Infection, inflammation & innate immunity in the paediatric CF airway

Submitted for MD(Res)

National heart and lung institute

Imperial College, London

Dr Rebecca Marie Thursfield
I declare that all the work in thesis is my own work. For some results, work was carried out by others and the results kindly been made available to me and this is clearly stated where applicable.

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.
This thesis focuses on infection and immunity within the airways in cystic fibrosis (CF), particularly the role of the antimicrobial peptides (part of the innate immune system) and their relationship to vitamin D status. Vitamin D response elements have been identified in the genes encoding the antimicrobial peptides cathelicidin (LL37) and human β defensins (HBD-2) and in-vitro vitamin D significantly induces expression of these peptides in both CF and non-CF bronchial epithelial cells.

As innate defence is pivotal to airway health and is one of the proposed ways that vitamin D deficiency contributes to worsening respiratory health, this thesis will consider first immunity of the normal airway and the interactions with vitamin D and then discuss the pathophysiology of CF and the role of vitamin D on the innate immune system within CF.

The role of vitamin D on infection and inflammation in the airways of infants with CF is explored and the impact of Vitamin D levels seen immunologically and functionally over the first year of life is described. Finally the role of vitamin D as an immunomodulatory molecule is explored in a greater range of CF disease severity and age.

Through the various parameters explored, in different CF patient populations, the conclusion remains the same; vitamin D deficiency is not associated with increased infection, greater inflammation nor a worse clinical outcome.

The possible reasons for the lack of any relationship are discussed in the final chapter; either a missed signal because the levels studied were on the low or high flat parts of a
sigmoid relationship thus effects seen only in really severe deficiency or because supra-high levels are needed to see any effect, the effect being lost in the inflammation seen within the CF airway or a true lack of relationship.
Acknowledgements

I would like to express my heartfelt gratitude to my three supervisors; Eric Alton for his words of wisdom, Andy Bush for his continued encouragement and input and particularly to Jane Davies for her unending advice and support over the past 4 ½ years. I am truly indebted to them for their sound wisdom and the time they have given to me in the planning, executing and writing up of this work.

I would also like to thank the lab team in the gene therapy department at NHLI, especially Samia Soussi for her patience in teaching me basic laboratory skills. I also thank Jackie Donovan for her advice and assistance with the Luminex experiments and general laboratory advice. I thank the consultants at the Royal Brompton Hospital for performing the bronchoscopies enabling me to obtain my samples. I am grateful to Harshil Bhayani for collecting the samples and Bushra Ahmed and Rishi Pabary for processing the samples collected in my absence. I am grateful to Lena Thia for making the CT and lung function results from her study available to me. I would particularly like to express my gratitude to the patients and their families for kindly consenting to participate in the research, without whom none of this would have been possible.

I would like to thank my family, especially my Mum for proof reading the first draft and my husband Ben and our daughter Molly for their support. Finally I would like to thank Alex Adams, colleague, fellow MD(Res) student and friend, for her assistance with the cytokine experiment making it a much more enjoyable day, a variety of different aspects of advice throughout the this period but mostly for her general support over the duration of our research.
Table of Contents

Abstract ............................................................................................................................................. 2
Acknowledgements ....................................................................................................................... 4
Table of Contents ........................................................................................................................... 5
List of Tables .................................................................................................................................. 14
List of Figures .................................................................................................................................. 15
List of Abbreviations ..................................................................................................................... 17

PART A: ........................................................................................................................................... 19

INTRODUCTION, HYPOTHESES, AIMS AND OBJECTIVES ..................................................... 19

1 CHAPTER 1: INFECTION, IMMUNE RESPONSE AND INNATE IMMUNITY ................. 20
1.1 Airway immunity ......................................................................................................................... 20
  1.1.1 Innate immune system ......................................................................................................... 20
  1.1.2 The innate immune response .............................................................................................. 22
  1.1.3 Antimicrobial peptides ....................................................................................................... 24
  1.1.4 Antimicrobial peptides in relation to vitamin D ................................................................. 24
  1.1.5 Defensins ........................................................................................................................... 24
  1.1.6 Cathelicidin ......................................................................................................................... 27

2 CHAPTER 2: CYSTIC FIBROSIS .......................................................................................... 31
2.1 CF – clinical disease .................................................................................................................. 31
  2.1.1 Clinical features of CF ....................................................................................................... 31
2.6 Non-CF chronic suppurative lung diseases ................................................................. 63
  2.6.1 Primary ciliary dyskinesia .................................................................................. 64
  2.6.2 Non-CF bronchiectasis ..................................................................................... 66

3 CHAPTER 3: VITAMIN D ......................................................................................... 68
  3.1 Vitamin D synthesis .............................................................................................. 68
  3.2 Classical actions of vitamin D .............................................................................. 69
  3.3 Non-classical actions of vitamin D ....................................................................... 70
  3.4 The role of vitamin D in airway defence ............................................................... 71
  3.5 Factors affecting Vitamin D levels ....................................................................... 76
  3.6 Vitamin D reference ranges ................................................................................. 77
  3.7 Vitamin D deficiency ........................................................................................... 79
  3.8 Vitamin D deficiency in CF .................................................................................. 79
  3.9 Vitamin D in respiratory health (non-CF) .............................................................. 80
  3.10 The role of Vitamin D in CF lung disease ............................................................ 82

4 CHAPTER 4: Hypothesis, aims and objectives .......................................................... 84
  4.1 Hypotheses ........................................................................................................... 84
  4.2 Aims ...................................................................................................................... 84
  4.3 Objectives ............................................................................................................ 85

