for the Voice100™, and were achieved
through modification of the electrostatic lenses and the flow conditions within the flow tube.
The increased ion signals gave the instrument improved sensitivity in comparison to its
predecessor.

The instrument was fitted with a Heated Inlet Extension (T0020, Syft Technologies Ltd, New
Zealand) 100 cm long, heated to a temperature of 120°C and supported by a support arm
attached to the Voice200™ SIFT-MS instrument. The Heated Inlet Extension was attached to
a Breath Head (T0033, Syft Technologies Ltd, New Zealand) (see Figure 4-1). This
comprised a stainless steel tube tapered at one end for the attachment of a disposable
respiratory filter (SureGuard, BIRD Healthcare, Australia), and at the other to accept a
pneumotachometer (RSS 100, Hans Rudolph Inc, USA), and heated to 40°C. The stainless
steel tube was held within an acetal casing, the base of which was secured to the Heated Inlet
Extension. A hole in the side of the tube allowed access for a sampling capillary protruding
from the Heated Inlet Extension.
The existing data acquisition software (Syft Technologies Ltd, New Zealand) for the Voice200™ SIFT-MS instrument was modified to communicate with the pneumotachometer via its serial port. This was achieved with a publicly available program (uCon, Microcross, USA) to control the serial communications with the pneumotachometer hardware. This software provided a Telnet Server interface that the data acquisition software used to enable and disable logging of breath profiles from the pneumotachometer. The data file format used by the data acquisition program was extended to permit saving of the pneumotachometer data along with the analyte concentration data. Each pneumotachometer data point and each SIFT-MS data point was provided with a time-point relative to the time at which the SIFT-MS instrument sample valve was opened. Thus, the measurements of both instruments were provided with time-points from the same internal timer. This eliminated the need for the input reference signal that was required when measurements were taken on separate timers using a Voice100™ SIFT-MS instrument and a pneumotachometer (see Section 3.2.5, Page 60).

4.2.2. SIFT-MS analysis of hydrogen sulphide

The H$_3$O$^+$ reagent ion is the only one available for the analysis of H$_2$S. The NO$^+$ reagent ion does not react with H$_2$S, and the O$_2$$^+$ reagent ion reacts to give H$_2$S$^+$, which reacts quickly.
with H$_2$O (Spanel and Smith, 2000b). The proton transfer reaction between H$_3$O$^+$ and H$_2$S is shown below:

$$\text{H}_3\text{O}^+ + \text{H}_2\text{S} \rightleftharpoons \text{H}_3\text{S}^+ + \text{H}_2\text{O}$$

The reaction between proton donor and acceptor can only proceed if the proton affinity of the latter is greater than that of the former. The proton affinities of H$_2$S and H$_2$O are 705 and 691 kJmol$^{-1}$ respectively, allowing the reaction to occur, and the concentration of H$_2$S can then be determined by monitoring the H$_3$S$^+$ product ion at $m/z$ 35 (Hunter and Lias, 1998; Spanel and Smith, 2000b). However, the 14 kJmol$^{-1}$ difference is less than the suggested 20 kJmol$^{-1}$ required for fast proton transfer from donor to recipient, and so any extra energy in the product ions can promote the reverse reaction (Bouchoux et al., 1996). This reaction has been studied previously, and the rate coefficient has been determined for the analysis of H$_2$S in humid air (Williams et al., 1998; Spanel and Smith, 2000b). In order to measure the concentration of H$_2$S in breath accurately, the instrument used for this study was calibrated using known concentrations of H$_2$S in humid air (see Section 4.2.4).

Monitoring cycles for breath analysis took 800 ms, and resulted in the acquisition of one data point, giving a sampling frequency of 1.3 Hz. Monitoring cycles for determining transit time and dynamic response were performed at 20 Hz.

4.2.3. SIFT-MS analysis of hydrogen cyanide

The analysis of hydrogen cyanide was performed using the H$_3$O$^+$ reagent ion in the proton transfer reaction shown below:

$$\text{H}_3\text{O}^+ + \text{HCN} \rightleftharpoons \text{H}_2\text{CN}^+ + \text{H}_2\text{O}$$

The proton affinity of HCN (713 kJmol$^{-1}$) is 22 kJmol$^{-1}$ greater than that of H$_2$O, and is sufficient to drive the reaction forward. This reaction has been studied in humid air, and the rate coefficient has been determined (Spanel et al., 2004). The concentration of HCN is then calculated from the product ion count of H$_2$CN$^+$ at $m/z$ 28 (Spanel et al., 2004). In order to measure the concentration of H$_2$S in breath accurately, the instrument used for this study was calibrated using known concentrations of H$_2$S in humid air (see Section 4.2.4).
Monitoring cycles for breath analysis took 600 ms, and resulted in the acquisition of one data point, giving a sampling frequency of 1.7 Hz. Monitoring cycles for determining transit time and dynamic response were performed at 20 Hz.

**4.2.4. Instrumental accuracy, repeatability and dynamic response**

The accuracy and repeatability of the instrument, for the measurement of H₂S and HCN, were determined using a custom permeation system consisting of a dilution apparatus (Syft Technologies Ltd., New Zealand) and permeation chamber (Dynacalibrator Model 150, VICI Metronics, USA), and permeation tubes of H₂S (Metronics, USA) and HCN (Kin-tek, USA) with known emission rates of 489 and 1307 ng/min respectively at 40°C. The system delivered a flow of a known concentration of H₂S or HCN in air at 100% relative humidity. H₂S at concentrations of 2.5 and 5.0 ppb were measured by the instrument on ten weekdays over four weeks. In addition, on four of those days, morning and afternoon measurements were made. HCN at concentrations of 760 and 1085 ppb were measured by the instrument on 27 days over 18 weeks. In addition, on four of those days, morning and afternoon measurements were made.

The dynamic response of the instrument was determined by measuring the time taken for the instrument to respond to step changes in H₂S and HCN concentration from the background level in the ambient air to a concentrations in humid air of 40 ppb for H₂S, and a concentrations of 475 ppb for HCN. The time taken between achieving a 10% and 90% response to the step change was measured when performing the same experimental method as described in Section 4.2.6.

**4.2.5. Breath analysis system**

The SIFT-MS instrument and pneumotachometer were configured to make simultaneous exhalation measurements (see Figure 4-1). For oral exhalations, a disposable mouthpiece with a respiratory filter (SureGuard, BIRD Healthcare, Australia) was connected to the proximal end of the Breath Head. For nasal exhalations, subjects wore a nasal mask (Flexifit™ 407, Fisher and Paykel Healthcare, New Zealand) attached to a respiratory filter (SureGuard, BIRD Healthcare, Australia) that was connected to the proximal end of the Breath Head. On the distal end of the Breath Head, the pneumotachometer and an adjustable flow-restrictor were attached in series. The flow measured by the pneumotachometer was displayed on a screen during exhalation so that a subject could exhale at a target expiratory
flow. The flow-restrictor had an inbuilt fan that could be operated between exhalations in order to purge the system, to expel exhaled breath from the system and prevent build-up of condensation.

4.2.6. Synchronisation of the SIFT-MS instrument and the pneumotachometer

As described above (see Section 4.2.1), the measurements of the SIFT-MS instrument and the pneumotachometer were provided with time-points from the same timer, and this obviated the need for an input reference signal to synchronise the instruments. However, in order to ensure the synchrony of the two instruments, it was necessary to determine any difference in transit time through the system between the expiratory flow signal measured by the pneumotachometer and the analyte signal measured by the SIFT-MS instrument. In order to do this, one port of a respiratory humidifier (HC150, Fisher and Paykel Healthcare, New Zealand) was connected to the disposable respiratory filter on the breath analysis system, and the second port was attached to a 1 litre syringe (Vitalograph, UK). Either H₂S (at a concentration of 70 ppb) or HCN (at concentrations of 300 to 400 ppb) was added to the humidifier. The syringe was emptied within 3 seconds, causing the humid air and the analyte to flow past the SIFT-MS inlet and through the pneumotachometer, displacing the ambient air. The flow was detected by the pneumotachometer, while the analyte was detected by the SIFT-MS instrument. The time-point at which flow was first detected by the pneumotachometer was measured, as was the time-point at which an increase in analyte concentration of >2 SDs occurred, and the difference between the two was calculated. The syringe injection was repeated 20 times, and the mean±SE transit time of the analyte was calculated relative to the time at which detection of expiratory flow was detected by the pneumotachometer.

4.2.7. Processing of data files

The analyte concentration data from the SIFT-MS instrument and the data from the pneumotachometer were saved and then processed as Excel 2007© files (Microsoft, USA). Using Visual Basic for Applications (Microsoft, USA), a number of macro programs were written for use with Excel 2007© in order to automate the processing of data files (see Appendix B).

On opening the first Excel 2007© file, the operator was instructed to create a workbook using a macro program (Pro1WorkbookCreate). The operator was then instructed to copy the SIFT-
MS analyte data file into one worksheet, to copy the pneumotachometer and m/z count data file into another worksheet, and to enter the SIFT-MS transit time for the analyte. The operator then ran another macro (ProcessAllData). This adjusted the time-points of the two instruments to account for the difference in transit times, and then identified the exhalations from within several minutes of data collection. These exhalations were then extracted and presented as shown in Table 4-1.

If the experiment required the curtailment of an individual’s exhalations to a uniform volume, a second file was then opened, and the data were copied into it. A further macro program (CurtAll) then curtailed all the exhalations performed by an individual to a volume predetermined by the operator. Curtailment of an individual’s exhalations to a uniform volume was applied when it was necessary to control for the variability of vital capacity between successive expiratory manoeuvres.

A final Excel 2007 file was then opened, and the data shown in Table 4-1 were copied into it. The operator then ran a macro program (BobOne) to calculate a number of exhalation characteristics and to calculate the flow and analyte concentration at various breath volume fractions (see Table 4-2).
Table 4-1 Example of processed exhalation data presented after adjustment for difference in transit time between the SIFT-MS instrument and pneumotachometer and after extraction from raw data file.

<table>
<thead>
<tr>
<th>SIFT-MS time (ms)</th>
<th>Analyte concentration (ppb)</th>
<th>Pneumotachometer time (ms)</th>
<th>Flow (l/min)</th>
<th>Pressure (cmH₂O)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16215</td>
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</table>
Table 4-2  Example of processed exhalation data presented as exhalation characteristics and expiratory flow and analyte concentration at various breath volume fractions.

<table>
<thead>
<tr>
<th>Exhalation characteristics</th>
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<tbody>
<tr>
<td>Duration (s)</td>
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<tr>
<td>Volume (l)</td>
</tr>
<tr>
<td>Mean flow (l/min)</td>
</tr>
<tr>
<td>Mean analyte conc. (ppb)</td>
</tr>
<tr>
<td>Max analyte conc. (ppb)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Flow and analyte conc. by breath volume fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breath fraction (%): Analyte (ppb): Flow (l/min)</td>
</tr>
<tr>
<td>0 to 10: 2.5: 4.0</td>
</tr>
<tr>
<td>10 to 20: 4.9: 13.0</td>
</tr>
<tr>
<td>20 to 30: 4.1: 13.0</td>
</tr>
<tr>
<td>30 to 40: 5.1: 12.0</td>
</tr>
<tr>
<td>40 to 50: 4.1: 10.9</td>
</tr>
<tr>
<td>50 to 60: 4.8: 10.7</td>
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<td>60 to 70: 3.7: 12.4</td>
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<td>60 to 80: 4.0: 12.3</td>
</tr>
<tr>
<td>80 to 100: 3.6: 5.5</td>
</tr>
</tbody>
</table>
4.2.8. Study design for testing of participants

*Hydrogen sulphide*

The experimental procedures were approved by the Upper South Regional Ethics Committee. Six consenting, healthy, non-smoking volunteers with no history of respiratory disease were studied. Volunteers attended three visits within a two month period. Study visits were performed between 10 a.m. and 12 p.m. for all 6 volunteers.

**Visit 1:** spirometry was measured, using an EasyOne™ spirometer (NDD, Switzerland), according to current ATS/ERS standards (Miller *et al.*, 2005), and a single-breath nitrogen washout test (SBN2) (Viasys, USA) was performed. Vital capacity achieved during the SBN2 test was within 200 ml of the volunteer’s vital capacity as measured by spirometry.

**Visit 2:** subjects refrained from eating or drinking anything other than water, and did not partake in exercise for at least an hour before being tested. After the subject had rested for five minutes, direct sampling of the oral and nasal cavity was performed in random order. For sampling of the oral cavity, an 80 mm length of 0.38mm internal diameter Teflon® tubing (Du Pont, USA) was connected to a particulate filter (Dismic-25JP, Advantec, Japan) and this was connected to the SIFT-MS sampling capillary on the Breath Head. The subject breathed tidally via the nose for 2 minutes before holding the end of the tubing in the mouth and maintaining a tight seal. The subject continued to breathe tidally via the nose while the concentration of H₂S inside the oral cavity was measured. For nasal sampling, the apparatus was modified with the addition of a nasal adaptor (Entsol nasal adaptor, Kenwood Therapeutics, USA) to the end of the tubing. The subject breathed tidally via the mouth for 2 minutes while wearing a nose clip. The nose clip was then removed, and the nasal adaptor was inserted into one nostril while the other nostril was pressed closed. The subject continued to breathe tidally via the mouth while the concentration of H₂S inside the nasal cavity was measured.

Following these oral and nasal manoeuvres, the breath analysis system was assembled as shown in Figure 4-1. Subjects then performed four vital capacity manoeuvres (A, B, C and D) in random order and at a target flow rate of 10 l/min (see Table 4-3). The concentration of H₂S in the exhaled breath was measured by the SIFT-MS instrument, and the exhalation flow and volume were measured by the pneumotachometer. The subject then repeated the four manoeuvres in random order.
Table 4-3 Four breathing manoeuvres were performed in random order.

<table>
<thead>
<tr>
<th>Manoeuvre</th>
<th>Two minutes of tidal breathing via</th>
<th>Inhale to vital capacity via</th>
<th>Exhale to residual volume into apparatus via</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mouth</td>
<td>Mouth</td>
<td>Mouth</td>
</tr>
<tr>
<td>B</td>
<td>Mouth</td>
<td>Mouth</td>
<td>Nose</td>
</tr>
<tr>
<td>C</td>
<td>Nose</td>
<td>Nose</td>
<td>Mouth</td>
</tr>
<tr>
<td>D</td>
<td>Nose</td>
<td>Nose</td>
<td>Nose</td>
</tr>
</tbody>
</table>

After completing the eight exhalation manoeuvres, the subject then rinsed his/her mouth with 5 ml of 3% hydrogen peroxide mouthwash (PSM Healthcare, New Zealand) for one minute. After a 5 minute interval, direct sampling of the oral and nasal cavities and the exhalation manoeuvres were repeated.

Visit 3: subjects refrained from exercise and eating or drinking anything other than water for at least an hour before being tested. After the subject had rested for five minutes, direct sampling of the oral and nasal cavities was performed in random order as described above. The subject then rinsed his/her mouth with 5 ml of 3% hydrogen peroxide mouthwash for one minute and, after a 5 minute interval, direct sampling of the oral and nasal cavities was repeated.

Following this, the breath analysis system was set up. The subject then performed three nasal exhalations into the breath analysis system at a target flow of 170 ml/s and three nasal exhalations at a target flow of 330 ml/s. The concentration of H$_2$S in the exhaled breath was measured by the SIFT-MS instrument, and the exhalation flow and volume were measured by the pneumotachometer. Each exhalation was preceded by 2 minutes of nasal tidal breathing and a nasal inhalation to total lung capacity, and each exhalation was to residual volume. The exhalations at the two target flows were performed in random order.

Lastly, the subject performed four nasal exhalations from total lung capacity to residual volume at 2 minute intervals. The concentration of H$_2$S in the four breaths was measured by the SIFT-MS instrument, and the exhalation flow and volume were measured by the pneumotachometer. In the intervening 2 minutes between exhalations, the subject performed nasal tidal breathing. For the first three exhalations, the subject inhaled *nasally* to total lung capacity. For the fourth exhalation, the subject inhaled *orally* to total lung capacity.
Hydrogen cyanide

The same six subjects also performed the same set of experiments for the analysis of hydrogen cyanide in exhaled breath. However, experiments were performed without the use of 3% hydrogen peroxide mouthwash. Therefore, at the second visit, there was no repetition of the direct sampling of HCN in the oral and nasal cavities and manoeuvres A to D after mouthwash. At the third visit, there was no repetition of the direct sampling of HCN in the oral and nasal cavities after mouthwash, but the experiment was otherwise unaltered.

4.2.9. Statistical analysis

Analyte concentrations in samples directly from the oral and nasal cavities were compared using the Wilcoxon signed-rank test. In order to compare the effects of different breathing manoeuvres on the concentration of an analyte in exhaled breath, two values for the analyte concentration in an exhalation were used: the mean-exhaled concentration and the end-exhaled concentration. The end-exhaled concentration was defined as the mean concentration measured in the last 20% by volume of an exhalation. Analysis of variance and paired t-tests were used to compare exhalations before or after mouthwash, with oral or nasal pre-test tidal breathing, and with oral or nasal exhalations. For the comparison of exhalations performed at target flows of 170 or 330 ml/s, exhalations of a volume less than 90% of an individual’s maximum exhaled volume were excluded, and the volumes of the remaining exhalations were curtailed to that of the smallest exhaled volume for analysis. Comparisons of the exhalations at the two different target flows were made by paired t-test. Analysis of variance and paired t-tests were used to compare the analyte concentration in different volume fractions of exhaled breath. Any difference in analyte concentration in repeated exhalations was determined by analysis of variance. Correlations were performed using Spearman’s rank correlation. Analyses were performed using SPSS 16.

4.3. Results – hydrogen sulphide

4.3.1. SIFT-MS instrument characteristics for the analysis of hydrogen sulphide

Measurements of H$_2$S in humid air were taken at known concentrations of 2.5 and 5.0 ppb on ten days over four weeks. On four of those days, morning and afternoon measurements were made. At known H$_2$S concentrations of 2.5 and 5.0 ppb, the H$_2$S concentrations measured by the SIFT-MS instrument were 2.2±0.7 and 4.5±0.7 ppb (mean±SD) respectively. The
accuracy of measurement of the known concentrations of 2.5 and 5.0 ppb were expressed as mean (95% confidence interval) percentage deviations from the known concentrations, and these were -13 (-3 to -24)% and -12 (-6 to -17)% respectively. There was no change in the accuracy of the measurements over the four weeks of testing. The precision of the instrument was expressed as inter-day and intra-day coefficients of variation of measurement of H₂S concentration. At the known H₂S concentration of 2.5 ppb, these were 22% and 25% respectively, and at the known H₂S concentration of 5.0 ppb, they were 13% and 15% respectively.

The 10-90% dynamic response time for the measurement of H₂S in humid air was measured ten times. For a step change from a mean H₂S concentration of 1.7 ppb in ambient air to a mean H₂S concentration of 40 ppb in humid air, the dynamic response time was 500±60 ms (mean±SE).

The difference in transit time through the system between the expiratory flow signal measured by the pneumotachometer and the analyte signal measured by the SIFT-MS instrument was measured 13 times using a known H₂S concentration in humid air of 70 ppb. The difference between the transit times of the two instruments was 720±40 ms (mean±SE).

4.3.2. Subject characteristics

The same six healthy non-smoking volunteers successfully completed all parts of the study protocols for the analysis of hydrogen sulphide in exhaled breath. Their characteristics are shown in Table 4-4. The volunteers had normal spirometry, and phase III slopes for the single-breath nitrogen washout test ranged from 0.58 to 1.13% N₂/l.

4.3.3. Concentration of hydrogen sulphide in ambient air

The ambient level of H₂S in the laboratory immediately before conducting experiments was 0.5 (0.3-0.9) ppb (median (IQR)).

4.3.4. Direct sampling of hydrogen sulphide from the oral and nasal cavities

Concentrations of H₂S measured directly from the mouth before and after mouthwash were 21.0 (1.7-60.8) ppb (median (range)) and 1.9 (1.3-6.2) ppb respectively. Concentrations of H₂S measured directly from the nose before and after mouthwash were 0.9 (0.4-2.1) ppb and 0.9 (0.5-1.6) ppb respectively. Concentrations of H₂S measured directly from the mouth and
nose before mouthwash were significantly different, as were the concentrations of H$_2$S measured directly from the mouth before and after mouthwash (see Figure 4-2).

**Table 4-4** Subject characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female/male</td>
<td>3/3</td>
</tr>
<tr>
<td>Age</td>
<td>36 (6)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>176 (7)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78 (9)</td>
</tr>
<tr>
<td>FEV$_1$ (L)</td>
<td>3.97 (1.01)</td>
</tr>
<tr>
<td>Percentage of predicted FEV$_1$</td>
<td>103 (6)</td>
</tr>
<tr>
<td>FVC (L)</td>
<td>4.94 (1.30)</td>
</tr>
<tr>
<td>Percentage of predicted FVC</td>
<td>106 (9)</td>
</tr>
<tr>
<td>SBN$<em>2$ S$</em>{III}$ (% N$_2$/L)</td>
<td>0.83 (0.21)</td>
</tr>
</tbody>
</table>

Abbreviations: FEV$_1$ = forced expiratory volume in one second; FVC = forced vital capacity; SBN$_2$ S$_{III}$ = single-breath nitrogen washout test phase III slope.

**Figure 4-2** Median and individual concentrations of H$_2$S measured by direct sampling from the mouth and nose before and after rinsing the mouth with 3% H$_2$O$_2$ mouthwash. *p<0.05.
4.3.5. Effect of oral vs. nasal breathing manoeuvres and effect of mouthwash

The mean expiratory flow for all manoeuvres was 148±18 (mean±SD) ml/s, and there was no difference in expiratory flow between different breathing manoeuvres. The mean exhaled volume for all manoeuvres was 4.32±1.40 litres (mean±SD). The mean exhaled volume of exhalation achieved for oral exhalations was 0.22 litres greater than that achieved for exhalations via the nose (p=0.02).

Figure 4-3 shows the effects of mouthwash and oral and nasal breathing on the concentration of H$_2$S in exhaled breath. A higher concentration of H$_2$S was observed in oral, compared with nasal, exhalations (oral vs. nasal mean-exhaled H$_2$S concentration (mean±SE): 2.4±0.5 ppb vs. 1.2±0.2 ppb, p=0.05; oral vs. nasal end-exhaled H$_2$S concentration: 2.5±0.5 ppb vs. 1.1±0.2 ppb, p=0.06). There was a significant interaction between pre-test tidal breathing and exhalation of vital capacity via mouth or nose (p=0.04 for mean H$_2$S concentration and p=0.02 for end-exhaled H$_2$S concentration), with higher H$_2$S concentrations observed in oral exhalations when preceded by nasal rather than oral tidal breathing.

There was a reduced concentration of H$_2$S in exhaled breath after mouthwash. Mean-exhaled H$_2$S concentration fell from 2.4±0.4 ppb to 1.2±0.2 ppb after mouthwash (p=0.01), and end-exhaled H$_2$S concentration fell from 2.4±0.4 ppb to 1.3±0.2 ppb after mouthwash (p=0.01).

The lowest mean-exhaled and end-exhaled concentrations of H$_2$S were observed in nasal exhalations after mouthwash (mean-exhaled H$_2$S concentration: 0.9±0.1 ppb; end-exhaled H$_2$S concentration: 0.9±0.2 ppb). Using this manoeuvre, there was no difference in the concentrations of H$_2$S in exhaled breath between manoeuvres with oral and nasal pre-test tidal breathing.
Figure 4-3  Mean (SE) H$_2$S concentrations for (1) 2 min of pre-test tidal breathing then inhalation to TLC via mouth (closed circles) or nose (open squares), followed by, (2) exhalation of vital capacity, via mouth (blue) or nose (red). Median and IQR for background H$_2$S levels are shown as a black line and grey area.
4.3.6. Effect of expiratory flow on hydrogen sulphide concentration in nasally-exhaled breath

Each volunteer rinsed his/her mouth with H$_2$O$_2$ mouthwash for one minute and then, after an interval of 5 minutes, completed six nasal exhalations from total lung capacity to residual volume: three exhalations at a target flow of 170 ml/s and three exhalations at a target flow of 330 ml/s performed in random order. All exhalations were preceded by at least 2 minutes of nasal tidal breathing and a nasal inhalation to total lung capacity. In total, 36 exhalations were performed (18 exhalations at each target flow) by the six volunteers. Six exhalations in four subjects were excluded because the exhaled volume was less than 90% of the maximum nasally-exhaled volume achieved by that subject, leaving 30 exhalations for analysis. For the six subjects, the mean curtailed volume of exhalation used for analysis was 4.19±1.27 litres (mean±SD). The mean volume of exhalation that was analysed for each individual was 93±1% (mean±SD) of the maximum nasally-exhaled volume achieved by that individual. The actual mean expiratory flows for target flows of 170 ml/s and 330 ml/s were 178±8 (mean±SE) and 305±16 ml/s respectively (p<0.001).

There was no difference in the concentrations of H$_2$S in exhalations performed at target flows of 170 ml/s and 330 ml/s: mean-exhaled H$_2$S concentrations were 0.9±0.3 ppb (mean±SE) and 1.0±0.3 ppb respectively, and end-exhaled H$_2$S concentrations were 1.0±0.3 ppb and 1.0±0.3 ppb respectively (see Figure 4-4). No interaction was observed between the effects of fraction of exhaled volume and expiratory flow on the concentration of H$_2$S in exhaled breath. Therefore, the data for the concentrations of H$_2$S at the two target expiratory flows were merged, and any effect of exhaled volume was explored: there was no difference in H$_2$S concentrations across the five fractions of exhaled volume shown in Figure 4-4.
Figure 4-4  (A) Mean (SE) H₂S concentrations for exhalations performed at target expiratory flows of 170 and 330 ml/s. Median and IQR for background H₂S levels are shown as a black line and grey area.  (B) Mean (SE) actual expiratory flows at target expiratory flows of 170 and 330 ml/s.
4.3.7. Effect of repetition of breathing manoeuvre

Each of the six volunteers rinsed his/her mouth with H\textsubscript{2}O\textsubscript{2} mouthwash for one minute and then, after at least 2 minutes of nasal tidal breathing, completed three nasal exhalations from total lung capacity to residual volume at a target flow of 170 ml/s. The three exhalations were performed at 2 minute intervals and were immediately preceded by a nasal inhalation to total lung capacity. During the intervening periods, the volunteer performed nasal tidal breathing.

For all exhalations, the mean-exhaled and end-exhaled concentrations of H\textsubscript{2}S were both 0.9±0.2 ppb (mean±SE), and there was no difference in either of these H\textsubscript{2}S concentrations in breath across the three repeated exhalations. The actual expiratory flow for all exhalations was 152±17 ml/s (mean±SD) and the exhaled volume was 4.59±1.35 litres, and there was no difference in exhaled flow or volume across the three repeated exhalations.

Given that the repetition of this breathing manoeuvre had no effect on the concentration of H\textsubscript{2}S in exhaled breath, within-session coefficients of variation were calculated from these data: the median intra-subject within-session CV for the mean-exhaled and end-exhaled H\textsubscript{2}S concentrations were 8.5% (IQR 6.0-11.1%) and 7.6% (IQR 2.6-16.2%) respectively.

4.3.8. Effect of oral vs. nasal inhalation on the concentration of H\textsubscript{2}S in nasally-exhaled breath

At the end of the above experiment (see Section 4.3.7), the volunteer performed nasal tidal breathing for a further 2 minutes, inhaled orally to total lung capacity, and then exhaled nasally to residual volume. A comparison was then made of the effects of oral vs. nasal inhalation on the concentration of H\textsubscript{2}S in nasally-exhaled breath.

There was no difference in exhaled H\textsubscript{2}S concentrations between the two breathing manoeuvres of oral vs. nasal inhalation (mean-exhaled concentration: 0.9±0.2 vs. 0.9±0.2 ppb, end-exhaled concentration: 0.9±0.2 vs. 0.9±0.2 ppb (mean±SE)). Expiratory flow was not different in exhalation manoeuvres of oral and nasal inhalation (146±19 ml/s (mean±SD) vs. 152±17 ml/s), but exhaled volume was 0.34 litres greater in exhalation manoeuvres of oral, compared with nasal, inhalation (4.59±1.35 vs 4.24±1.36 litres, p<0.01).
4.3.9. Relationship between the concentration of H$_2$S in nasally-exhaled breath and sources of contamination

Using the breathing manoeuvre described in Section 4.3.7, there was a positive correlation between the concentration of H$_2$S in exhaled breath and in the ambient air ($r=0.93$, $p=0.01$ for both mean-exhaled and end-exhaled H$_2$S). No significant correlation was observed between the concentration of H$_2$S in exhaled breath and in gas sampled directly from the oral cavity either before or after H$_2$O$_2$ mouthwash.

4.4. Results – hydrogen cyanide

4.4.1. SIFT-MS instrument characteristics for the analysis of hydrogen cyanide

Measurements of HCN in humid air were taken at known concentrations of 760 and 1085 ppb on 27 days over 18 weeks. On four of those days, morning and afternoon measurements were made. At known HCN concentrations of 760 and 1085 ppb, the HCN concentrations measured by the SIFT-MS instrument were 737±68 and 1201±138 ppb (mean±SD) respectively. The accuracy of measurement of the known concentrations of 760 and 1085 ppb were expressed as mean (95% confidence interval) percentage deviations from the known concentrations, and these were -3 (-6 to 0)% and +11 (+8 to +22)% respectively. There was no change in the accuracy of the measurements over the two months of testing. The precision of the instrument was expressed as inter-day and intra-day coefficients of variation of measurement of HCN concentration. At the known HCN concentration of 760 ppb, these were 9% and 6% respectively, and at the known HCN concentration of 1085 ppb, they were 12% and 4% respectively.

The 10-90% dynamic response time for the measurement of HCN in humid air was measured ten times. For a step change from a mean HCN concentration of 2.5 ppb in ambient air to a mean HCN concentration of 475 ppb in humid air, the dynamic response time was 620±50 ms (mean±SE).

The difference in transit time through the system between the expiratory flow signal measured by the pneumotachometer and the analyte signal measured by the SIFT-MS instrument was measured ten times using known HCN concentrations in humid air of 300-400 ppb and was calculated to be 700±30 ms (mean±SE).
4.4.2. Subject characteristics

Six healthy non-smoking volunteers successfully completed all parts of the study protocols for analysis of hydrogen cyanide and in exhaled breath. Their characteristics are shown in Table 4-5. The volunteers had normal spirometry, and phase III slopes for the single-breath nitrogen washout test ranged from 0.58 to 1.13% N₂/l.

Table 4-5 Subject characteristics.

<table>
<thead>
<tr>
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<td>Weight (kg)</td>
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<td>FEV₁ (L)</td>
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<td>Percentage of predicted FEV₁</td>
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</tr>
<tr>
<td>FVC (L)</td>
<td>4.97 (1.25)</td>
</tr>
<tr>
<td>Percentage of predicted FVC</td>
<td>104 (11)</td>
</tr>
<tr>
<td>SBN₂ S₃Ⅲ (% N₂/l)</td>
<td>0.82 (0.20)</td>
</tr>
</tbody>
</table>

Abbreviations: FEV₁ = forced expiratory volume in one second; FVC = forced vital capacity; SBN₂ S₃Ⅲ = single-breath nitrogen washout test phase III slope.

4.4.3. Concentration of hydrogen cyanide in ambient air

Ambient levels of HCN in the laboratory immediately before conducting experiments were 2.3 (2.1-2.7) ppb (median (IQR)).

4.4.4. Direct sampling of hydrogen cyanide from the oral and nasal cavities

The concentrations of HCN measured directly from the mouth and nose were 64.3 (57.1-81.4) ppb (median (range)) and 25.1 (14.2-32.8) ppb respectively (p<0.05) (see Figure 4-5).
Figure 4-5 Median and individual concentrations of HCN measured by direct sampling from the mouth and nose.

4.4.5. Effect of oral vs. nasal breathing manoeuvres

The mean expiratory flow for all manoeuvres was $178\pm16$ (mean±SD) ml/s, and there was no difference in expiratory flow between different breathing manoeuvres. The mean exhaled volume for all manoeuvres was $4.21\pm1.20$ litres (mean±SD), and no significant difference was observed between manoeuvres.

Figure 4-6 shows the effects of oral and nasal breathing on the concentration of HCN in exhaled breath. A higher mean-exhaled and end-exhaled HCN concentration was observed in oral, compared with nasal, exhalations (oral vs. nasal mean-exhaled HCN (mean±SE): $5.0\pm0.7$ ppb vs. $2.6\pm0.4$ ppb, $p<0.01$; oral vs. nasal end-exhaled HCN: $4.5\pm0.6$ ppb vs. $2.4\pm0.3$ ppb, $p<0.01$). The interaction between pre-test tidal breathing and exhalation of VC via mouth or nose approached significance for the mean-exhaled HCN concentration ($p=0.07$), with higher HCN concentrations observed in oral exhalations when preceded by nasal rather than oral tidal breathing. No such interaction was observed for the end-exhaled HCN concentration.