PART B: METHODS ........................................................................................................ 86

5 CHAPTER 5: Patients and Methods ......................................................................... 87
  5.1 Patients ............................................................................................................... 87
    5.1.1 Patient selection ............................................................................................. 87
    5.1.2 Group 1: Cystic fibrosis ................................................................................ 87
    5.1.3 Group 2: Non-CF chronic suppurative lung disease (CSDL) ......................... 88
5.1.4 Group 3: Healthy Controls .................................................................88

5.2 Fibreoptic bronchoscopy .................................................................89
  5.2.1 Royal Brompton Hospital bronchoscopy programme .....................89
  5.2.2 Indications for bronchoscopy .......................................................89
  5.2.3 Consent .......................................................................................90
  5.2.4 Safety considerations .................................................................90
  5.2.5 Bronchoscopy Protocol ...............................................................92

5.3 Samples collected at bronchoscopy ................................................93
  5.3.1 BALF collection and processing .................................................93
  5.3.2 Sample suitability .......................................................................94
  5.3.3 Blood samples ..........................................................................94

5.4 Clinical patient data .......................................................................94

5.5 Lung function ..................................................................................95

5.6 Vitamin D measurement ...............................................................95
  5.6.1 Vitamin D assay ........................................................................95
  5.6.2 Comparing vitamin D results when performed using different methods ..............................................96
  5.6.3 Assessing stability of vitamin D over time ..................................97
  5.6.4 Conclusions of establishing methodology for vitamin D assay ..........................................................101
  5.6.5 Definition of vitamin D deficiency ..............................................103

5.7 Cytokine analysis ...........................................................................104
  5.7.1 Choice of assay ..........................................................................104
  5.7.2 Choice of cytokine ......................................................................105
  5.7.3 MSD assay ................................................................................106
  5.7.4 MSD detection ranges and standard curves ................................107

5.8 HBD-2 ELISA ................................................................................108
  5.8.1 HBD-2 measurement .................................................................108
  5.8.2 Establishing reproducibility .......................................................109
    5.8.2.1 Intra-assay reproducibility ..................................................109
5.8.2.2 Inter-run analysis.................................................................110
5.8.2.3 “Fresh” vs “old” samples .....................................................111
5.8.2.4 Dilution experiments...............................................................112
5.8.2.5 Outcome of HBD2 methodology experiments .......................116

5.9 LL-37 ELISA ...................................................................................116
5.9.1 LL-37 measurement ....................................................................116
5.9.2 Establishing reproducibility ..........................................................117

5.10 Microbiology of BALF samples ....................................................119

5.11 Statistical analyses.........................................................................119

PART C: STUDY SPECIFIC INTRODUCTION, METHODS, RESULTS AND DISCUSSION
..................................................................................................................................................120

6 Chapter 6: The role of Vitamin D in infection and inflammation in the airways
of infants diagnosed on new born screening .........................................................121

6.1 Introduction.....................................................................................121
6.1.1 Early lung disease outcome measures ...........................................121
6.1.2 Inflammatory changes .................................................................122

6.2 Hypotheses......................................................................................123

6.3 Methods............................................................................................123

6.4 Results ............................................................................................124
6.4.1 Subjects .........................................................................................124
6.4.2 Nutritional status of NBS CF infants .............................................127
6.4.3 Clinical symptoms ........................................................................127
6.4.4 Infection ........................................................................................127
6.4.5 Inflammation ................................................................................128
6.4.6 Relationship between infection and inflammation.........................131
7.4.2 Lung function
7.4.3 Infection at 3 months does not predict infection and inflammation at 12 months
7.4.4 Inflammation at 3 months is persistent at 12 months
7.4.5 Neutrophilic airway inflammation at 3 months is not associated with functional change at 3 or 12 months
7.4.6 Infection at 3 months correlates with functional change at 3 and 12 months
7.4.7 CT scores
7.4.8 Vitamin D

7.5 Discussion
7.5.1 Statement and interpretation of principal findings
7.5.2 Strengths of the study
7.5.3 Limitations of the study
7.5.4 Findings of the study in relation to other studies

7.6 Conclusions

8 Chapter 8: The role of vitamin D in airway infection and inflammation
8.1 Introduction
8.2 Hypotheses
8.3 Methods
8.3.1 Subjects
8.3.2 Clinical data collection
8.3.3 Statistical analysis
8.4 Results
8.4.1 Subjects
8.4.2 Vitamin D levels
8.4.3 Age
8.4.4 Season
8.4.5 Pancreatic status
8.4.6 Growth ..................................................................................................................................................... 177
8.4.7 Spirometry ............................................................................................................................................. 178
8.4.8 Intravenous antibiotics ......................................................................................................................... 181
8.4.9 Infection .................................................................................................................................................. 181
8.4.10 Cellular and soluble markers of inflammation .................................................................................... 186
8.4.11 Analyses with alternative vitamin D categories ................................................................................... 189
8.4.12 Antimicrobial peptides .......................................................................................................................... 189
  8.4.12.1 Antimicrobial peptides and vitamin D ............................................................................................. 190
  8.4.12.2 Antimicrobial peptides and airway infection and inflammation ....................................................... 190
  8.4.12.3 Antimicrobial peptides and lung function ...................................................................................... 190
8.5 Discussion ............................................................................................................................................. 197
  8.5.1 Statement and interpretation of principle findings ................................................................................. 197
    8.5.1.1 Overall findings .................................................................................................................................. 197
    8.5.1.2 Vitamin D level .................................................................................................................................... 197
    8.5.1.3 Antimicrobial peptides ................................................................................................................... 199
  8.5.2 Strengths of the study ............................................................................................................................ 202
  8.5.3 Weaknesses of the study .......................................................................................................................... 203
  8.5.4 Findings in relation to other studies ....................................................................................................... 205
    8.5.4.1 Vitamin D levels .................................................................................................................................. 205
    8.5.4.2 Clinical and immunological effects .................................................................................................... 207
    8.5.4.3 Bacteriology ...................................................................................................................................... 209
    8.5.4.4 Antimicrobial peptides ................................................................................................................... 211
  8.5.5 Conclusions ........................................................................................................................................... 214