The lowest mean-exhaled and end-exhaled concentrations of HCN were observed in nasal exhalations (mean-exhaled HCN concentration: $2.6\pm0.4$ ppb; end-exhaled H$_2$S concentration: $2.4\pm0.3$ ppb). Using this manoeuvre, there was no difference in the concentrations of H$_2$S in exhaled breath between manoeuvres with oral and nasal pre-test tidal breathing.
Figure 4-6 Mean (SE) HCN concentrations for (1) 2 min of pre-test tidal breathing then inhalation to TLC via mouth (closed circles) or nose (open squares), followed by, (2) exhalation of vital capacity, via mouth (blue) or nose (red). Median and IQR for background HCN levels are shown as a black line and grey area.

4.4.6. Effect of expiratory flow on hydrogen cyanide concentration in nasally-exhaled breath

Each volunteer completed six nasal exhalations from total lung capacity to residual volume: three exhalations at a target flow of 170 ml/s and three at a target flow of 330 ml/s (see Figure 4-7). All exhalations were preceded by at least 2 minutes of nasal tidal breathing and a nasal inhalation to total lung capacity. In total, 18 exhalations were performed at each target flow. Four exhalations from three volunteers were excluded because the exhaled volume was less than 90% of the maximum nasally-exhaled volume achieved by that subject, leaving 32 exhalations for analysis. For the six subjects, the curtailed volume of exhalation used for analysis was 4.18±1.42 litres (mean±SD). The volume of exhalation that was analysed for each individual was 93±2% (mean±SD) of the maximum nasally-exhaled volume achieved by that individual. The actual expiratory flows for target flows of 170 ml/s and 330 ml/s were 177±11 (mean±SE) and 313±13 ml/s respectively (p<0.001).

The mean-exhaled concentrations of HCN in exhalations were different at target expiratory flows of 170 ml/s and 330 ml/s: 2.1±0.3 ppb (mean±SE) and 1.8±0.2 ppb respectively (p=0.05). End-exhaled concentrations of HCN of 2.0±0.3 ppb and 1.7±0.2 ppb, at expiratory flows of 170 and 330 ml/s respectively, were not significantly different (see Figure 4-7 A).
At both target expiratory flows, there was no difference in H$_2$S concentrations across the five fractions of exhaled volume shown in Figure 4-7 B.

**Figure 4-7 (A)** Mean (SE) HCN concentrations for exhalations performed at target expiratory flows of 170 and 330 ml/s. Median and IQR for background HCN levels are shown as a black line and grey area. 

**Figure 4-7 (B)** Mean (SE) actual expiratory flows at target expiratory flows of 170 and 330 ml/s.
4.4.7. Effect of repetition of breathing manoeuvre

Each of the six volunteers completed three nasal exhalations from total lung capacity to residual volume at a target flow of 170 ml/s. The three exhalations were performed at 2 minute intervals and were immediately preceded by a nasal inhalation to total lung capacity. During the intervening periods, the volunteer performed nasal tidal breathing.

For all exhalations, the mean-exhaled concentration and end-exhaled concentrations of HCN were 2.15±0.5 ppb and 2.0±0.4 ppb (mean±SE) respectively and there was no difference in either of these across the three repeated exhalations. The actual expiratory flow for all exhalations was 175±30 ml/s (mean±SD) and the exhaled volume was 4.22±1.37 litres, and there was no difference in exhaled flow or volume across the three repeated exhalations.

Given that the repetition of this breathing manoeuvre had no effect on the concentration of HCN in exhaled breath, within-session coefficients of variation were calculated from these data: the median intra-subject within-session CV for the mean-exhaled and end-exhaled HCN concentrations were 10.5% (IQR 7.0-14.7%) and 9.7% (IQR 8.5-20.5%) respectively.

4.4.8. Effect of oral vs. nasal inhalation on the concentration of HCN in nasally-exhaled breath

At the end of the above experiment (effect of repetition of breathing manoeuvre), the volunteer performed nasal tidal breathing for a further 2 minutes, inhaled orally to total lung capacity, and then exhaled nasally to residual volume. A comparison was then made of the effects of oral vs. nasal inhalation on the concentration of HCN in nasally-exhaled breath.

An oral inhalation to TLC before exhalation of vital capacity gave a slightly higher mean-exhaled HCN concentration than nasal inhalation (oral vs. nasal inhalation: 2.4±0.6 vs. 2.1±0.5 ppb, p=0.01), but no significant difference in end-exhaled HCN concentration (oral vs. nasal inhalation: 2.3±0.5 vs. 2.0±0.4 ppb, p=0.12) (see Figure 4-8). Expiratory flow was not different in exhalation manoeuvres of oral and nasal inhalation (179±26 ml/s (mean±SD) vs. 175±20 ml/s), and there was no significant difference in exhaled volume between oral and nasal inhalation manoeuvres (4.43±1.10 vs. 4.22±1.36 litres).
Figure 4-8 Mean (SE) HCN concentrations in exhaled breath after 2 minutes of tidal breathing via the nose, then inhalation to TLC via the mouth (blue closed circles) or nose (red open squares), followed by exhalation of vital capacity, at a target expiratory flow of 10 l/min, via the nose. Median and IQR for background HCN levels are shown as a black line and grey area.

4.4.9. Relationship between the concentration of HCN in nasally-exhaled breath and sources of contamination

Using the breathing manoeuvre described in Section 4.4.7, there was a positive correlation between the concentration of HCN in mean-exhaled breath and in the ambient air (r=0.81, p<0.05), but no significant correlation was observed between the concentration of HCN in end-exhaled breath and in the ambient air. No significant correlations were observed between the concentration of HCN in exhaled breath and in gas sampled directly from the oral or nasal cavities.

4.5. Discussion

The aims of this study were to characterise the accuracy, repeatability, dynamic response and transit time of the SIFT-MS instrument for the measurement of the concentration of H$_2$S and HCN in humid air. Secondly, experiments were performed to determine the effects of expiratory flow, volume, and oral or nasal passage on the concentration of these volatile compounds in exhaled breath, so that appropriate breathing manoeuvres could be devised for the analysis of these compounds in exhaled breath. The analysis of both hydrogen sulphide
and hydrogen cyanide in exhaled breath originating from the lower respiratory tract required a breathing manoeuvre that minimised or eliminated contamination by H₂S and HCN originating directly from the mouth.

4.5.1. Instrument characteristics

The SIFT-MS instrument was appropriate for the measurement of H₂S at the concentrations and humidity present in breath. At concentrations of H₂S similar to those seen in exhaled breath, the mean measured concentration was 12% to 13% less than the actual concentration, and this did not alter over the four weeks of testing. The inter-day and intra-day coefficients of variation for the measurement of the H₂S concentration were 21% and 17% respectively. These were considerably higher than the previously obtained inter-day and intra-day coefficients of variation for measurement of acetone concentration of 5.6% and 0.0% respectively using a Voice100™ instrument (Section 3.3.1, Page 65). Analyte concentration and the related product ion count rate have previously been identified as strong determinants of variation in SIFT-MS measurement (Boshier et al., 2010). Given that the acetone concentrations used for determining measurement variation were three orders of magnitude greater than the H₂S concentrations used for similar experiments, this result was therefore to be expected. The dynamic response of the instrument was acceptable for the measurement of H₂S and HCN as, in both cases, it was less than 10% of the duration of any of the exhalations analysed (Bates et al., 1983).

Experiments to determine the HCN measurement characteristics of SIFT-MS were limited by the lack of commercially available standard HCN concentrations at the low levels required to replicate concentrations in breath. There was a significant difference between the accuracies of HCN measurements taken at 760 and 1085 ppb suggesting a lack of linearity. Furthermore, measurements at the higher concentration were less accurate than measurements at the lower concentration. The SIFT-MS technique is designed for the analysis of gases at low concentrations. At high analyte concentrations, accuracy is limited by an increasing departure from linearity, which occurs when the count rate of the SIFT-MS reagent ion is depleted by more than 10% of the initial count rate (Smith and Spanel, 2005a). Previously, the departure from linearity has been described as occurring at analyte concentrations of 10 ppm and above (Smith and Spanel, 2005a). The exact concentration at which this occurs may depend on a number of instrument characteristics and sampling conditions, and could possibly have occurred at a lower analyte concentration in this experiment. Ideally, future experiments
to assess the accuracy of the SIFT-MS instrument would be performed using lower standard HCN concentrations in order to avoid this problem.

At the HCN concentrations tested in this experiment, the inter-day and intra-day coefficients of variation of measurement of HCN concentration were 9-12% and 4-6% respectively. As explained in the previous paragraph, it was not possible to test the precision of the SIFT-MS instrument using standard HCN concentrations similar to those present in exhaled breath. A previous experiment used samples of breath collected in Tedlar bags to determine the within-session coefficient of variation for the measurement of HCN (Boshier et al, 2010). The median concentration of HCN in the bags was 9 ppb (although the accuracy of this concentration was not be confirmed with standard concentrations of HCN), and the within-session coefficient of variation for the measurement of HCN was 19%. This figure is not dissimilar to the intra-day and inter-day coefficients of variation for the measurement of H_2S at similar low concentrations seen in this study.

The measurements of the SIFT-MS instrument and the pneumotachometer were provided with time-points from the same internal timer, and this greatly simplified the synchronisation of the two instruments, making breath analysis experiments quicker and easier to perform. Furthermore, the software written to process the data files (See Section 4.2.7 and Appendix B) greatly reduced the time taken to process breath analysis results. Both of these modifications to the breath analysis system were of great practical significance, because they permitted larger experiments with greater numbers of participants.

**4.5.2. Hydrogen sulphide in exhaled breath**

The use of hydrogen peroxide mouthwash prior to testing, and exhalation via the nose were both associated with a lower concentration of H_2S in exhaled breath. Using a manoeuvre that incorporated the prior use of H_2O_2 mouthwash and a nasal exhalation, there was no difference between the H_2S levels in exhalations preceded by two minutes of tidal breathing via mouth or nose. There was no difference between the concentrations of H_2S at mean expiratory flows of 178 and 305 ml/s and, over the course of an exhalation, H_2S concentration did not change with exhaled volume. Using a nasal exhalation preceded by the use of H_2O_2 mouthwash, the median within-session coefficients of variation for the mean-exhaled and end-exhaled concentrations of H_2S of were 8.5% (IQR 6.0-11.1%) and 7.6% (IQR 2.6-16.2%) respectively.
Using a breathing manoeuvre of nasal exhalation with prior use of H\textsubscript{2}O\textsubscript{2} mouthwash, the concentration of H\textsubscript{2}S in exhaled breath was similar to, and correlated with, the concentration of H\textsubscript{2}S in the ambient air. Furthermore, no relationship was observed between the concentration of H\textsubscript{2}S in a nasal exhalation after H\textsubscript{2}O\textsubscript{2} mouthwash, and the concentration of H\textsubscript{2}S in gas directly sampled from the oral cavity. While these findings suggested that the oral source of H\textsubscript{2}S contamination had been successfully eliminated, they also indicated that, at least in healthy subjects, the ambient air could be a source of H\textsubscript{2}S contamination. In order to reduce contamination of exhaled breath by H\textsubscript{2}S in the ambient air, H\textsubscript{2}S-scrubbed air could have been considered as a breathing source prior to testing, in the same way that nitric oxide-free air is used prior to testing in the measurement of F\textsubscript{E}NO (Silkoff et al, 2004). However, the H\textsubscript{2}S concentrations observed in the ambient air and exhaled breath were already close to the limit of detection of the instrument. Therefore, H\textsubscript{2}S-scrubbing of the inspired air might have been of little benefit. Because exhaled H\textsubscript{2}S concentrations were not tested in subjects with airway inflammation, it was uncertain as to whether the H\textsubscript{2}S concentrations in ambient air would hinder analysis by contaminating exhalations. Further work in this thesis aimed to establish exhaled H\textsubscript{2}S levels in airway inflammation (see Chapter 5, Page 111).

Because the concentration of H\textsubscript{2}S in a nasal exhalation with prior use of H\textsubscript{2}O\textsubscript{2} mouthwash was similar to the H\textsubscript{2}S concentration in the ambient air, it was difficult to draw conclusions about the physiology of H\textsubscript{2}S exhalation from the lower respiratory tract. While expiratory flow and volume did not affect the concentration of H\textsubscript{2}S in exhaled breath, it may be that any such effects were unobserved because of interference from the concentration of H\textsubscript{2}S in the ambient air. For the same reason, it was not possible to determine a definitive exhalation manoeuvre for the analysis of H\textsubscript{2}S in exhaled breath, because any requirements for the control of expiratory flow and volume were uncertain. Likewise, it was not possible to conclude whether the sampling of the end-exhaled breath or the whole (mean-exhaled) breath, was more appropriate, because the effects of expiratory flow and volume were uncertain.

The increased concentration of H\textsubscript{2}S directly sampled from the mouth, compared with the nose, was consistent with the known source of H\textsubscript{2}S from oral bacteria (Rosenberg and McCulloch, 1992). On direct sampling from the six volunteers, the oral cavity contained a higher concentration of H\textsubscript{2}S than the nasal cavity: 21.0 (1.7-60.8) ppb (median (range)) vs. 0.9 (0.4-2.1) ppb, p<0.05. The concentration of H\textsubscript{2}S directly sampled from the nasal cavity has not been previously documented. The use of hydrogen peroxide mouthwash reduced the concentration of H\textsubscript{2}S in the oral cavity from 21.0 (1.7-60.8) ppb (median (range)) down to 1.9
(1.3-6.2) ppb, p<0.05. This ten-fold decrease in \( \text{H}_2\text{S} \) concentration was of a similar order of magnitude to the twenty-fold decrease seen in a previous study (Suarez et al, 2000).

The higher concentration of \( \text{H}_2\text{S} \) observed in end-exhaled breath from the mouth, compared with the nose (2.5±0.5 ppb (mean±SE) vs. 1.1±0.2 ppb, p=0.06), was consistent with a previous SIFT-MS study, in which multiple samples of breath exhaled via the mouth and nose were studied in two individuals (Pysanenko et al, 2008). However, in that study, the concentration of \( \text{H}_2\text{S} \) in oral exhalations was ten-fold greater than in nasal exhalations – a much greater difference than seen in this study. This difference might be explained by the much higher levels of \( \text{H}_2\text{S} \) on direct sampling of the oral cavity in the two individuals in the previous study, or possibly because of differences in sampling methodology.

For the analysis of \( \text{H}_2\text{S} \) in exhaled breath, a nasal exhalation, preceded by the use of \( \text{H}_2\text{O}_2 \) mouthwash, eliminated the contamination of the breath by \( \text{H}_2\text{S} \) produced in the mouth. Using such a manoeuvre, concentrations of \( \text{H}_2\text{S} \) in exhaled breath correlated with those in the ambient air, suggesting that ambient air was also a potential source of contamination. At the low levels of exhaled \( \text{H}_2\text{S} \) observed using this manoeuvre, it was not possible to determine the effects of expiratory flow and volume.

4.5.3. Hydrogen cyanide in exhaled breath

Exhalation from total lung capacity to residual volume via the nose, rather than the mouth, was associated with a lower concentration of HCN in exhaled breath. There was no difference between the HCN levels in nasal exhalations preceded by two minutes of tidal breathing via mouth or nose. However, after 2 minutes of tidal breathing via the nose, an inhalation to TLC via the mouth was associated with an increased concentration of HCN in the subsequent nasally exhaled breath when compared to an inhalation to TLC via the nose. The concentration of HCN in nasally exhaled breath was higher at a mean expiratory flow of 177 ml/s compared with a mean expiratory flow of 313 ml/s and, over the course of an exhalation, HCN concentration did not change with exhaled volume. Using a breathing manoeuvre of 2 minutes of nasal tidal breathing, a nasal inhalation to TLC and a nasal exhalation to RV, the median within-session coefficients of variation for the mean-exhaled and end-exhaled concentrations of \( \text{H}_2\text{S} \) of were 10.5% (IQR 7.0-14.7%) and 9.7% (IQR 8.5-20.5%) respectively.
HCN produced in the mouth affected the concentration of HCN in the exhaled breath. This was most obvious in the observation that HCN concentrations were higher in oral vs. nasal exhalations, and this was consistent with a lower concentration of HCN directly sampled from the nasal, compared with the oral, cavity. Furthermore, after 2 minutes of tidal breathing via the nose, an inhalation to TLC via the mouth was associated with an increased concentration of HCN in the subsequent nasally exhaled breath when compared to an inhalation to TLC via the nose. This suggested that HCN sequestered in the mouth during 2 minutes of tidal breathing could contaminate a subsequent nasal exhalation if inhaled. Finally, the concentration of HCN in mean-exhaled breath was higher at the lower target expiratory flow. While this finding might have been related to interaction between HCN and the lower airways, it was also consistent with an increased amount of HCN diffusing from the oral cavity into the oropharynx over the course of a longer exhalation at a lower expiratory flow.

There was no established method to diminish the concentration of HCN in the mouth, in contrast to the case of H$_2$S in exhaled breath, in which H$_2$O$_2$ mouthwash reduced the concentration of H$_2$S in the mouth (Suarez et al., 2000). However, using only a manoeuvre comprising a nasal inhalation to TLC and exhalation to RV, it was possible to obtain levels of exhaled breath that were similar to and correlated with HCN levels in the ambient air, whereas no correlation between HCN levels in nasally exhaled breath and samples directly from the mouth were observed. This suggested that, as for H$_2$S, the oral source of HCN contamination had been successfully eliminated, but the ambient air could also be a source of HCN contamination. In order to reduce contamination of exhaled breath by HCN in the ambient air, HCN-scrubbed air could have been considered as a breathing source prior to testing. This might have been more effective than in the case of H$_2$S, given that the HCN concentrations in ambient air were well above the limit of detection of the instrument. Exhaled HCN concentrations were not tested in subjects with airway inflammation, and it was therefore uncertain as to whether the HCN concentrations in ambient air would hinder analysis by contaminating exhalations. Further work in this thesis aimed to establish exhaled HCN levels in airway inflammation (see Chapter 5, Page 111).

Because the concentration of HCN in a nasal exhalation was similar to the concentration of HCN in the ambient air, and also because of potential contamination of nasal exhalations by HCN originating from the oral cavity, it was difficult to draw conclusions about the physiology of HCN exhalation from the lower respiratory tract. Contamination from the oral cavity and the ambient air may have masked any effect (or lack of effect) of expiratory flow
or volume. Similarly, it was not possible to determine a definitive exhalation manoeuvre for the analysis of HCN in exhaled breath, because any requirements for the control of expiratory flow and volume were uncertain. It was also not possible to conclude whether the sampling of the end-exhaled breath or the whole (mean-exhaled) breath, was more appropriate, because the effects of expiratory flow and volume were uncertain.

The higher concentration of HCN in gas sampled directly from the mouth, compared with the nose, was consistent with the known oral production of HCN by salivary peroxidase (Lundquist et al., 1988). The higher concentration of HCN observed in orally exhaled breath compared to nasally exhaled breath was similar to the findings of a previous study of three individuals (Wang et al., 2008). In the previous study, the concentration of HCN in oral exhalations was between two and fourteen times greater than in nasal exhalations—a greater difference than the two-fold difference seen in this study. This might be explained by the differences in sampling methodology, given that the sampling methodology used by Wang et al. (2008) was similar to that used by Pysanenko et al. (2008) for the sampling of HCN via mouth and nose, and that there was a consistently greater magnitude of difference in analyte concentrations observed between those studies and this thesis.

For the analysis of HCN in exhaled breath, a nasal inhalation followed by a nasal exhalation, eliminated the contamination of the breath by HCN produced in the mouth. Using this manoeuvre, concentrations of HCN in exhaled breath correlated with those in the ambient air, suggesting that ambient air was also a potential source of contamination. Because of potential contamination by HCN in the ambient air, it was not possible to determine the effects of expiratory flow and volume.

4.6. Summary

The SIFT-MS instrument was appropriate for the measurement of H$_2$S at the concentrations and humidity present in breath. Experiments to determine the characteristics of SIFT-MS for the analysis of HCN were limited by the lack of commercially available standard HCN concentrations at the low levels required to replicate concentrations in breath.

For the analysis of H$_2$S in exhaled breath, a nasal exhalation, preceded by the use of H$_2$O$_2$ mouthwash, eliminated the contamination of the breath by H$_2$S produced in the mouth. For
the analysis of HCN in exhaled breath, a nasal inhalation followed by a nasal exhalation, eliminated the contamination of the breath by HCN produced in the mouth. Using these breathing manoeuvres, concentrations of H$_2$S and HCN in exhaled breath correlated with H$_2$S and HCN levels in the ambient air, suggesting that ambient air was also a potential source of contamination. Because of potential contamination by H$_2$S and HCN in the ambient air, it was not possible to determine the effects of expiratory flow and volume on the concentrations of H$_2$S and HCN in exhaled breath.
5.

Hydrogen Sulphide and Hydrogen Cyanide in Exhaled Breath as Inflammatory Biomarkers in COPD and Asthma

5.1. Introduction

Breath biomarkers of airway inflammation would be invaluable in the diagnosis and treatment of airway diseases such as asthma and COPD. To date the only such biomarker in use is exhaled nitric oxide, a surrogate marker for eosinophilic airway inflammation, but there may be other biomarkers yet to be defined. Hydrogen sulphide (H$_2$S) and hydrogen cyanide (HCN) in exhaled breath are both candidate gaseous markers of airway inflammation. These two compounds have been implicated in inflammatory processes in vitro, and there is some in vivo evidence that inflammatory changes in COPD are associated with changes in serum H$_2$S concentration (Stelmaszynska, 1986; Chen et al, 2005; Zhang and Bhatia, 2008).

Selected ion flow tube – mass spectrometry has the potential to be a major advance in breath analysis, with the ability to detect trace gases on-line at concentrations down to individual parts per billion. The SIFT-MS analysis of H$_2$S and HCN in exhaled breath has been investigated and described (see Chapter 4, Page 77). For each compound, the differential effects of oral and nasal exhalation have been explored, along with the effects of expiratory flow and volume. Appropriate exhalation manoeuvres have been devised to minimise the contamination of breath from the lower respiratory tract by the oral reservoirs of both H$_2$S and HCN.

While H$_2$S and HCN have been implicated in the inflammatory process, their roles have not been defined. They have been shown to be associated with neutrophilic inflammation, but
their role in other inflammatory phenotypes is unknown. In this pilot study, the levels of H$_2$S and HCN in the exhaled breath of subjects with neutrophilic COPD and eosinophilic asthma were explored. The primary aim of this study was to determine whether each candidate marker was elevated in the exhaled breath of patients with neutrophilic and/or eosinophilic airway inflammation compared to control groups. The secondary aim was to examine the relationship between each candidate marker and the established markers of airway inflammation, exhaled nitric oxide, induced sputum eosinophils and neutrophils.

5.2. Methods

5.2.1. COPD study participants

Six subjects with COPD and six control subjects were recruited. Inclusion criteria for all subjects were: age greater than 45 years; smoking history of > 10 pack years; ex-smokers for > 6 months and no other significant co-morbidity. Additional inclusion criteria for subjects with COPD were: persistent symptoms of chronic airflow obstruction; post-bronchodilator FEV$_1$/FVC < 70% and < lower limit of normal; sputum neutrophil percentage ≥ 61%; sputum eosinophil percentage < 3%; and no oral steroid in previous 4 weeks. Additional inclusion criteria for control subjects were: no respiratory symptoms; no chronic respiratory disease; FEV$_1$/FVC ≥ 70% and ≥ lower limit of normal.

5.2.2. Asthma study participants

Six subjects with asthma and six control subjects were recruited. Inclusion criteria for all subjects were: smoking history of < 1 pack year and ex-smoker for > 6 months. Diagnostic criteria for subjects with asthma were > 5 year history of doctor-diagnosed asthma, and either significant bronchodilator reversibility (FEV$_1$ increase of ≥ 12% and ≥ 200ml) or a positive methacholine challenge (PC$_{20}$ < 4 mg/ml). Inclusion criteria for control subjects were: no respiratory symptoms; no chronic respiratory disease; FEV$_1$ ≥ 80% predicted; FEV$_1$/FVC ≥ lower limit of normal.

5.2.3. Study procedures

The experimental procedures were approved by the Upper South Regional Ethics Committee. Participants attended a single visit (unless undergoing withdrawal of ICS – see Section 5.2.4 below) at which they performed a fixed sequence of assessments: FEno measurement; SIFT-
MS analysis of HCN in exhaled breath; throat swab; SIFT-MS analysis of H2S in exhaled breath; nasopharyngeal swab; spirometry; and sputum induction.

5.2.4. Withdrawal of inhaled corticosteroid

Selected patients with stable asthma on inhaled corticosteroid attended for two visits: at the first, they underwent clinical assessment and withdrawal of inhaled corticosteroid (Jones et al., 2001; Smith et al., 2005b). They were then monitored at least twice a week by telephone for review of symptoms and peak expiratory flow rate. Members of the Canterbury Respiratory Research Group were available 24 hours a day by telephone for patient queries and concerns. After a three week interval, or until loss of control, whichever was shorter, they attended the visit described in Section 5.2.3 above. This enabled assessment of corticosteroid-naïve eosinophilic airway inflammation. Inhaled corticosteroid was restarted immediately after the visit.

Criteria for loss of control were any of (Jones et al., 2001):

1. A fall in the mean (over last 7 days) morning peak expiratory flow rate (PEFR) of greater than 10% from baseline, or a fall in either morning or evening PEFR on two consecutive days to 80% of baseline or less.
2. Mean daily bronchodilator use of greater than three puffs more than during run-in.
3. Nocturnal wakening with asthma symptoms on three nights or more per week greater than during the run-in.
4. Disagreeable or distressing asthma symptoms.
5. Fall in FEV1 of >20% from baseline or >40% of predicted value.

5.2.5. Nitric oxide measurement

FE\textsubscript{NO} was measured using an on-line chemiluminescence analyser (Aerocrine AB, Solna, Sweden) according to current recommendations (1999). See Section 2.2.3, Page 36 for further details of the procedure.

5.2.6. SIFT-MS analysis

The breath analysis system was assembled, using a Voice200™ SIFT-MS instrument (Syft Technologies Ltd, New Zealand), as described in Section 4.2.5, Page 82 and a SIM scan for the analysis of HCN was performed (see Section 4.2.3, Page 81). The subject donned a nasal mask (Flexifit™ 407, Fisher and Paykel Healthcare, New Zealand) attached to a respiratory
filter (SureGuard, BIRD Healthcare, Australia) that was connected to the proximal end of the Breath Head (T0033, Syft Technologies Ltd, New Zealand). The subject inhaled nasally to total lung capacity, then exhaled nasally into the breath analysis system, at a target expiratory flow of 170 ml/s, to residual volume. The concentration of HCN in the exhaled breath was measured by the SIFT-MS instrument, and the expiratory flow and volume were measured by the pneumotachometer. This manoeuvre was repeated performed in triplicate.

For the analysis of H$_2$S in exhaled breath, the subject rinsed their mouth with 5 ml of 3% hydrogen peroxide mouthwash for one minute. After a 5 minute interval, a SIM scan for the analysis of H$_2$S was performed by the SIFT-MS instrument (see Section 4.2.2, Page 80) and the breathing manoeuvre described above for HCN analysis was repeated in triplicate.

5.2.7. Throat and nasopharyngeal swabs

Swabs of the oropharynx (Copan Italia, Italy), and the nasopharynx (Medical Wire and Equipment, UK) were taken and cultured to define colonisation by bacterial respiratory pathogens (Lieberman et al, 2007).

5.2.8. Spirometry

Any inhaled bronchodilators were withheld for 6 to 24 hours before attendance at the research clinic. Spirometry was measured using an EasyOne™ spirometer (NDD, Switzerland) according to current international standards, and subjects with COPD also performed post-bronchodilator spirometry 15 minutes after 400 μg of inhaled salbutamol (Miller et al, 2005). See Section 2.2.4, Page 36 for further details of the procedure.

5.2.9. Sputum induction and processing

Sputum induction and processing were undertaken as previously described (Aldridge et al, 2000). See Section 2.2.7, Page 37 for further details of the procedure.

5.2.10. Statistical analysis

Comparisons between the patient group and the control group were made by independent $t$-test. Correlations were performed using Spearman’s rank correlation. Comparisons of the relationships between sputum neutrophils and H$_2$S in exhaled breath in the patient group and the control group were performed by univariate analysis of variance. Analyses were performed using SPSS 16.
5.3. Results

5.3.1. Subject characteristics

Table 5-1 shows the characteristics of the six patients with COPD and six control subjects, all of whom completed all parts of the study. Three patients with COPD had moderate disease and three patients had severe disease according to GOLD classification (GOLD, 2008). Two patients with COPD had significant reversibility of their airway obstruction in response to 400 μg of inhaled salbutamol (FEV₁ increase of > 12% and > 0.2 litres). No respiratory pathogens were cultured from throat or nasopharyngeal swabs in the COPD group. In the control group, respiratory pathogens were cultured from nasopharyngeal swabs taken from two subjects: one showed scanty growth of *S aureus* and the other showed a moderate growth of *M catarrhalis*. One COPD patient was taking 400 μg of inhaled beclomethasone once daily. The remainder of the COPD patients were not taking any inhaled corticosteroid. The six patients with COPD had neutrophilic sputum samples (sputum neutrophil proportion > 61%), but no sputum eosinophilia (sputum eosinophil proportion > 3%) (Simpson *et al*, 2006; Siva *et al*, 2007). The six control subjects had neither sputum neutrophilia, nor sputum eosinophilia.

Table 5-2 shows the characteristics of the six patients with asthma and six control subjects. Two subjects in each group were unable to expectorate a sputum sample, but all other parts of the study were completed by all subjects. All asthma subjects had been diagnosed by a doctor. Four subjects demonstrated significant reversibility of their airway obstruction in response to 400 μg of inhaled salbutamol (FEV₁ increase of >12% and >0.2 litres), and the other two subjects had a previous positive methacholine challenge test. Three asthma patients were not taking regular inhaled corticosteroid, two asthma patients were taking inhaled flixotide 125 μg twice daily, and one asthma patient was taking oral prednisone 5mg once daily. The two subjects taking inhaled flixotide had this medication withheld for three weeks before performing the study procedures. The subject taking oral prednisone was not withdrawn from this medication. Two of the four asthma patient who provided sputum samples had sputum eosinophilia (sputum eosinophil proportion >3%). A further asthma patient had off-steroid sputum neutrophilia (sputum neutrophil proportion >61%). None of the four control subjects who provided sputum samples demonstrated any sputum eosinophilia or neutrophilia.
Table 5-1  Characteristics of patients with COPD and control subjects.

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<th>Patients’ characteristics</th>
<th>COPD (n=6)</th>
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<tr>
<td>Sex (male/female)</td>
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<tr>
<td>Age (yrs)</td>
<td>69 (63-75)</td>
<td>72 (70-74)</td>
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<tr>
<td>BMI (kg/m³)</td>
<td>30 (26-35)</td>
<td>25 (22-27)</td>
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<tr>
<td>No. of pack yrs</td>
<td>38 (28-48)</td>
<td>37 (29-45)</td>
</tr>
<tr>
<td>FE_{NO} (ppb) *</td>
<td>13.7 (10.0-24.3)</td>
<td>12.3 (6.9-22.9)</td>
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Pre-bronchodilator spirometry

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<tr>
<td>FEV₁/FVC (%)</td>
<td>44 (33-54)</td>
<td>74 (67-81)</td>
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<td>FEV₁ (l)</td>
<td>1.16 (0.91-1.40)</td>
<td>2.40 (1.97-2.82)</td>
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<td>Percentage of predicted FEV₁</td>
<td>42 (33-50)</td>
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<tr>
<td>FVC (l)</td>
<td>2.73 (2.31-3.14)</td>
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Post-bronchodilator spirometry

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<td>FEV₁/FVC (%)</td>
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<tr>
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<td>FVC (l)</td>
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Sputum characteristics *

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<th>Controls (n=6)</th>
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<td>Macrophages (%)</td>
<td>21.5 (6.2-27.4)</td>
<td>42.3 (33.9-52.7)</td>
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<tr>
<td>Neutrophils (%)</td>
<td>74.0 (71.4-85.2)</td>
<td>50.3 (39.0-60.2)</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>1.3 (0.4-2.7)</td>
<td>5.3 (1.9-7.8)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.6 (0.2-1.1)</td>
<td>0.0 (0.0-0.3)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.4 (0.2-2.7)</td>
<td>0.3 (0.1-0.6)</td>
</tr>
</tbody>
</table>

The data are expressed as mean (95% confidence interval) unless otherwise stated.

*FE_{NO} and sputum characteristics are expressed as median (interquartile range).
Table 5-2 Characteristics of patients with asthma and control subjects.