Part D: Conclusions ........................................................................................................................................ 215

9 Chapter 9: Final discussion, conclusions and future work ................................................................. 216
  9.1 Principal findings .................................................................................................................................... 216
9.2 Strengths of the study ................................................................. 218
9.3 Weaknesses of the study ............................................................... 219
9.4 Future work .................................................................................. 220
  9.4.1 Vitamin D .................................................................................. 220
  9.4.2 Early inflammation .................................................................. 221
9.5 Conclusions .................................................................................. 222

10 References ..................................................................................... 223
List of Tables

Table 1: Concentrations required for LL-37 killing .................................................................28
Table 2: Summary of mutation classes ...............................................................................39
Table 3: Immune cells in CF lung disease (from (97)) ..........................................................50
Table 4: Immunomodulatory effects of 1,25(OH)D3 ...............................................................72
Table 5: Difference between clinical and research results ..................................................101
Table 6: Various cut-off values used for vitamin d deficiency .............................................103
Table 7: Results obtained from “fresh” and “old” samples” ................................................112
Table 8: The results of the serum samples assayed ..............................................................114
Table 9: Patient demographics ..........................................................................................126
Table 10: Comparison of infants with culture +ve and culture –ve BALF ............................132
Table 11: Correlation of vitamin D with antimicrobial peptides ........................................133
Table 12: Longitudinal change in lung function by infection state on BALF .........................156
Table 13: Patient demographics to compare the included patients with entire potentially included population .........................................................................................................................168
Table 14: Patient demographics ........................................................................................171
Table 15: Inflammatory markers and antimicrobial peptide levels of the 3 groups ...............173
Table 16: Nutritional health of the cf cohort by vitamin d status .........................................178
Table 17: Clinical parameters by vitamin D status ..............................................................181
Table 18: Cellular and soluble markers of inflammation of the cf cohort by bacterial culture status .................................................................188
Table 19: Cellular and soluble markers of inflammation of the cf cohort by vitamin d status .................................................................189
List of Figures

Figure 1: Proposed mechanisms of LL-37 induced antimicrobial activity ................................................................. 29
Figure 2: Flow chart to illustrate the downstream consequences of CFTR mutations. ........................................ 41
Figure 3: Electron microscope images of cilia showing a) normal cilia, b) outer dynein arm defect and c) inner dynein arm and microtubular disorganisation ................................................................................................................. 65
Figure 4: Chemical structure of vitamin D and its active metabolites ........................................................................... 69
Figure 5: Activation of vitamin D ......................................................................................................................................................... 69
Figure 6: a) Increase in LL37 mRNA following treatment with 1,25(OH)2D3 b) a decrease in bacterial colonies was seen following exposure to 1,25(OH)2D3 ........................................................................................................ 71
Figure 7: Scandanavian CF nutritional study outcomes ........................................................................................................... 83
Figure 8: 25(OH)D2 levels over time ......................................................................................................................................................... 94
Figure 9: 25(OH)D results performed on separate occasions ................................................................................................. 94
Figure 10: Bland Altman plot of agreement of 25(OH)D2 levels ................................................................................................. 95
Figure 11: Consort diagram to show determination of vitamin D samples used in the study ................................................ 102
Figure 12: Layout of luminex multiassay plate ................................................................................................................................. 103
Figure 13: HBD-2 ELISA standard curve ............................................................................................................................. 109
Figure 14: HBD-2 duplicate measurements – intra-assay reproducibility ........................................................................... 106
Figure 15: HBD-2 levels (pg/ml) of samples assayed on more than one run .................................................................................. 111
Figure 16: Comparison of serum samples assayed ..................................................................................................................... 114
Figure 17: Dilution curves of 2 samples with concentrations before and after adjustment for the dilution 111
Figure 18: Standard curves of LL-37 assay ................................................................................................................................. 113
Figure 19: Agreement between a) duplicate samples, b) standards and c) bland-altman bias plot of standards ................................................................................................................................................................................ 114
Figure 20: Pie chart of bacterial organisms isolated on BALF in NBS CF infants .......................................................... 129
Figure 21: BALF cell counts of the 3 patient groups .................................................................................................................. 130
Figure 22: Inflammation according to microbiologic status ....................................................................................................... 131
Figure 23: Vitamin D by BALF culture status .............................................................................................................................. 134
Figure 24: Consort diagram to show patients included in NBS longitudinal study .................................................. 143

Figure 25: BALF neutrophil differential at 12months according to culture status on 3 month bronchoscopy
......................................................................................................................................................................................................................... 151

Figure 26: Infection at 12months according to infection status on 3 month bronchoscopy ............................. 152

Figure 27: Inflammation at 12months according to inflammation status on 3 month bronchoscopy ............ 153

Figure 28: Lung function at 3 months of age .............................................................................................................................. 155

Figure 29: Difference in lung function at 12 months of age between infants culture positive and culture
negative at 3 months .............................................................................................................................................................................. 157

Figure 30: Vitamin D and change in lung function .......................................................................................................................... 159