<table>
<thead>
<tr>
<th>Patients’ characteristics</th>
<th>All subjects</th>
<th>Subjects able to provide sputum sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asthma (n=6)</td>
<td>Controls (n=6)</td>
</tr>
<tr>
<td></td>
<td>Asthma (n=4)</td>
<td>Controls (n=4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td>2/2</td>
<td>2/2</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>41 (30-52)</td>
<td>41 (29-54)</td>
</tr>
<tr>
<td></td>
<td>28 (26-31)</td>
<td>24 (22-25)</td>
</tr>
<tr>
<td>BMI (kg/m(^3))</td>
<td>27 (24-30)</td>
<td>24 (23-26)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of pack yrs</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FE(_{NO}) (ppb) *</td>
<td>35.4 (20.5-48.2)</td>
<td>18.4 (17.9-26.0)</td>
</tr>
<tr>
<td></td>
<td>36.2 (16.6-62.8)</td>
<td>18.2 (16.7-22.3)</td>
</tr>
<tr>
<td>Pre-bronchodilator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FE(_V_1)/FVC (%)</td>
<td>63 (50-76)</td>
<td>78 (76-80)</td>
</tr>
<tr>
<td></td>
<td>55 (43-66)</td>
<td>78 (75-80)</td>
</tr>
<tr>
<td>FE(_V_1) (l)</td>
<td>2.66 (1.84-3.47)</td>
<td>3.81 (2.94-4.67)</td>
</tr>
<tr>
<td></td>
<td>2.28 (1.43-3.12)</td>
<td>3.93 (2.90-4.95)</td>
</tr>
<tr>
<td>Percentage of predicted</td>
<td>74 (52-95)</td>
<td>101 (92-109)</td>
</tr>
<tr>
<td></td>
<td>63 (42-84)</td>
<td>99 (88-110)</td>
</tr>
<tr>
<td>FVC (l)</td>
<td>4.09 (3.32-4.86)</td>
<td>4.89 (3.73-6.05)</td>
</tr>
<tr>
<td></td>
<td>3.95 (2.98-4.92)</td>
<td>5.06 (3.69-6.42)</td>
</tr>
<tr>
<td>Post-bronchodilator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FE(_V_1)/FVC (%)</td>
<td>67 (53-81)</td>
<td>62 (50-74)</td>
</tr>
<tr>
<td></td>
<td>2.99 (2.18-3.81)</td>
<td>2.75 (1.78-3.73)</td>
</tr>
<tr>
<td>Percentage of predicted</td>
<td>83 (62-104)</td>
<td>76 (53-100)</td>
</tr>
<tr>
<td>FVC (l)</td>
<td>4.21 (3.33-5.09)</td>
<td>4.26 (3.13-5.39)</td>
</tr>
<tr>
<td>Sputum characteristics *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>28.5 (25.1-33.3)</td>
<td>50.6 (37.4-62.2)</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>50.8 (41.4-60.0)</td>
<td>35.5 (28.4-44.5)</td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>5.3 (4.1-10.2)</td>
<td>9.4 (6.7-20.7)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>0.6 (0.1-1.0)</td>
<td>0.0 (0.0-0.1)</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>5.6 (1.4-23.6)</td>
<td>0.1 (0.0-0.2)</td>
</tr>
</tbody>
</table>

The data are expressed as mean (95% confidence interval) unless otherwise stated. *FE\(_{NO}\) and sputum characteristics are expressed as median (interquartile range).

5.3.2. Exhaled hydrogen sulphide in COPD

Comparison of post-mouthwash, nasally-exhaled \(H_2S\) in COPD and control groups

The ambient level of \(H_2S\) was 1.1 (0.9-1.2) ppb (median (IQR)). There was no significant difference in mean expiratory flow (118±5 vs. 132±8 ml/s (mean ± SE)) and exhaled volume (2.3±0.3 vs. 2.9±0.5 litres) between the COPD and control groups, although the actual expiratory flows for both groups were significantly below the target expiratory flow of 170 ml/s. There was no significant difference in the mean-exhaled concentration of \(H_2S\) between the COPD and control groups (2.2±0.4 vs. 2.3±0.3 ppb respectively (mean ± SE)), and no difference in end-exhaled concentration of \(H_2S\) (2.1±0.4 vs. 2.5±0.3 ppb respectively) (see Figure 5-1 and Figure 5-2).
Figure 5-1 Mean-exhaled and end-exhaled concentrations of H$_2$S in the post-mouthwash, nasally-exhaled breath of patients with COPD and control subjects. Mean and individual values are shown for each group.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure51.png}
\caption{Mean-exhaled and end-exhaled concentrations of H$_2$S in the post-mouthwash, nasally-exhaled breath of patients with COPD and control subjects. Mean and individual values are shown for each group.}
\end{figure}

Figure 5-2 (A) Mean (SE) H$_2$S concentrations in the post-mouthwash, nasally exhaled breath of the COPD and control groups. The ambient H$_2$S concentration is shown as median (black line) and inter-quartile range (grey area). (B) Mean (SE) expiratory flow for the COPD and control groups at a target expiratory flow of 170 ml/s.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure52.png}
\caption{(A) Mean (SE) H$_2$S concentrations in the post-mouthwash, nasally exhaled breath of the COPD and control groups. The ambient H$_2$S concentration is shown as median (black line) and inter-quartile range (grey area). (B) Mean (SE) expiratory flow for the COPD and control groups at a target expiratory flow of 170 ml/s.}
\end{figure}
Relationship between $H_2S$ in exhaled breath and sputum neutrophils

When the COPD and control groups were analysed together, there was no correlation between $H_2S$ in exhaled breath and the percentage of sputum neutrophils (correlation of mean-exhaled $H_2S$ with percentage of sputum neutrophils $r_s$=-0.06, $p=0.85$; correlation of end-exhaled $H_2S$ with percentage of sputum neutrophils $r_s$=-0.24, $p=0.46$). However, in the six patients with COPD, there was a strong and statistically significant negative correlation between the concentration of $H_2S$ in exhaled breath and the percentage of neutrophils in sputum ($r_s$=-0.89, $p=0.02$) (see Figure 5-3). In the six control subjects, there was a positive correlation of borderline significance between the concentration of $H_2S$ in exhaled breath and the percentage of neutrophils in sputum ($r_s$=0.77, $p=0.07$). For the concentrations of both mean-exhaled and end-exhaled $H_2S$, the slopes of the lines of best fit for the control group and the COPD group were significantly different ($p=0.001$), indicating a difference in the relationship between exhaled $H_2S$ and sputum neutrophils between the two groups. In patients with COPD, there was a negative correlation of borderline significance between the concentration of $H_2S$ in exhaled breath and the absolute number of neutrophils per ml of sputum ($r_s$=-0.77, $p=0.07$) and, in control subjects, no association was observed between exhaled $H_2S$ and the absolute number of neutrophils per ml.

Figure 5-3  (A) Mean-exhaled and (B) end-exhaled concentrations of $H_2S$ plotted against percentage sputum neutrophils in six patients with COPD and six control subjects. The slopes of the lines of best fit for the COPD and control groups were significantly different ($p=0.001$).
Chapter Five

**Relationship between the concentration of $H_2S$ in exhaled breath and biomarkers of airway eosinophilia**

Whether the COPD patients and controls were analysed together or apart, there was no significant correlation between the concentration of $H_2S$ in exhaled breath and either the percentage of eosinophils in sputum or the FE$_{NO}$ measurement (see Table 5-3).

**Table 5-3** Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of $H_2S$ in exhaled breath and the percentage of eosinophils in the sputum and the FE$_{NO}$ measurement in patients with COPD and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th>Sputum eosinophils (%)</th>
<th>FE$_{NO}$ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=12)</td>
</tr>
<tr>
<td>Mean-exhaled $H_2S$</td>
<td>-0.11 (0.73)</td>
</tr>
<tr>
<td>End-exhaled $H_2S$</td>
<td>-0.12 (0.71)</td>
</tr>
</tbody>
</table>

**Relationship between $H_2S$ in exhaled breath, sputum neutrophils and spirometric measurements**

In the COPD group, the pre-bronchodilator FEV$_1$ correlated positively with the concentration of $H_2S$ in exhaled breath and negatively with the percentage of neutrophils in sputum (see Table 5-4). A negative correlation between FEV$_1$ values and sputum neutrophils was also seen when all subjects were analysed together. Whether the COPD patients and controls were analysed together or apart, there was no significant correlation between the concentration of $H_2S$ in exhaled breath and FVC.
Table 5-4  Spearman’s rank correlation coefficients ($r_s$) for the correlations of FEV$_1$ parameters with the concentration of H$_2$S in exhaled breath and the sputum neutrophil percentage in patients with COPD and control subjects. The data are expressed as $r_s$ (p value). Significant correlations (p<0.05) are shown in bold.

<table>
<thead>
<tr>
<th></th>
<th>All (n=12)</th>
<th>COPD (n=6)</th>
<th>Control (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV$_1$ % predicted FEV$_1$</td>
<td>FEV$_1$ % predicted FEV$_1$</td>
<td>FEV$_1$ % predicted FEV$_1$</td>
</tr>
<tr>
<td>Mean-exhaled H$_2$S</td>
<td>0.25 (0.43)</td>
<td>0.83 (0.04)</td>
<td>0.71 (0.11)</td>
</tr>
<tr>
<td>End-exhaled H$_2$S</td>
<td>0.42 (0.18)</td>
<td>0.83 (0.04)</td>
<td>0.71 (0.11)</td>
</tr>
<tr>
<td>Sputum neutrophil %</td>
<td>-0.78 (&lt;0.01)</td>
<td>-0.89 (0.02)</td>
<td>-0.66 (0.16)</td>
</tr>
</tbody>
</table>

**Relationship between the concentration of H$_2$S in exhaled breath and ambient air**

See Figure 5-4. There was a significant positive correlation between the mean-exhaled concentration of H$_2$S and the concentration of H$_2$S in the ambient air ($r_s=0.63$, p=0.03). The positive correlation between the concentration of H$_2$S in end-exhaled breath and ambient air did not reach significance ($r_s=0.52$, p=0.09).

Figure 5-4  (A) Mean-exhaled and (B) end-exhaled H$_2$S concentration plotted against ambient H$_2$S concentration in six patients with COPD (red circles) and six control subjects (blue squares).
5.3.3. **Exhaled hydrogen sulphide in asthma**

*Comparison of exhaled H\textsubscript{2}S in asthma and control groups*

The ambient level of H\textsubscript{2}S was 0.9 (0.8-1.3) ppb (median (IQR)). There was no significant difference in mean expiratory flow between the asthma and control groups (153±22 vs. 162±20 ml/s). However, there was a significantly lower exhaled volume in the asthma group compared to the control group (2.9±0.3 vs. 4.2±0.6 litres, p<0.01). There was no significant difference in mean-exhaled concentration of H\textsubscript{2}S between the asthma and control groups (2.1±0.2 vs. 2.2±0.2 ppb respectively (mean ± SE)), and no difference in end-exhaled concentration of H\textsubscript{2}S (2.2±0.2 vs. 2.4±0.3 ppb respectively) (see Figure 5-5 and Figure 5-6).

**Figure 5-5** Mean-exhaled and end-exhaled concentrations of H\textsubscript{2}S in the nasally-exhaled breath of patients with asthma and control subjects after mouthwash. Mean and individual values are shown for each group.
Figure 5-6  (A) Mean (SE) H$_2$S concentrations in the exhaled breath of the asthma and control groups. The ambient H$_2$S concentration is shown as median (black line) and inter-quartile range (grey area).  (B) Mean (SE) expiratory flow for the asthma and control groups at a target expiratory flow of 170 ml/s.

A.

B.

**Relationship between H$_2$S in exhaled breath and biomarkers of airway eosinophilia**

Figure 5-7, Figure 5-8 and Table 5-5 show the relationships between the concentration of H$_2$S in exhaled breath and the percentage of eosinophils in the sputum and the FE$_{NO}$ measurement in patients with asthma and control subjects. Combining the four subjects from each group who successfully provided a sputum sample, there was no correlation between exhaled H$_2$S and sputum eosinophil percentage (see Table 5-5). Nor was there any significant correlation between H$_2$S in exhaled breath and sputum eosinophils in the group of four control subjects ($r_s$=-0.63, p=0.37). However, in the group of four patients with asthma there was a positive correlation between the concentration of H$_2$S in exhaled breath and the percentage of sputum eosinophils ($r_s$=1.00, p<0.05). Positive correlations between the concentration of H$_2$S in exhaled breath and the FE$_{NO}$ measurement in patients with asthma and control subjects, separately and together, did not achieve significance (see Table 5-5 and Figure 5-8).
Figure 5-7  (A) Mean-exhaled and (B) end-exhaled concentrations of H\textsubscript{2}S plotted against percentage sputum eosinophils in four patients with asthma (green circles) and four control subjects (purple squares). There was a significant positive correlation between sputum eosinophil percentage and concentration of exhaled H\textsubscript{2}S in patients with asthma ($r_s=1.00$, $p<0.05$).

Figure 5-8  (A) Mean-exhaled and (B) end-exhaled concentrations of H\textsubscript{2}S plotted against FE\textsubscript{NO} in six patients with asthma (green circles) and six control subjects (purple squares).
Table 5-5  Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of H$_2$S in exhaled breath and the percentage of eosinophils in the sputum and the F$_{ENO}$ measurement in patients with asthma and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th>Sputum eosinophils (%)</th>
<th>Fe$_{ENO}$ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=8)</td>
</tr>
<tr>
<td>Mean-exhaled H$_2$S</td>
<td>0.01 (0.98)</td>
</tr>
<tr>
<td>End-exhaled H$_2$S</td>
<td>0.17 (0.69)</td>
</tr>
</tbody>
</table>

Relationship between H$_2$S in exhaled breath and sputum neutrophils

There was no significant correlation between H$_2$S in exhaled breath and sputum neutrophils in the asthma and control groups either separately or when combined (see Table 5-6).

Table 5-6  Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of H$_2$S in exhaled breath and the percentage of neutrophils in the sputum in patients with asthma and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th>Sputum neutrophils (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=8)</td>
</tr>
<tr>
<td>Mean-exhaled H$_2$S</td>
<td>-0.45 (0.26)</td>
</tr>
<tr>
<td>End-exhaled H$_2$S</td>
<td>-0.41 (0.32)</td>
</tr>
</tbody>
</table>

Relationship between the concentration of H$_2$S in exhaled breath and spirometric measurements

No significant correlation was observed between the concentration of H$_2$S in exhaled breath and either FEV$_1$ or FVC (see Table 5-7). In the asthma group, there was no correlation between the concentration of H$_2$S in exhaled breath and the percentage change in FEV$_1$ after 400 μg of inhaled salbutamol.
Table 5-7  Spearman’s rank correlation coefficients (r_s) for correlation of the concentration of H_2S in exhaled breath with FEV_1 and FVC in patients with asthma and control subjects. The data are expressed as r_s (p value).

<table>
<thead>
<tr>
<th></th>
<th>All (n=12)</th>
<th>Asthma (n=6)</th>
<th>Controls (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV_1</td>
<td>FVC</td>
<td>FEV_1</td>
</tr>
<tr>
<td>Mean-exhaled H_2S</td>
<td>0.17</td>
<td>-0.08</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(0.60)</td>
<td>(0.81)</td>
<td>(0.87)</td>
</tr>
<tr>
<td>End-exhaled H_2S</td>
<td>0.13</td>
<td>-0.04</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>(0.69)</td>
<td>(0.91)</td>
<td>(0.87)</td>
</tr>
</tbody>
</table>

Relationship between the concentration of H_2S in exhaled breath and ambient air

There was no significant correlation between the concentration of H_2S in mean-exhaled or end-exhaled breath and the concentration of H_2S in the ambient air (r_s=-0.09, p=0.78 and r_s=-0.17, p=0.61 respectively) (see Figure 5-9).

Figure 5-9  (A) Mean-exhaled and (B) end-exhaled H_2S concentration plotted against ambient H_2S concentration in six patients with asthma (green circles) and six control subjects (purple squares).
5.3.4. Exhaled hydrogen cyanide in COPD

**Comparison of exhaled HCN in COPD and control groups**

The ambient level of HCN was 1.5 (1.3-2.2) ppb (median (IQR)). A significant difference in mean expiratory flow was observed between the COPD and control groups (118±8 vs. 141±4 ml/s, p=0.04), and the actual expiratory flows for both groups were significantly below the target expiratory flow of 170 ml/s. There was no significant difference in exhaled volume (2.3±0.2 vs. 3.0±0.4 litres) between the COPD and control groups. There was no significant difference in mean-exhaled concentration of HCN between the COPD and control groups (3.4±0.3 vs. 3.1±0.4 ppb respectively (mean ± SE)), and no difference in end-exhaled concentration of HCN (3.5±0.3 vs. 3.3±0.6 ppb respectively) (see Figure 5-10 and Figure 5-11).