Figure 31: Vitamin D levels of the 3 patient groups ............................................................................................................................ 172

Figure 32: Vitamin D level by age .......................................................................................................................................................... 175

Figure 33: Vitamin D levels in pancreatic sufficient and insufficient children ................................................. 177

Figure 34: Vitamin D and lung function ................................................................................................................................. 175

Figure 35: Vitamin D level of culture positive and culture negative patients ................................................. 183

Figure 36: Vitamin D level of Pseudomonas aeruginosa positive and negative patients ................................................. 185

Figure 37: Vitamin D level of staphylococcus aureus positive and negative patients ................................................. 185

Figure 38: BALF cellular inflammation seen in the 3 groups ............................................................................................. 187

Figure 39: Vitamin D and HBD-2 ..................................................................................................................................................... 186

Figure 40: Vitamin D and LL-37 ...................................................................................................................................................... 192

Figure 41: LL37 level in culture positive and culture negative CF patients ................................................................. 193

Figure 42: LL37 and inflammatory markers .............................................................................................................................. 195

Figure 43: LL37 and HBD-2 and FEV1 in CF patients ............................................................................................................. 196
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Annual assessments</td>
</tr>
<tr>
<td>AREST CF</td>
<td>Australian Respiratory Early Surveillance Team for CF</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFF</td>
<td>Cystic Fibrosis Foundation</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CoV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>CSLD</td>
<td>Chronic suppurrative lung disease</td>
</tr>
<tr>
<td>CXR</td>
<td>Chest x-ray</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;0.5&lt;/sub&gt;</td>
<td>Forced expiratory volume in 0.5 seconds</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FRC</td>
<td>Functional resistance capacity</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GA</td>
<td>General anaesthesia</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GORD</td>
<td>Gastro-oesophageal reflux disease</td>
</tr>
<tr>
<td>GOSH</td>
<td>Great Ormond Street Hospital</td>
</tr>
<tr>
<td>HBD-</td>
<td>Human β-defensin -</td>
</tr>
<tr>
<td>hCAP18</td>
<td>Human cationic antimicrobial protein 18</td>
</tr>
<tr>
<td>HDS-6</td>
<td>Human α-defensins 5 and 6</td>
</tr>
<tr>
<td>HNP 1-4</td>
<td>Human α-defensins 1-4</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HRCT</td>
<td>High-resolution computerised tomography</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IKK</td>
<td>IκBKinase</td>
</tr>
<tr>
<td>IL-</td>
<td>Interleukin -</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulated factors</td>
</tr>
<tr>
<td>iRT</td>
<td>Immune reactive trypsinogen</td>
</tr>
<tr>
<td>IVAB</td>
<td>Intravenous antibiotics</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LCFC</td>
<td>London Cystic Fibrosis Collaboration</td>
</tr>
<tr>
<td>LCI</td>
<td>Lung clearance index</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LL-37</td>
<td>Cathelicidin</td>
</tr>
<tr>
<td>LLD</td>
<td>Lower limit of detection</td>
</tr>
<tr>
<td>LMA</td>
<td>Laryngeal mask airway</td>
</tr>
<tr>
<td>MBW</td>
<td>Multiple-breath washout</td>
</tr>
<tr>
<td>MCC</td>
<td>Mucociliary clearance</td>
</tr>
<tr>
<td>MEF25</td>
<td>Maximal expiratory flow at 25% VC</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitres</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSD</td>
<td>Meso scale discovery</td>
</tr>
<tr>
<td>NBS</td>
<td>New born screening</td>
</tr>
<tr>
<td>NFkB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>nmol</td>
<td>Nanomoles</td>
</tr>
<tr>
<td>NTM</td>
<td>Non-tuberculous mycobacteria</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PCD</td>
<td>Primary ciliary dyskinesia</td>
</tr>
<tr>
<td>PCL</td>
<td>Periciliary layer</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognising receptors</td>
</tr>
<tr>
<td>RBH</td>
<td>Royal Brompton Hospital</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised control trial</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RVRTC</td>
<td>Raised lung volume rapid thoracoabdominal compression technique</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory leukocyte protease inhibitors</td>
</tr>
<tr>
<td>TB</td>
<td>Mycobacterium tuberculosis</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>ULD</td>
<td>Upper limit of detection</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D binding protein (VDBP)</td>
</tr>
<tr>
<td>VDRE</td>
<td>Vitamin D response elements</td>
</tr>
</tbody>
</table>
PART A:

INTRODUCTION, HYPOTHESES, AIMS AND OBJECTIVES
CHAPTER 1: INFECTION, IMMUNE RESPONSE AND INNATE IMMUNITY

1.1 Airway immunity

1.1.1 Innate immune system

The airway requires an effective immune system as over 11,000 litres of only partially filtered air enter the adult airway each day (2). Once the upper airway has been bypassed, the initial defences are expulsive coughing and the mucociliary escalator. The latter comprises motile cilia, the airway surface liquid and mucus, which combine to expel inspired pathogens and debris from the airways thereby preventing infection and airway damage.

The epithelium of the upper and lower respiratory tract is largely comprised of ciliated cells, each possessing 100-200 cilia. The cilia beat in a co-ordinated fashion in order to expel inhaled particles and pathogens. In normal individuals each cilium is made up of 9 doublet tubules arranged around a central tubule pair. The outer doublet tubules have inner and outer dynein arms and radial spokes associated with them to maintain structural integrity.