**Figure 5-10** Mean-exhaled and end-exhaled concentrations of HCN in the nasally-exhaled breath of patients with COPD and control subjects. Mean and individual values are shown for each group.
**Figure 5-11** (A) Mean (SE) HCN concentrations in the exhaled breath of the COPD and control groups. The ambient HCN concentration is shown as median (black line) and inter-quartile range (grey area). (B) Mean (SE) expiratory flow for the COPD and control groups at a target expiratory flow of 170 ml/s.
Relationship between HCN in exhaled breath and sputum neutrophils

Whether the COPD and control groups were analysed together or separately, there was no correlation between the concentration of HCN in exhaled breath and the percentage of neutrophils in sputum (see Table 5-8 and Figure 5-12). Nor was any association observed between the concentration of HCN in exhaled breath and the absolute number of neutrophils per ml of sputum.

Table 5-8  Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of neutrophils in the sputum in patients with COPD and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th>Sputum neutrophils (%)</th>
<th>All (n=12)</th>
<th>COPD (n=6)</th>
<th>Controls (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean-exhaled HCN</td>
<td>0.08</td>
<td>-0.49</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>(0.80)</td>
<td>(0.33)</td>
<td>(0.62)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>-0.06</td>
<td>-0.66</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>(0.86)</td>
<td>(0.16)</td>
<td>(0.70)</td>
</tr>
</tbody>
</table>

Figure 5-12  (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against percentage sputum neutrophils in six patients with COPD (red circles) and six control subjects (blue squares).
Chapter Five

Relationship between the concentration of HCN in exhaled breath and biomarkers of airway eosinophilia

Whether the COPD patients and controls were analysed together or apart, there was no significant correlation between the concentration of HCN in exhaled breath and either the percentage of eosinophils in the sputum or the FE\(_{NO}\) measurement (see Table 5-9).

Table 5-9 Spearman’s rank correlation coefficients (r\(_s\)) for the relationships between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum and the FE\(_{NO}\) measurement in patients with COPD and control subjects. The data are expressed as r\(_s\) (p value).

<table>
<thead>
<tr>
<th>Sputum eosinophils (%)</th>
<th>FE(_{NO}) (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=12)</td>
</tr>
<tr>
<td>Mean-exhaled HCN</td>
<td>0.02 (0.95)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>-0.19 (0.56)</td>
</tr>
</tbody>
</table>

Relationship between the concentration of HCN in exhaled breath and spirometric measurements

Whether COPD patients and controls were analysed together or apart, there was no significant correlation between the concentration of HCN in exhaled breath and either FE\(_V1\) or the percentage of predicted FE\(_V1\). In the COPD group, there was no correlation between the concentration of HCN in exhaled breath and either the FE\(_V1\) or percentage of predicted FE\(_V1\) after 400 μg of inhaled salbutamol.

When the COPD and control groups were analysed together, there was no correlation between the concentration of HCN in exhaled breath and FVC. However, in the COPD group alone, there was a significant correlation between the concentration of HCN in exhaled breath and FVC (see Table 5-10). This correlation was not present after inhalation of 400 μg of inhaled salbutamol.
Table 5-10  Spearman’s rank correlation coefficients ($r_s$) for correlation of the concentration of HCN in exhaled breath with FVC in patients with COPD and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th></th>
<th>All (n=12)</th>
<th>COPD (n=6)</th>
<th>Controls (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pre-bronchodilator</td>
<td>Post-bronchodilator</td>
</tr>
<tr>
<td>Mean-exhaled HCN</td>
<td>-0.25 (0.43)</td>
<td>-0.83 (0.04)</td>
<td>-0.66 (0.16)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>-0.08 (0.81)</td>
<td>-0.66 (0.16)</td>
<td>-0.37 (0.47)</td>
</tr>
</tbody>
</table>

Relationship between the concentration of HCN in exhaled breath and ambient air

In Figure 5-13, a positive correlation is shown between the end-exhaled concentration of HCN and the concentration of HCN in the ambient air ($r_s=0.60$, $p=0.04$). The positive correlation between the concentration of HCN in mean-exhaled breath and ambient air did not reach significance ($r_s=0.51$, $p=0.09$).

Figure 5-13  (A) Mean-exhaled and (B) end-exhaled HCN concentration plotted against ambient HCN concentration in six patients with COPD (red circles) and six control subjects (blue squares).
5.3.5. Exhaled hydrogen cyanide in asthma

*Comparison of exhaled HCN in asthma and control groups*

The ambient level of HCN was 1.7 (1.4-2.0) ppb (median (IQR)). There was no significant difference in mean expiratory flow (164±8 vs. 164±7 ml/s) and exhaled volume (3.2±0.3 vs. 4.3±0.6 litres) between the asthma and control groups. There was no significant difference in mean-exhaled concentration of HCN between the asthma and control groups (4.8±0.4 vs. 4.4±0.8 ppb respectively (mean ± SE)), and no difference in end-exhaled concentration of HCN (4.7±0.5 vs. 4.4±0.9 ppb respectively) (see Figure 5-14 and Figure 5-15).

**Figure 5-14** Mean-exhaled and end-exhaled concentrations of HCN in the nasally-exhaled breath of patients with asthma and control subjects. Mean and individual values are shown for each group.
Figure 5-15  (A) Mean (SE) HCN concentrations in the exhaled breath of the asthma and control groups. The ambient HCN concentration is shown as median (black line) and inter-quartile range (grey area). (B) Mean (SE) expiratory flow for the asthma and control groups at a target expiratory flow of 170 ml/s.
**Relationship between HCN in exhaled breath and biomarkers of airway eosinophilia**

Figure 5-16, Figure 5-17 and Table 5-11 show the relationships between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum and the \( \text{FE}_{\text{NO}} \) measurement in patients with asthma and control subjects. Whether the two groups were analysed together or apart, there was no significant correlation between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum or the \( \text{FE}_{\text{NO}} \) measurement.

**Figure 5-16** (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against percentage sputum eosinophils in four patients with asthma (green circles) and four control subjects (purple squares).

**Figure 5-17** (A) Mean-exhaled and (B) end-exhaled concentrations of HCN plotted against \( \text{FE}_{\text{NO}} \) in six patients with asthma (green circles) and six control subjects (purple squares).
Table 5-11  Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of eosinophils in the sputum and the $\text{FE}_{\text{NO}}$ measurement in patients with asthma and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th></th>
<th>Sputum eosinophils (%)</th>
<th>$\text{FE}_{\text{NO}}$ (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=8) Asthma (n=4)</td>
<td>Controls (n=4)</td>
</tr>
<tr>
<td>Mean-exhaled HCN</td>
<td>0.06 (0.89) 0.80 (0.20) -0.11 (0.90)</td>
<td>0.52 (0.08) 0.60 (0.21) 0.64 (0.17)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>0.28 (0.51) 0.80 (0.20) -0.11 (0.90)</td>
<td>0.45 (0.14) 0.66 (0.16) 0.38 (0.46)</td>
</tr>
</tbody>
</table>

Relationship between HCN in exhaled breath and sputum neutrophils

There was no significant correlation between HCN in exhaled breath and sputum neutrophils in the asthma and control groups either separately or when combined (see Table 5-12).

Table 5-12  Spearman’s rank correlation coefficients ($r_s$) for the relationships between the concentration of HCN in exhaled breath and the percentage of neutrophils in the sputum in patients with asthma and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th></th>
<th>Sputum neutrophils (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All (n=8) Asthma (n=4) Controls (n=4)</td>
</tr>
<tr>
<td>Mean-exhaled HCN</td>
<td>-0.14 (0.74) 0.00 (1.00) -0.40 (0.60)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>-0.02 (0.96) 0.00 (1.00) -0.20 (0.80)</td>
</tr>
</tbody>
</table>
Relationship between the concentration of HCN in exhaled breath and spirometric measurements

No significant correlation was observed between the concentration of HCN in exhaled breath and either FEV$_1$ or FVC (see Table 5-13). In the asthma group, there was no correlation between the concentration of HCN in exhaled breath and the percentage change in FEV$_1$ after 400 μg of inhaled salbutamol.

Table 5-13  Spearman’s rank correlation coefficients ($r_s$) for correlation of the concentration of HCN in exhaled breath with FEV$_1$ and FVC in patients with asthma and control subjects. The data are expressed as $r_s$ (p value).

<table>
<thead>
<tr>
<th></th>
<th>All (n=12)</th>
<th>Asthma (n=6)</th>
<th>Controls (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FEV$_1$</td>
<td>FVC</td>
<td>FEV$_1$</td>
</tr>
<tr>
<td>Mean-exhaled HCN</td>
<td>-0.26</td>
<td>0.29</td>
<td>-0.26</td>
</tr>
<tr>
<td></td>
<td>(0.42)</td>
<td>(0.37)</td>
<td>(0.62)</td>
</tr>
<tr>
<td>End-exhaled HCN</td>
<td>-0.43</td>
<td>-0.39</td>
<td>-0.37</td>
</tr>
<tr>
<td></td>
<td>(0.17)</td>
<td>(0.21)</td>
<td>(0.47)</td>
</tr>
</tbody>
</table>

Relationship between the concentration of HCN in exhaled breath and ambient air

Figure 5-18 shows there was no significant correlation between the concentration of HCN in mean-exhaled or end-exhaled breath and the concentration of HCN in the ambient air ($r_s=-0.43$, $p=0.16$ and $r_s=-0.46$, $p=0.14$ respectively).

Figure 5-18  (A) Mean-exhaled and (B) end-exhaled HCN concentration plotted against ambient HCN concentration in six patients with asthma (green circles) and six control subjects (purple squares).
5.4. Discussion

In this study of small numbers of subjects, in the case of both COPD and asthma, there was no difference in the concentration of either hydrogen sulphide or hydrogen cyanide in exhaled breath between the patient and control groups. There was evidence, however, that the concentrations of H$_2$S and HCN in breath were associated with the levels of known biomarkers of airway inflammation. There was a strong and statistically significant negative correlation between exhaled H$_2$S and sputum neutrophils in the COPD patient group, and a positive correlation between exhaled H$_2$S and sputum neutrophils of borderline significance in the COPD control group. In the COPD patient group, exhaled HCN and the percentage of neutrophils in sputum were also negatively correlated ($r_s$=-0.49 to -0.66), although this relationship did not achieve statistical significance. In the asthma patient and control groups, positive correlations of $r_s=0.6$-0.7 were observed between the concentration of H$_2$S in exhaled breath and the $F_{E\text{NO}}$ measurement, although these correlations did not achieve statistical significance. In the group of four asthma patients who successfully provided a sputum sample, there was a significant positive correlation between exhaled H$_2$S and the percentage of sputum eosinophils. Exhaled HCN and biomarkers of eosinophilic airway inflammation were also positively correlated in the asthma patient group ($r_s=0.6$ to 0.8), but these relationships did not achieve statistical significance. The concentrations of both H$_2$S and HCN in nasally-exhaled breath were close to, and demonstrated some positive correlations with, the concentrations of these volatiles in ambient air, suggesting that ambient air may be an important source of inaccuracy in their measurement.

5.4.1. Exhaled hydrogen sulphide in COPD and asthma

While the concentration of H$_2$S in exhaled breath did not differ between patient groups and their controls, there was some evidence of an association between the concentration of H$_2$S in exhaled breath and other markers of airway inflammation. In the COPD group, there was a strong and significant negative correlation between the concentration of H$_2$S in exhaled breath and the percentage of neutrophils in the sputum, while in the control group there was a positive correlation between the two that reached borderline significance. The implication of this finding is not clear, although the negative correlation between exhaled H$_2$S and sputum neutrophils in COPD seen in this study is consistent with the findings of previous studies that have shown a negative correlation between the concentration of H$_2$S in serum and the proportion of neutrophils in sputum (Chen et al., 2005; Chen et al., 2008). In the control group, a positive correlation between the two might represent a normal relationship, whereas a
negative correlation in the COPD group might indicate a disruption of that relationship. While there is limited value in speculating on the nature of any disruption of such a relationship, there are possible explanations for these findings. For example, it is possible that, in the COPD group, neutrophilic inflammation is inappropriately stimulated by a mechanism unrelated to H$_2$S, resulting in the suppression of H$_2$S production in the lower respiratory tract. This pattern is frequently seen in the setting of endocrine disease, when inappropriate autonomous production of a hormone causes suppression of its normal stimulating hormone (for example, in hyperthyroidism thyroid stimulating hormone is suppressed by the abnormal autonomous production of thyroxine). It should also be borne in mind that H$_2$S has been shown variously to have vasodilator, vasoconstrictor and bronchodilator properties (Kubo et al, 2007a; Lim et al, 2008; Wang, 2009; Olson et al, 2010). Therefore, the association of exhaled H$_2$S with the proportion of neutrophils in sputum may be due to changes in these properties in COPD rather than any direct connection to inflammation.

There was evidence of an association between the concentration of H$_2$S in exhaled breath and biomarkers of eosinophilic airway inflammation. In both the asthma patient and control groups, positive correlations of $r_s=0.6-0.7$ were observed between the concentration of H$_2$S in exhaled breath and the FE$_{NO}$ measurement, although these correlations did not achieve statistical significance. In the group of four asthma patients who successfully provided a sputum sample, there was a significant positive correlation between exhaled H$_2$S and the percentage of sputum eosinophils. This pilot work was not sufficiently powered to detect anything other than strong correlations of $r_s>0.8$ within the individual groups. In previous studies of steroid-naïve asthma, the Spearman’s rank correlation coefficient ($r_s$) for the association between the FE$_{NO}$ measurement and sputum eosinophils has been 0.45 to 0.48 (Jatakanon et al, 1998; Berlyne et al, 2000), and FE$_{NO}$ has proven to be a useful biomarker of eosinophilic airway inflammation. Clearly, further work is required, with greater numbers of study participants, in order to determine any relationship between the concentration of H$_2$S in exhaled breath and the FE$_{NO}$ measurement.

The concentration of H$_2$S in exhaled breath and ambient air were positively correlated in the COPD patient and control groups, but no such correlation was observed in the asthma patient and control groups. The reason for this difference was unclear, but the possible association between exhaled H$_2$S and the ambient concentration of H$_2$S indicated that the exhalation manoeuvre might be improved by the inhalation of H$_2$S-scrubbed air prior to breath analysis.
As observed in Section 4.5.2, Page 105, however, the H\textsubscript{2}S concentrations observed in the ambient air and exhaled breath were already close to the limit of detection of the instrument, so H\textsubscript{2}S-scrubbing of the inspired air might have been of little benefit. Further work will be required to determine whether the inhalation of H\textsubscript{2}S-scrubbed air prior to breath analysis usefully eliminates contamination from the ambient air. If so, experiments will then be needed to determine the optimum length of time spent breathing H\textsubscript{2}S-scrubbed air before breath analysis. This will depend on whether the association between the concentrations of exhaled and ambient H\textsubscript{2}S is mediated by a washin-washout effect, in which H\textsubscript{2}S is inhaled then exhaled straight back out again, or whether the kinetics of inhalation and exhalation are more complex and a longer duration of time is required to reach steady state.

In the COPD group, the concentration of H\textsubscript{2}S in exhaled breath and the percentage of neutrophils in sputum correlated positively and negatively with the pre-bronchodilator FEV\textsubscript{1} respectively. Similar but weaker and non-significant correlations were seen with the pre- and post-bronchodilator percentage of predicted FEV\textsubscript{1} and with the post-bronchodilator FEV\textsubscript{1}. A negative correlation between the percentage of neutrophils in sputum and the percentage of predicted FEV\textsubscript{1} has previously been observed (O'Donnell et al, 2004; Singh et al, 2010). Further work, with a greater number of study participants, will be required to define the relationship between exhaled H\textsubscript{2}S and spirometric measurements.