The respiratory epithelium is lined by a layer of airway surface liquid that is made up of a periciliary layer and a mucous layer. The cilia beat within the periciliary layer and, in health, the height of the cilia is approximately the same as the height of this fluid layer, providing an optimal environment for the beat pattern and mucous clearance. Results of cell culture experiments suggest that both the periciliary layer and the mucous layer travel at the same
rate, up to 20 mm/min and this movement aids the expulsion of particles from the airway (3).

If a pathogen overcomes the body’s barrier defence systems, the immune response is triggered. This can be classified into two main parts; the innate and adaptive immune systems. The innate immune system does not rely on previous exposure, and has a more generic response to invading pathogens, whilst the adaptive immune system has memory functions and has organism-specific responses, either as a result of a previous encounter or immunisation. Antibodies are produced, and if subsequent exposure occurs, the acquired immune system is able to trigger a response involving production of these specific antibodies. Because of these differences, it was previously thought that the acquired immune system was a sophisticated system, and the innate immune system crude and clumsy. However, a better understanding of the innate immune system has led to the discovery that this is not the case, that this is also an elaborate and sophisticated system, signalling in what way and for how long the adaptive immune system should respond (4).

The innate immune system can activate the adaptive immune system via a variety of pathways, and recognised pathogens also trigger the adaptive immune system directly. In health, the two systems work together in order to provide protection against invading pathogens.

As acquired immunity develops throughout life with exposure to, or vaccination against, disease older children and adults have a better system in place able to cope with a greater diversity of organisms. The innate immunity therefore plays a greater role in immunity in younger children, as this is present from birth.
1.1.2 The innate immune response

As well as providing a mechanical defence the respiratory epithelium has a major role within the innate defence system.

Organisms that are not cleared by the airway barrier defences are detected and recognised by the innate immune system via specific molecular patterns on the surface of the pathogen, its pathogen-associated-molecular patterns (PAMPs). The PAMPs are detected by pattern recognising receptors (PRRs), small proteins expressed by innate immune cells.

Pattern recognition receptors (PRR) are a diverse group of receptors that can be divided into at least five different families: the toll-like receptors (TLR), c-type leptin receptors, NOD-like receptors, RIG-like helicase receptors and scavenger receptors. Others are likely to be identified as this is an area of active research. PRRs reside in the plasma membrane and endosomal compartments, the exact location varying with the type. TLRs, which are located in the cytoplasmic membrane, are the most frequently encountered PRR in the respiratory tract. Once the PRR has become engaged by encountering its PAMP, the receptor switches from its quiescent state to an activated one. Binding to TLRs leads to the expression of various cytokines, antimicrobial peptides and proteins through activation of nuclear factor kB (NFkB) or interferon regulated factors (IRF). The exact pathway triggered will vary with the TLR to which PAMPs are bound (5, 6).

The functions of the innate immune system can be broadly classified into two categories; phagocytosis by cellular components, and pathogen destruction by soluble components. Macrophages are present in most tissues in a quiescent state and engagement of PRR
on the plasma membrane by the PAMP of an invading organism switches on genes enabling the macrophage to perform new functions. The activated macrophage is now optimised for engulfing and killing micro-organisms. As well as phagocytosis, macrophages also secrete cytokines, chemokines, complement, and lysosome and act as an antigen presenting cell to the adaptive immune system. The secreted chemokines and cytokines allow neutrophils, normally restricted to the blood, to invade tissues by increasing vascular permeability. Neutrophils aid pathogen killing by direct phagocytosis and they also produce numerous soluble immune factors including cytokines, proteases and defensins. The expressed cytokines are crucial for B-cell functioning therefore neutrophils have a role in both the innate and the adaptive immune systems. Recent evidence suggests that neutrophils are involved in the active induction of resolution of inflammation by the conversion of leukotriene B4 to lipoxin A4, which inhibits neutrophilic recruitment and their contribution to the synthesis of resolvins, lipid mediators of inflammation resolution (7).

Sustained neutrophilic inflammation causes uncontrolled release of the toxic granule contents of the neutrophil, oxidants, serine and metalloproteases (7). Therefore, it is not only important that an inflammatory response is mounted, but that it is regulated to prevent it becoming exaggerated. Failure to initiate this inflammatory reaction, to sustain it, or to halt it appropriately, can result in lung disease.
1.1.3 Antimicrobial peptides

The antimicrobial peptides are soluble innate immune factors present in the airway surface liquid that have direct and indirect actions on a variety of bacteria, viruses and fungi.

Antimicrobial peptides are comprised of between 50 -150 amino acids, are positively charged and contain both hydrophilic and hydrophobic components enabling them to be soluble both in lipid membranes and water. Numerous antimicrobial peptides have been identified in humans including larger proteins such as lysozyme, lactoferrin and cathepsin G and smaller peptides such as secretory leukocyte protease inhibitors (SLPI), RNase 7, defensins and cathelicidins.

1.1.4 Antimicrobial peptides in relation to vitamin D

Previous work has found that the promoter regions of the genes for two of these peptides, human-β-defensin -2 (HBD-2) and cathelicidin (LL-37) contain vitamin D response elements (VDRE) suggesting a role for vitamin D in their regulation and for this reason these are the two peptides focussed on in the work of this thesis.

1.1.5 Defensins

First identified in 1985 (8) defensins have now been divided into 3 categories, alpha, beta and theta; however the latter have not been found in humans (9) and so will not be discussed here. All peptides within the family have a similar role but there are
specificities of structure, function and biological properties within each group making it unique.

Defensins are antimicrobial peptides active against a wide range of pathogens including gram positive and gram negative bacteria, fungi and enveloped and non-enveloped viruses (5). The mechanism of action is not fully understood but it appears that defensins alter the permeability of the invading pathogen cell membrane and have both bactericidal and bacteriostatic effects (2). For viral killing, there is either a direct effect against the virus itself by disruption of the envelope or interaction with the glycoproteins at the virus cell surface inhibiting replication; or modulatory effects on the target of the virus increasing epithelial cell expression of defensin mRNA and acting as a chemotactic agent (10). The antimicrobial properties are greatly affected by the environment and the activity of all the host defence peptides is greatest in environments of low ionic strength. The activity of human β-defensin -1(HBD-1) and to lesser extent HBD-2 is reduced in the presence of sodium ions meaning a far higher concentration of the peptide is required for killing ability; magnesium and calcium ions have a detrimental effect on the antimicrobial activity of α-defensins. Such environments are commonly seen in-vivo in healthy subjects raising questions about the physiological relevance of these peptides, which reside in environments sub-optimal for their antimicrobial activity. The activity of these peptides is also affected by a reduced pH, and a reduction in airway surface liquid (ASL) pH from 8 to 6.8 rendered HBD-3 ineffective to Staphylococcus aureus (S. aureus) and the synergistic effects of various antimicrobial peptides are also reduced by a decrease in the environmental pH (11). It has been shown that in addition to their direct antimicrobial action, they have immunomodulatory effects
The number of α and β defensins varies between species; there are currently 6 α and 4 β defensins identified in humans (12). The human α-defensins (HNP1-4) are expressed mainly in neutrophils, where they are stored in the azurophil granules (4, 13), although they are also produced to a lesser extent by eosinophils. Macrophages are able to store α-defensin, and in addition to storage, they acquire more by ingestion of apoptotic neutrophils. If *Mycobacterium tuberculosis* (TB) and α-defensins are both ingested by a macrophage, they are initially transported to the early endosome where the defensin contributes to inhibition of mycobacterial growth (14). Alpha defensins are chemotactic, stimulate IL-8 production by human bronchial epithelial cells and cause aggregation of bacteria and viruses to facilitate phagocytosis by macrophages and neutrophils. Alpha defensins 5 and 6 (HD5-6) are expressed only in the small intestine by Paneth cells (2, 10, 12).

The β-defensins are widely expressed in epithelial cells, including those lining the respiratory tract (8). HBDs are secreted onto the cell surface in their mature form and are thereby active at the time of secretion (14). HBD-1 is constitutively expressed whilst HBD-2, 3 and 4 are up-regulated in response to infection or inflammatory stimuli. HBD-2 is most highly expressed in lungs, HBD-3 in skin and tonsils and HBD-4 in the testes and stomach. Although it is known that HBD-2 is expressed by the respiratory epithelium in the proximal airways, the epithelial expression and level of defensins at different airway generations has not been evaluated (14). HBD-2 is chemotactic and causes migration of other inflammatory cells including neutrophils (thereby increasing the levels of neutrophil-derived α-defensins and cathelicidin), and causing mast cells to migrate and to become activated (9, 14, 15).
1.1.6 Cathelicidin

Cathelicidin is secreted by neutrophils, macrophages and epithelial cells including those of the respiratory tract (14) and has a similar role to defensins in lung immunity (8). There is considerable variation in structure amongst cathelicidins but they are grouped together because they are all stored as inactive prepropeptides, with a signal residue, an N-terminus peptide and then a smaller, highly variable C-terminus peptide (16-18). The structural heterogeneity between cathelicidins also translates into variable activity of the peptides, which have differing functions and mechanisms of action. Cathelicidins have now been identified in chicken and fish, as well as in all mammals in which they have been sought. Their presence in hag fish suggests that they have been conserved through evolution (17, 19). Although many different cathelicidins exist in nature, only one is found in humans, human cationic antimicrobial protein 18 (hCAP18). This cathelicidin has an alpha-helical structure (18, 19), which is the most common structure seen in this family, and which is also seen in cows, monkeys, mice, rabbits, guinea pigs, sheep, pigs and horses (16, 20). As with all cathelicidins, the human cathelicidin, hCAP18, is stored intracellularly as the inactive prepeptide. Once secreted from the cell, the C-terminus is cleaved by the enzyme protease 3. The cleaved product consists of 37 amino acids, the first 2 of which are leucine (21), hence its name LL-37. The terms hCAP18 and LL-37 are sometimes used interchangeably, but in this thesis, hCAP-18 refers, more accurately, to the inactive prepeptide and LL-37 the smaller, active peptide. Alpha-helical cathelicidins are active against a wide range of pathogens including gram positive and negative bacteria, fungi, parasites and enveloped viruses (16, 18, 20). The concentration required for killing varies between pathogens.
(Table 1) with salmonella being killed at relatively low concentrations, whereas

*Streptococcus* group B, *E. coli* and *Pseudomonas aeruginosa* require a much higher concentration (17).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Concentration required for killing (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmonella</td>
<td>2.8 – 6</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>16</td>
</tr>
<tr>
<td><em>E. Coli</em></td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>Streptococcus group B</em></td>
<td>&gt;32</td>
</tr>
</tbody>
</table>

Table 1: Concentrations required for LL-37 killing

LL-37 activity against two viruses has been investigated: it has minimal activity against herpes simplex but does reduce replication of vaccinia virus (17). Activity of LL-37 is very much affected by its environment (22) with its antimicrobial activities being reduced by the presence of plasma, and by sodium, magnesium and calcium ions (19). However investigators disagree as to the extent of the reduction. Some studies found that activity in high salt environments is reduced, but less so than the reduction in activity of other antimicrobial peptides (17) whilst other studies found no demonstrable antimicrobial activity at all in normal physiological conditions (23). Its activity has been found to be reduced by apolipoprotiens and by low environmental pH (12).