The concentration of H\textsubscript{2}S in exhaled breath may be a biomarker that has a relationship with both neutrophilic and eosinophilic inflammation and, given the results of this study, these relationships merit further investigation. Firstly, the exhalation manoeuvre requires refinement in order to minimise the effect of ambient H\textsubscript{2}S on the concentration of H\textsubscript{2}S in exhaled breath. Secondly, the role of H\textsubscript{2}S in airway inflammatory processes requires further study, as does the relationship between serum and exhaled H\textsubscript{2}S. Thirdly, the findings in this work require confirmation in a larger study, particularly because this pilot study was not powered to determine the significance of anything other than strong correlations of \( r > 0.8 \) within the individual groups. Fourthly, while exhaled H\textsubscript{2}S may not be of use as a diagnostic marker, it may have value as a prognostic marker, but this will require longitudinal studies. In summary, this study showed that exhaled H\textsubscript{2}S is a promising biomarker of airway inflammation, and is worthy of further and more extensive study.
5.4.2. Exhaled hydrogen cyanide in COPD and asthma

In both COPD and asthma, no difference was observed between the concentration of HCN in exhaled breath in the patient and control groups. This implies that exhaled HCN may be of little or no value as a diagnostic marker for these airway diseases. In the COPD group, exhaled HCN and the percentage of neutrophils in sputum were negatively correlated ($r_s = -0.49$ to $-0.66$), but this relationship was not statistically significant. Exhaled HCN and biomarkers of eosinophilic airway inflammation were positively correlated in the asthma group ($r_s = 0.6$ to $0.8$), but these relationships were not statistically significant either. This pilot study was not powered to determine the significance of anything other than strong correlations of $r_s > 0.8$ within the individual groups. As described in Section 5.4.1, the association between $FE_{NO}$ measurements and sputum eosinophils of $r_s = 0.45$-$0.48$ (Jatakanon et al., 1998; Berlyne et al., 2000), has proven sufficiently strong for $FE_{NO}$ to be a useful biomarker of eosinophilic airway inflammation. Greater numbers of study participants will be required to determine an association of this strength between the concentration of HCN in exhaled breath and biomarkers of neutrophilic and eosinophilic inflammation.

The concentrations of HCN in exhaled breath and ambient air were positively correlated in the patient and control groups, and these correlations approached or achieved statistical significance. As for $H_2S$, the possible association between the exhaled and ambient concentrations of HCN indicates that the exhalation manoeuvre might be improved by the inhalation of HCN-scrubbed air prior to breath analysis, but further work will be required to determine the optimum time spent breathing HCN-scrubbed air before breath analysis.

In order to determine any future role for exhaled HCN as a biomarker of airway inflammation, future studies will require refinement of the breathing manoeuvre performed during breath analysis to lessen the effect of ambient HCN on the concentration of HCN in exhaled breath. In addition, studies of greater numbers of participants will be required to determine conclusively whether any useful association exists between exhaled HCN and proven biomarkers of neutrophilic and eosinophilic airway inflammation.
5.5. Summary

In the case of both COPD and asthma, there was no difference in the concentration of either H$_2$S or HCN in exhaled breath between the patient and control groups.

**H$_2$S in exhaled breath**

In the COPD patient group, there was a strong and significant negative correlation between the concentration of H$_2$S in exhaled breath and the proportion of neutrophils in the sputum, while in the COPD control group a positive correlation between the two achieved borderline significance. In the asthma patient group, there was evidence of a positive correlation between the concentration of H$_2$S in exhaled breath and established markers of eosinophilic airway inflammation. These data suggest that the concentration of H$_2$S in exhaled breath may be worthy of further study as a biomarker of airway inflammation.

**HCN in exhaled breath**

The concentration of HCN in exhaled breath was negatively correlated with the proportion of neutrophils in sputum in the COPD patient group, and positively correlated with biomarkers of eosinophilic airway inflammation in the asthma patient group. These correlations were strong but did not achieve significance, and further studies are justified to determine whether the concentration of HCN in exhaled breath is a biomarker of airway inflammation.
Chapter Six

6.

Discussion

The analysis of volatile biomarkers of airway inflammation in breath is an attractive concept. Firstly, inflammatory biomarkers may be more closely related to the underlying inflammatory disease processes than the physiological markers that are currently used, and therefore they may be valuable clinical tools in the diagnosis and monitoring of airway inflammatory diseases. Secondly, breath analysis takes advantage of an easily obtainable sample and is agreeable to patients because it is non-invasive and takes little time. The development of clinical breath analysis has been slow, however. This is due to the complex and interacting challenges involved in developing the analysis of an individual volatile compound, including: the instrumentation required for the identification and measurement of analytes at the parts-per-billion level and lower; the physiology of exhalation; and the relationship between the proposed biomarker and the underlying condition (Risby and Solga, 2006; Stockley, 2007).

Work undertaken for this thesis investigated the analysis of volatile compounds in exhaled breath as biomarkers of airway inflammation. Firstly, a study was performed with the aim of determining the utility of $\text{FE}_{\text{NO}}$ measurement as a predictor of the response to corticosteroid in COPD. A second study aimed to determine the accuracy and repeatability of the selected ion flow tube – mass spectrometry (SIFT-MS) technique for the measurement of trace volatile compounds, and to determine the effects of expiratory flow, volume and the effect of the oral or nasal route on the concentration of a volatile compound in exhaled breath. Initially, these experiments were performed using acetone as a model volatile compound. Similar experiments were then performed using hydrogen sulphide ($\text{H}_2\text{S}$) and hydrogen cyanide (HCN) as potential biomarkers of airway inflammation. The aim of the final study was to determine the concentrations of $\text{H}_2\text{S}$ and HCN in the exhaled breath of patients with asthma and COPD compared to control groups, and to investigate any relationship between these volatile compounds and currently accepted biomarkers of neutrophilic and eosinophilic airway inflammation.
The potential for the analysis of volatile biomarkers of airway inflammation is perhaps best demonstrated by the use of $\text{FeNO}$ measurement in asthma and other airway diseases. Nitric oxide is synthesised in the airways, and $\text{FeNO}$ measurement is clinically useful because it correlates with eosinophilic airway inflammation and this inflammation is associated with a positive response to treatment with corticosteroid (Taylor et al., 2006). A low $\text{FeNO}$ (<25ppb) predicts the absence, and a high $\text{FeNO}$ (50ppb) predicts the presence, of a response to corticosteroid (Pavord et al., 2008). $\text{FeNO}$ measurement is therefore helpful to the clinician when there is diagnostic uncertainty. However, the use of $\text{FeNO}$ measurement to predict a response to corticosteroid has its limitations: mid-range $\text{FeNO}$ measurements are difficult to interpret because the performance of $\text{FeNO}$ in predicting eosinophilic airway inflammation is only “fair” (Lex et al., 2006), and there is overlap between the $\text{FeNO}$ ranges seen in healthy subjects (Olin et al., 2007) and patients with airway inflammation (Kostikas et al., 2008). The role of $\text{FeNO}$ measurement in the therapeutic monitoring of eosinophilic asthma has also been explored, but the results of studies comparing treatment algorithms with and without $\text{FeNO}$ measurements have not been definitive (See Section 1.2.6, Page 16). Therefore, while it is of some use, $\text{FeNO}$ is an imperfect predictor of response to corticosteroid in airway inflammation, and our understanding of its utility in the management of asthma, is incomplete.

In COPD, $\text{FeNO}$ measurement presents an opportunity to identify the minority of patients who respond to corticosteroid. There is evidence that steroid-responsive patients (demonstrated by increased airway calibre and improved health-related quality of life in response to corticosteroid) are more likely to be characterised by the presence of eosinophilic airway inflammation (Pizzichini et al., 1998; Brightling et al., 2000; Brightling et al., 2005; Leigh et al., 2006). Furthermore, a significant correlation has previously been reported between the percentage of eosinophils in sputum and $\text{FeNO}$ levels in patients with COPD ($r_s = 0.65$) (Rutgers et al., 1999). These studies and others (see Section 1.2.7, Page 17) suggest that $\text{FeNO}$ may have potential as a predictor of response to corticosteroid in stable COPD.

The results of the study undertaken for this thesis demonstrate that $\text{FeNO}$ is a weak predictor of short-term response to oral corticosteroid in patients with stable, moderately severe COPD. $\text{FeNO}$ measurements in patients with COPD were a weak predictor for reversibility of airflow obstruction, and did not predict improvements in functional exercise capacity or health-related quality of life, with corticosteroid therapy. The weak predictive utility of $\text{FeNO}$ was reflected in an area under the receiver operator characteristic curve of 0.69 (a value above 0.8 denoting a strong predictor (Hanley and McNeil, 1982)), and a modest positive predictive value of 67%
Chapter Six

at the optimum cut-point for predicting an increase in FEV\(_1\) (>50ppb). A low FE\(_{\text{NO}}\) (<25ppb) helpfully predicted the absence of a response to corticosteroid, however, with a high negative predictive value of 87%. Given that only around 20% of patients demonstrate steroid responsiveness (Weir et al., 1990; Weir and Burge, 1993), this information could help the clinician to avoid the unnecessary prescription of ICS treatment. This is a useful finding, given that the use of ICS in undifferentiated COPD patients provides little or no benefit (Suissa and Barnes, 2009), results in no reduction in mortality (Drummond et al., 2008) and may increase the risk of pneumonia (Drummond et al., 2008; Singh et al., 2009).

In order to establish the role of FE\(_{\text{NO}}\) in COPD definitively, future work should explore the utility of FE\(_{\text{NO}}\) measurement for predicting the long-term response to corticosteroid in COPD. Long-term ICS treatment is more likely to benefit COPD patients whose pre-treatment airway inflammation includes a significant eosinophilic component (Siva et al., 2007), and FE\(_{\text{NO}}\) measurements may be useful because they are a surrogate marker for sputum eosinophil counts. Future work will need to examine the utility of FE\(_{\text{NO}}\) for predicting the response to ICS of long-term outcomes such as mortality, exacerbation rates and rate of decline in FEV\(_1\).

Any future study designs should take into account the effect of dropout during steroid-withdrawal. In this study, 13 patients (16% of the recruited volunteers) were unable to tolerate the cessation of ICS during the run-in phase, resulting in a potential selection bias and underestimation of the beneficial effects of prednisone. Other studies incorporating steroid-withdrawal in COPD patients demonstrate a similar proportion of patients unable to discontinue ICS treatment (O'Brien et al., 2001; van der Valk et al., 2002). While this group of patients has proved to be a potential source of bias in several of the randomised controlled trials upon which ICS treatment recommendations in COPD are based (Suissa and Barnes, 2009), they present less of a problem in routine clinical practice. Clearly, if a patient is unable to tolerate the withdrawal of ICS, that patient should recommence treatment. Given that so many patients with COPD are already taking ICS treatment, it is important to ensure that future studies are designed pragmatically, so that they not only answer a clinically useful question, but are also relevant to the current practising environment. For example, in patients taking ICS who successfully withdraw their ICS treatment, does FE\(_{\text{NO}}\) measurement predict a response to treatment? In patients not taking ICS, does FE\(_{\text{NO}}\) measurement predict a response to treatment? It should be noted that such questions themselves are not answered simply, because there is an interaction between these two patient groups: in clinical practice, some patients who are currently steroid-free will have previously trialled ICS treatment but then
discontinued treatment when they perceived no benefit. Nevertheless, it is important that future studies are not biased by COPD patients unable to tolerate withdrawal of ICS treatment during the run-in.

A greater understanding of the cause of eosinophilic airway inflammation in stable COPD would be helpful in defining the role of $\text{Fe}_{\text{NO}}$ measurement. While the relationships between $\text{Fe}_{\text{NO}}$, sputum eosinophils and the inflammatory response to steroid seen in this study were similar to those seen in asthma (Taylor et al, 2006), the underlying cause of the eosinophilic inflammation may be different from the type 1 hypersensitivity response seen in eosinophilic asthma. While some patients with stable COPD may simply have concomitant eosinophilic asthma, an alternative explanation for their eosinophilic airway inflammation may relate to viral infection and persistence. Acute exacerbations of COPD precipitated by acute viral infection are associated with an increase in both eosinophilic airway inflammation and $\text{Fe}_{\text{NO}}$ (Papi et al, 2006), and recent evidence suggests that viruses such as respiratory syncytial virus (RSV) may persist in the lungs of patients with stable COPD (Sikkel et al, 2008). Furthermore, the persistence of RSV in stable COPD is associated with an increased rate of FEV$_1$ decline (Wilkinson et al, 2006). It is not yet known whether subclinical persistence of RSV, or any other virus, is associated with eosinophilic inflammation or elevated $\text{Fe}_{\text{NO}}$ in stable COPD, but this relationship should be explored. A positive association might lead to the use of markers of eosinophilic inflammation in COPD not only for determining a patient’s steroid responsiveness, but also their viral status and disease prognosis.

Additional volatile biomarkers of eosinophilic airway inflammation would be useful adjuncts to $\text{Fe}_{\text{NO}}$ measurement. The development of volatile biomarkers of neutrophilic airway inflammation would also be valuable, given the lack of such markers at present and, more generally, the lack of adequate biomarkers for diagnosis, monitoring and prognosis in COPD. Two potential markers that have been investigated previously are carbon monoxide and hydrocarbons in exhaled breath. While marked elevations in the levels of exhaled carbon monoxide have been observed in smokers, smaller elevations in levels have been noted in non-smokers with respiratory diseases including asthma, COPD and cystic fibrosis (Paredi et al, 2002; Zhang et al, 2010). Hydrocarbons in exhaled breath, derived from lipid peroxidation, may be increased when there is an excess of pro-oxidative free radicals relative to antioxidants (Buszewski et al, 2007). Increased levels of hydrocarbons have also been observed in the breath of patients with asthma, COPD and cystic fibrosis (Paredi et al, 2002). At present, the role of CO in the inflammatory process remains uncertain (Zhang et al, 2010),
and the methodology for the analysis of hydrocarbons in breath requires further work (Larstad et al., 2007; Gorham et al., 2009), and neither of these potential markers has yet been established as a clinical tool for the assessment of airway inflammation.

Work not presented for this thesis has examined SIFT-MS analysis of gaseous chloramines and bromamines in exhaled breath as biomarkers of airway inflammation (Senthilmohan et al., 2008). These compounds are derived from hypochlorous acid and hypobromous acid, which are produced by activated neutrophils and eosinophils (Klebanoff, 2005). Results were initially promising (Senthilmohan et al., 2008), but further study has failed to demonstrate conclusive evidence of chloramines and bromamines in exhaled breath (Epton et al., 2009; Hu et al., 2010).

Given the limitations of FE\textsubscript{NO} measurement and the lack of other exhaled biomarkers of airway inflammation, it is clear that novel breath markers of airway inflammation would be valuable. Studies performed for this thesis examined the potential of H\textsubscript{2}S and HCN as biomarkers of airway inflammation. H\textsubscript{2}S is elevated in the serum of patients with stable COPD, and correlates positively with serum nitric oxide, and negatively with percentage sputum neutrophils (Chen et al., 2005). However, the role of H\textsubscript{2}S in inflammation has not been fully defined (Zhang and Bhatia, 2008). The production of HCN by activated neutrophils has previously been demonstrated (Stelmaszynska, 1985), but there has been little additional work on the role of HCN in inflammation since the mid 1980s. There has been renewed interest, however, in the role of HCN in inflammation since the discovery that HCN is elevated in the sputum of patients infected with \textit{P. aeruginosa} (Ryall et al., 2008). The potential use of H\textsubscript{2}S and HCN in exhaled breath as biomarkers of airway inflammation has not previously been explored.

Both H\textsubscript{2}S and HCN in exhaled breath are suitable for analysis by SIFT-MS (Spanel and Smith, 2000b; Spanel et al., 2004) – an analytical technique that provides an opportunity to examine concentrations of volatile compounds on-line and in real time (see Section 1.5, Page 25). In order to examine these compounds in breath using SIFT-MS, it was necessary to determine the accuracy, repeatability and dynamic response time of the SIFT-MS instrument for each compound. Furthermore, it was necessary to synchronise the SIFT-MS measurements of the concentration of an exhaled volatile compound with measurements of expiratory flow and volume taken by a pneumotachometer. Synchronisation of the two instruments allowed investigation of the exhalation physiology of H\textsubscript{2}S and HCN, and also
allowed the establishment of an optimum breathing manoeuvre for analysis of each compound. Initially, these experiments were successfully performed using acetone as a model volatile compound, and an appropriate manoeuvre for the analysis of acetone in exhaled breath was determined. This was a useful piece of work in itself, as the analysis of acetone in exhaled breath has potential clinical applications (Galassetti et al., 2005; Pabst et al., 2007). In addition, it provided a model for the development of volatile compound analysis using SIFT-MS. The same instrument characteristics were then defined for the analysis of \( \text{H}_2\text{S} \) and HCN using SIFT-MS, and the instrument was successfully synchronised with a pneumotachometer for their analysis in breath.

When developing breathing manoeuvres for the analysis of both \( \text{H}_2\text{S} \) and HCN in exhaled breath, the most noticeable findings were the elevated level of both of these compounds in the oral cavity, and the effect that this source of both volatiles had on the concentration in exhaled breath. While this finding was unsurprising and consistent with previous work on both volatiles (Pysanenko et al., 2008; Wang et al., 2008), it vindicated the approach taken in this study of a systematic examination of the effects of oral and nasal breathing, and changing expiratory flow and volume, on the concentration of a volatile in exhaled breath. With the benefit of hindsight, it may seem an obvious approach to take, but previous studies by others have been performed in which the effect of breathing manoeuvre on the concentration of a volatile compound in breath has not been considered. Some of these studies have since been shown to be flawed. For example, some studies using oral exhalations for the sampling of exhaled HCN and ammonia are of limited value (Turner et al., 2006a; Enderby et al., 2009b), given that the mouth is a major source of both volatiles (Wang et al., 2008). Factors such as oral contamination of exhaled breath, which are unrelated to airway disease, but capable of changing concentration of a volatile compound in exhaled breath must be understood in order to develop robust clinical tests.