LL-37 expression is up-regulated by certain bacteria, but conversely, the peptide is broken down by proteases produced by some bacteria, including *Pseudomonas aeruginosa* (*P. aeruginosa*) (16, 18). LL-37 has been shown to alter membrane permeability and different mechanisms are proposed, but the exact mechanism is unclear. The amphipathicity of
LL-37 is important in its action of membrane disruption and the three main proposed mechanisms are barrel-stave, carpet and toroidal pore models (18, 19).

Figure 1: Proposed mechanisms of LL-37 induced antimicrobial activity (18).
LL-37 may form cylindrical pores (B); it may coat the surface of the cell until the cell membrane dissolves (C); or it may coat the cell surface, until curvature of the cell membrane occurs and pores form.

One postulated mechanism is that LL-37 attaches perpendicular to the membrane and forms large cylindrical pores therein (Figure 1B). Alternatively, and more likely, it coats the surface until a critical concentration is reached when it acts to either dissolve the membrane (Figure 1C) or causes curvatures that form toroidal pores (Figure 1D).

In addition to antimicrobial activity, as with defensins, LL-37 exhibits immunomodulatory properties (8, 12, 14, 19) and these properties appear less influenced by the environment (23). These properties include chemoattraction, stimulation of gene expression of
various cytokines and chemokines and altering bacterial signalling by binding to LPS disabling its usual inflammatory trigger (19).
CHAPTER 2: CYSTIC FIBROSIS

2.1 CF – clinical disease

2.1.1 Clinical features of CF

CF is a multi-system disease and whilst lung disease accounts for the majority of morbidity in CF, and respiratory failure is the cause of death in over 90% of CF patients, there is also significant pancreatic, liver and gastrointestinal (GI) involvement and infertility in the majority of males. Lung involvement occurs from an early age with intermittent then chronic bacterial infection, inflammation and eventual bronchiectasis, fibrosis and death from respiratory failure. Infection is frequently seen in the airways of people with CF (24) with particular susceptibility to certain bacteria including \textit{S. aureus}, \textit{P. aeruginosa} and \textit{Burkholderia cepacia (B.cepacia)}; the use of molecular detection techniques has revealed much more diverse bacterial populations present in the lower airway than hitherto suspected (more details in section 2.3). The organisms most commonly isolated change with age; in infancy and in the pre-school years, \textit{S. aureus} and \textit{Haemophilus influenzae (H. influenzae)} are most commonly cultured with a tendency for these organisms to be replaced by \textit{P. aeruginosa} by the teenage years (25), up to 80% of adults having isolated this pathogen. Chronic \textit{P. aeruginosa} is associated with a worse outcome, as are \textit{B. cenocepacia}, \textit{Achromobacter xylosoxidans} (24), and certain non-tuberculous mycobacteria (NTMs), particularly \textit{Mycobacterium abscessus}. There remains debate as to whether MRSA is associated with worse clinical outcomes (26) but recent data shows that MRSA colonisation in CF adolescents correlated with worse pulmonary function (27). It was recognised in
the 1990s that cross-infection occurred within CF centres (28-31) and subsequently segregation and isolation was introduced and is now a standard of care in UK CF centres (32).

In the pancreas, cystic fibrosis transmembrane regulator (CFTR) is predominantly located at the apical membrane of the cells that line the small pancreatic ducts. Defective CFTR leads to mucus plug formation leading to pancreatic injury, the consequence of which is failure of secretion of pancreatic enzymes and bicarbonate to the GI tract and therefore an inability to absorb fat manifested by steatorrhoea and failure to gain weight. Most individuals with CF (85%) are pancreatic insufficient from birth whilst pancreatic endocrine dysfunction commonly occurs later in life at a median of 20 years. Affected individuals develop CF related diabetes and this occurs in approximately one third of CF patients.

2.1.2 Diagnosis of CF

CF can be detected on screening in the newborn period by the detection of elevated immune reactive trypsin (iRT) in a routinely collected blood spot, usually combined with genetic testing (33). IRT is elevated at birth in individuals with CF and remains so for approximately 8 weeks. The elevation of this protein in the blood was first described in 1979 (34) and screening was introduced shortly after this in some parts of the world including Australia, France and in the UK in East Anglia but it was not until 30 years later, in 2007, that screening became routine in the whole of the UK (35-37). Following this screening test, infants deemed to have a likely diagnosis of CF are referred for sweat testing and clinical
review at a CF centre. The iRT assay is a screening, rather than diagnostic, test and false negatives and false positives will occur; there is thought to be an approximately 3-6% false negative rate (38-41). In order to minimise such errors, most centres employ a 3 or 4 step process testing a combination of iRT and DNA mutation analysis (33). The experience of East Anglia after 30 years of screening was that incorporating DNA mutation analysis in to the screening process improved the positive predictive value from 67 to 86% (38). The children missed on new born screening (NBS), and children born prior to 2007, are diagnosed following clinical suspicion from symptoms including cough, respiratory tract infections and failure to thrive.

Once referred for testing, either through NBS detection or on clinical suspicion, a sweat test is performed. This allows measurement of the sweat chloride concentration and a diagnosis of CF is made with a sweat chloride of ≥ 60 mmol/L in a properly performed sweat test (42). Sweat chloride levels <30 are considered normal and ≥30 - <60 are borderline and further testing in the way of genotyping (42, 43), gene sequencing (44) or nasal potential difference measurements (45) are recommended (43).