Having developed a breathing manoeuvre that minimised the contribution of \( \text{H}_2\text{S} \) or HCN from the oral cavity to exhaled breath originating from the lower respiratory tract, it was found that the concentrations of \( \text{H}_2\text{S} \) and HCN in the exhaled breath of healthy subjects correlated strongly with the concentration in the ambient air. While it was encouraging that contamination from the oral cavity had been limited to the extent that a lesser source of contamination was detectable in the breath, the ambient source of contamination needs to be addressed. Levels of \( \text{H}_2\text{S} \) in exhaled breath were approximately twice the levels seen in the ambient air, while levels of HCN in exhaled breath were similar to those seen in the ambient
The concentrations of both volatile compounds in the ambient air were therefore greater than 25% of the concentrations in breath, and above the threshold previously described as acceptable in order to avoid significant error due to the subject not being in steady state with his or her environment (Risby and Solga, 2006). The levels of H$_2$S and HCN were not elevated in patients with COPD and asthma, and so the concentrations of both volatile compounds in the ambient air remained greater than 25% of the concentrations in breath in these groups. These findings suggest that further refinement of the manoeuvre may be necessary, using air scrubbed of H$_2$S and HCN as a breathing source prior to testing. While this may prove a useful modification to the breathing manoeuvre, it may not be necessary for a subject to reach steady state with his or her environment before performing breath analysis for H$_2$S or HCN. In the case of FE$_{NO}$ measurement, ambient levels of NO may sometimes be much higher than the levels in exhaled breath, but a single inhalation of NO-scrubbed air prior to testing is sufficient for breath analysis. This is because NO binds avidly to haemoglobin, which acts as a sink for NO on inspiration (Gow and Stamler, 1998). H$_2$S and HCN also bind to haemoglobin (Park and Nagel, 1984; Brunori et al, 1992), so it may act as a sink for these volatiles as well. Further studies are required to establish the inhalation and exhalation kinetics of H$_2$S and HCN.

The concentrations of H$_2$S and HCN in exhaled breath were examined in patients with COPD and asthma. The nasal breathing manoeuvres that were established for their analysis were simple and easily performed by patients, and the concentrations of H$_2$S and HCN in the exhaled breath of patient groups with asthma and COPD and in the exhaled breath of control groups were shown to be similar. However, a negative correlation was demonstrated between the concentration of H$_2$S in exhaled breath and the proportion of neutrophils in the sputum of patients with COPD, while a positive correlation between these two variables approached significance in the control group. This finding is consistent with previous studies of the relationship between serum H$_2$S and the proportion of neutrophils in sputum (Chen et al, 2005; Chen et al, 2008), and clearly merits further exploration, as discussed in Section 5.4.1, Page 137. The prospect that H$_2$S in exhaled breath might be developed into a biomarker of neutrophilic airway inflammation is an exciting one. Furthermore, the relationship between serum and exhaled H$_2$S and neutrophilic inflammation may be worthy of investigation in other respiratory diseases, such as cystic fibrosis and pneumonia. When designing future studies, the complexity of inflammatory processes should not be underestimated, and the interactions between inflammatory mediators should be considered. H$_2$S should not only be studied as an inflammatory marker in isolation; its relationships with other markers must also
be considered. For example, an association between serum NO and serum H$_2$S has previously been noted in COPD (Chen et al, 2005), and the two compounds are known to interact in their roles as vasoactive mediators (Whiteman et al, 2006; Kubo et al, 2007b). It may be that the relationship between the two compounds is of more relevance as a biomarker than the absolute concentration of either one or the other. Within the COPD and asthma patient groups, a number of non-significant relationships between the concentrations of H$_2$S and HCN in exhaled breath and established inflammatory biomarkers were observed, and studies of greater numbers of subjects will be required to determine whether or not these are significant (see Section 5.4.2, Page 140). More broadly, further in vitro and in vivo work will be required to fully elucidate the role of H$_2$S and HCN in airway inflammation and to fulfil the previously suggested requirements of a biomarker in inflammatory airway disease: that it must be central to the pathophysiological process or must be a clear surrogate of that process; it must vary only with events known to relate to disease progression, and must predict progression; those individuals with a higher value at baseline must have either an increased risk of disease onset or greater disease severity; and the biomarker must also be sensitive to interventions that are known to be effective (Stockley, 2007).

While the work performed in this thesis was restricted to the role of volatile compounds in exhaled breath as biomarkers of airway inflammation, the concentrations of NO, H$_2$S and HCN in exhaled breath may all have potential roles as biomarkers in respiratory infections. Acute exacerbations of COPD precipitated by acute viral infection are associated with an increase in FE$_{NO}$ (Papi et al, 2006), and FE$_{NO}$ measurement has recently been used to predict the response to treatment in patients with acute exacerbations of COPD (Antus et al, 2010). Serum H$_2$S is lower in patients with pneumonia than in control subjects, and predicts the need for antibiotic treatment (Chen et al, 2009b). HCN has been detected in the headspace above cultures of P aeruginosa (Carroll et al, 2005), and cyanide has been found in the sputum of cystic fibrosis patients infected with P aeruginosa (Ryall et al, 2008; Sanderson et al, 2008). As with the inflammatory diseases examined for this thesis, it is important that future work elucidates the exhalation physiology of these compounds and their biological roles within the infective process (Miekisch et al, 2004; Stockley, 2007).

In conclusion, work undertaken for this thesis explored the role of volatile compounds in exhaled breath as biomarkers of airway inflammation. FE$_{NO}$ measurement is a useful but imperfect predictor of short-term response to corticosteroid in COPD. Further work is justified to determine its role in COPD as a predictor of long-term response to corticosteroid,
and to increase our understanding of the underlying causes of eosinophilic airway inflammation in COPD, so that further applications of FE\textsubscript{NO} measurement might be developed. The potential of H\textsubscript{2}S and HCN in exhaled breath as additional biomarkers of airway inflammation was explored and, while levels of H\textsubscript{2}S and HCN in exhaled breath were not elevated in COPD and asthma, there was evidence of relationships between these volatile compounds and established markers of both neutrophilic and eosinophilic inflammation. Further studies are required to determine strength and significance of these relationships, but the prospect that exhaled H\textsubscript{2}S and HCN may be biomarkers of airway inflammation is an exciting one.
References


Ballantyne, B. (1983). The influence of exposure route and species on the acute lethal toxicity and tissue concentrations of cyanide. Developments in the science and practice of toxicology : proceedings of the Third International Congress on Toxicology held in


References


References


References


References


Appendix A

Publications Resulting from this Thesis

Original articles


Conference abstracts


Appendix B

Macro Programs

Using Visual Basic for Applications (Microsoft, USA), a number of macro programs were written for use with Excel 2007© in order to automate the processing of data files obtained from the Voice200™ SIFT-MS and the pneumotachometer (see Section 4.2.7, Page 83). The programming is documented below for the four macro programs: Pro1WorkbookCreate; ProcessAllData; CurtAll; and BobOne.

Sub Pro1WorkbookCreate()
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
After:=Sheets(Sheets.Count)
Sheets.Add
Sheets("Sheet1").Select
Sheets("Sheet1").Name = "SIFT"
Sheets("Sheet2").Select
Sheets("Sheet2").Name = "SIFTpneumo"
Sheets("Sheet3").Select
Sheets("Sheet3").Name = "Pneumofull"
Sheets("Sheet4").Select
Sheets("Sheet4").Name = "Process1"
Sheets("Sheet5").Select
Sheets("Sheet5").Name = "Process2"
Sheets("Sheet6").Select
Sheets("Sheet6").Name = "Process3"
Sheets("Sheet7").Select
Sheets("Sheet7").Name = "Process4"
Sheets("Sheet8").Select
Sheets("Sheet8").Name = "Process5"
End Sub

Sub ProcessAllData()
Application.StatusBar = "Now processing file"
Application.ScreenUpdating = False
Process1Data
Process1XData
Process2Data
Process3Data
Process4Data
Process5Data
ProcessDelete
Application.ScreenUpdating = True
Application.StatusBar = False
End Sub

Sub Process1Data()
FindData1
CopySIFTData
CopySIFTPneumo
FindSummary
DelSummary
Process2a
Process2a1
Process2a2
End Sub

Sub FindData1()
Sheets("SIFT").Select
Range("A1").Select
End Sub

Sub CopySIFTData()
ActiveCell.Offset(1, 0).Range("A1").Select
Range("A1").Select
ActiveSheet.Paste
End Sub

Sub CopySIFTPneumo()
ActiveSheet.Paste
End Sub

Sub FindSummary()
End Sub

Sub DelSummary()
ActiveCell.Offset(-1, 0).Range("A1").Select
Selection.ClearContents
End Sub

Sub Process2a()
Columns("J:J").Select
Selection.Copy
Columns("H:H").Select
ActiveSheet.Paste
End Sub

Sub CopySIFTData1()
ActiveCell.Offset(0, 0).Range("A1").Select
Range("A1").Select
ActiveSheet.SpecialCells(xlLastCell).Select
Selection.Copy
Sheets("Process1").Select
ActiveSheet.Paste
End Sub
ActiveSheet.Paste
Range("A1").Select
End Sub
Sub Process2a1()
Range("D1").Select
ActiveCell.FormulaR1C1 = 1 = "Time"
Range("E1").Select
ActiveCell.FormulaR1C1 = 1 = "Analyte"
Range("D2").Select
Sheets("Process1").Select
Range("A2").Select
End Sub
Sub Process2a2()
Do While ActiveCell.Value <> ""
Sheets("Process2").Select
ActiveCell.FormulaR1C1 = 1 = "Process1\R[-3]\Process2\R2C1"
ActiveCell.Offset(0, 1).Select
ActiveCell.FormulaR1C1 = 1 = "Process1\R[-2]"
ActiveCell.Offset(1, -1).Select
Sheets("Process1").Select
ActiveCell.Offset(1, 0).Select
Loop
End Sub
Sub Process1Xa()
Range("I2").Select
Sub Process1Xb()
End Sub
Sub Process1Xc()
Range("I2").Select
Sub Process1Xd()
Do While ActiveCell.Value <> ""
Sheets("Process2").Select
ActiveCell.FormulaR1C1 = 1 = "Process1\R[-5]\Process2\R2C1"
ActiveCell.Offset(0, 1).Select
ActiveCell.FormulaR1C1 = 1 = "Process1\R[-4]\Process2\R2C1"
ActiveCell.Offset(1, -1).Select
Sheets("Process1").Select
ActiveCell.Offset(1, 0).Select
Loop
End Sub
Sub Process2a5()
Range("A1").Select
Sub Process2a6()
StateCells(xlLastCell).Select
End Sub
Sub Process1Xf()
Sheets("Process2").Select
Range("A1").Select
End Sub
Appendix B

Range("K1").Select
ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 2).Columns("A:A").EntireColumn.Select
  Selection.Find(What:="2",
    After:=ActiveCell, Lookin:=xlFormulas,
    LookAt :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
  ActiveCell.Offset(1, 0).Range("A1:G60000").Select
  Selection.ClearContents
  ActiveCell.Offset(-1, 0).Range("A1").Select
  ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
  ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Pressure"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Flow"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Volume"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = "Pn Time"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Exhalations"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "S Time"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = 
  "=SUM(C[1])"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Analyte"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = "Flow"
ActiveCell.Select
  Loop
ActiveCell.FormulaR1C1 = "Pressure"
Do While ActiveCell.Value > 0.5
  ActiveCell.Offset(0, 3).Columns("A:F").EntireColumn.Select
  Selection.SpecialCells(xlCellTypeBlanks).
  Select
  Selection.Delete Shift:=xlUp
  ActiveCell.Offset(0, 7).Range("A1").Select
  ActiveCell.FormulaR1C1 = "Volume"
ActiveCell.Select
  Loop
ActiveCell.Offset(0, 1).Select
End Sub
Sub ProcessDelete()
  Sheets("Process5").Select
  Sheets("Process5").Name = 
  "Exhalations"
  Application.DisplayAlerts = False
  Sheets("Process4").Delete
  Sheets("Process3").Delete
  Sheets("Process2").Delete
  Sheets("Process1").Delete
  Sheets("PneumoFull").Delete
  Sheets("SIFTpneumo").Delete
  Sheets("SIFT").Delete
  Application.DisplayAlerts = True
End Sub
Sub CurtAll()
  Application.StatusBar = "Now processing file"
  Application.ScreenUpdating = False
  Curt1
  Curt2
  Curt4
  Curt5
  Curt6
  Curt7
  Curt8
  Application.ScreenUpdating = True
  Application.StatusBar = False
End Sub
Sub Curt1()
  Sheets("Sheet1!").Select
  Range("E2").Select
End Sub
Sub Curt2()
  Do While ActiveCell.Value <> 
  ActiveCell.Offset(0, 6).Select
  ActiveCell.FormulaR1C1 = 
  "=IF(RC[-1]:R2C1=1)"
  ActiveCell.Offset(1, -6).Select
  Loop
  Curt3
End Sub
Sub Curt3()
  ActiveCell.Columns("A:A").EntireColumn.Select
  Selection.Find(What:="S Time",
    After:=ActiveCell, Lookin:=xlValues,
    MatchCase:=False,
    SearchFormat:=False).Activate
  ActiveCell.Offset(1, 9).Select
  Loop
  Range("E2").Select
End Sub
Sub Curt4()
  Range("E2").Select
  Do While ActiveCell.Value <> 
  ActiveCell.Offset(0, 6).Columns("A:A").EntireColumn.Select
  Selection.Find(What:="1",
    After:=ActiveCell, Lookin:=xlValues,
    LookAt _
    :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
  ActiveCell.Offset(1, -6).Select
  ActiveCell.FormulaR1C1 = "Exhalations"
  Application.ClearContents
  ActiveCell.Columns("A:A").EntireColumn.Select
  Selection.Find(What:="S Time",
    After:=ActiveCell, Lookin:=xlFormulas,
    LookAt _
    :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
  ActiveCell.Columns("A:A").EntireColumn.Select
  Selection.Find(What:="1",
    After:=ActiveCell, Lookin:=xlValues,
    LookAt _
    :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
  ActiveCell.Columns("A:A").EntireColumn.Select
  Selection.Find(What:="S Time",
    After:=ActiveCell, Lookin:=xlFormulas,
    LookAt _
    :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
  ActiveCell.Columns("A:A").EntireColumn.Select
  Selection.Find(What:="1",
    After:=ActiveCell, Lookin:=xlValues,
    LookAt _
    :=xlPart, SearchOrder:=xlByRows,
    SearchDirection:=xlNext, MatchCase:=
    False,
    SearchFormat:=False).Activate
Appendix B

ActiveCell.FormulaR1C1 = "=MAX(C[-11])"
Range("U2").Select
ActiveCell.FormulaR1C1 = "=SUM(C[-7]-SUM(C[3])"
ActiveCell.Offset(0, 1).Range("A1").Select
End Sub

Sub Ex1d()
Range("A3").Select
Do While ActiveCell.Value = "" "
ActiveCell.Offset(0, 14).Range("A1").Select
ActiveCell.FormulaR1C1 = "=RC[-7]-SUM(C[3])"
ActiveCell.Offset(0, 1).Range("A1").Select
End Sub

Sub Ex1e()
Range("A5").Select
ActiveCell.FormulaR1C1 = "=SUM(C[-7]-SUM(C[3])"
ActiveCell.Offset(0, 1).Range("A1").Select
End Sub

Sub Ex1e2()
Range("A1").Select
ActiveCell.FormulaR1C1 = "=SUM(C[-7]-SUM(C[3])"
ActiveCell.Offset(0, 14).Range("A1").Select
End Sub

Sub Ex1f()
Range("A7").Select
ActiveCell.FormulaR1C1 = "=SUM(C[-7]-SUM(C[3])"
ActiveCell.Offset(0, 14).Range("A1").Select
End Sub

Sub Ex1g()
Range("A9").Select
ActiveCell.FormulaR1C1 = "=SUM(C[-7]-SUM(C[3])"
ActiveCell.Offset(0, 14).Range("A1").Select
End Sub
Range("N4").Select
ActiveCell.FormulaR1C1 = "=MAX(C[-12])"
End Sub

Sub Ex1i()
Range("M3").Select
ActiveCell.FormulaR1C1 = "=(RC[-3]-R[-1]*0.15)"
End Sub

Sub Ex2i()