2.1.3 Introduction of new born screening in UK

Screening started in East Anglia and Trent in 1980s (38, 46) and in Wales in 1996 whilst the rest of the UK began screening much later with routine nationwide screening occurring from October 2007. The reason for this delay was the lack of evidence to support a better outcome with screening. The diagnosis of CF is made at a younger age with screening
than diagnosis on clinical presentation; median age 1 month versus 6 months in the UK and 2 weeks versus 14 months in the US (47). Whilst it is clear that screening does lead to earlier diagnosis, whether this translated to a clinical benefit was undetermined. With this lack of certainty it was argued that screening might offer no advantage over clinical diagnosis and one study found worse clinical respiratory outcomes in screened babies compared with those diagnosed later (48).

Shortly after the turn of the century, more evidence was emerging suggesting there was a clinical benefit (49-53) and following this screening became routine although there is no universally agreed system and the screening testing protocols vary between countries (54).

### 2.1.4 Benefits of newborn screening

As discussed above, whether earlier diagnosis was beneficial was initially unclear. The evidence is growing that there are improved respiratory outcomes in the paediatric population but the strongest evidence relates to nutritional data, which has highlighted the importance of improved nutritional state after NBS.

One of the benefits of screening is thought be the early identification of infants with disease in order to start treatments before the onset of symptoms and airway inflammation and subsequent airway destructive changes. However, in one cohort of 170 screened infants, 48% were already displaying some symptoms by the time of diagnosis at a median age of 3 weeks (39) and 2 other large cohorts found presence of airflow obstruction at 3 months of
The Wisconsin randomised controlled trial of screening found more *P. aeruginosa* in their screened infants compared with those diagnosed clinically (48). This increased infection rate was thought to be due to cross infection with children with established CF in one of their centres and would therefore expect to be abolished by segregation. This study did not find any pulmonary advantage of screening by the age of 6 years, and this was thought to be due to the increased *P. aeruginosa* rate in this group. Not all centres found more *P. aeruginosa* in the screened infants; a study in UK found less *P. aeruginosa* in the screened infants (57) and an Australian study found less bacteria in the airways of those diagnosed on newborn screening (58). The earlier acquisition of *P. aeruginosa* in the non-screened infants in these studies was found to correlate with a poorer clinical outcome with lower lung function, less good nutritional status and poorer survival (59). Other centres found no difference in *P. aeruginosa* rates between their screened and non-screened cohorts (60, 61).

Evaluation of the CF patients in Australia, who have been screening for CF for over 20 years, found that adolescents who had been diagnosed on screening had a better forced expiratory volume in 1 second (FEV\(_1\)) and forced vital capacity (FVC) at transition to the adult clinic than their clinically diagnosed counterparts (59).

In terms of structural lung damage, an Australian study found better chest x-ray (CXR) scores in the screened cohort (59). Worse CXR scores were seen in follow-up chest radiographs in the Wisconsin cohort in the screened cohort (48), but scores correlated with *P. aeruginosa* infection rate, which as discussed, was higher in the screened infants most
likely due to cross-infection within clinics.

The most convincing benefit for screening comes from nutritional data. Studies have shown that screening leads to better early nutrition, as treatments, including pancreatic enzyme replacement treatment, are started earlier. Unsurprisingly, screened infants had a higher height and weight percentage compared with those diagnosed clinically, as less time had passed prior to introduction of treatments during which they risk becoming malnourished. What was more surprising was that this difference persisted and was present on analysis of nutritional status at the age of 10 years (51) and at time of transition to adult care (59).

The most recent data from the Wisconsin study examined clinical outcomes of the individuals, now aged 20 years old and found that the infants with better nutrition in early life had better clinical status at 20 years of age (62). The importance of nutritional status was also highlighted in a recent prospective observational study of the US CF registry patients born 1989-1992 (63). This identified that nutritional status at age 4 has an impact on health at age 18 years with weight-for-age percentile predicting lung function in later life. Those with the highest FEV\textsubscript{1} at 18 years of age had a weight-for-age percentile >50\textsuperscript{th} centile at 4 years of age, and mortality in this group was also lower. Despite this survival and lung function advantage there was no difference in \textit{P. aeruginosa} acquisition between the weight-for-age percentile groups.

The screened infants, with better nutritional status, were also found to have better health related quality of life in their teenage years (64). Better nutrition would be expected to result in a better quality of life, which would hopefully impact on general well being.
Screening of infants with CF leads to earlier diagnosis and the ability to introduce treatments from early on before significant symptoms have occurred, and there is also evidence of better survival. A significant improvement in survival was seen in the Netherlands with screening; survival at age 11 years increased from 65% the previous cohort of non-screened children to 94% in the screened cohort. Clearly treatments overall have improved since publication of this paper in 1995, and we would not expect a survival of just 65% in non-screened children now, but nonetheless it showed that, at that time, screening offered a clinical advantage. More recent survival outcome data come from the Australian cohort. There was no survival advantage of screening at transition from paediatric to adult care, but by the age of 25 years, there was a difference in survival of 20% between screened and non-screened individuals (59).

One big problem with such studies is the methodology used. There is only one randomised control trial (RCT) of screening (51) and all the others have inferior methodology and pre- and post- screening results are complicated by better treatments available with time. This important factor should be remembered when considering the results of pre and post screening comparative studies.
2.2 Cystic fibrosis - pathophysiology

2.2.1 CF genetic mutations

CF occurs in the presence of two CF disease causing mutations and is inherited as an autosomal recessive disease with a prevalence of approximately 1:2,500 and a carrier rate of around 1:25 in the UK Caucasian population. It is estimated that there are approximately 60,000-70,000 people with CF worldwide (65). It is caused by a defect in the CFTR gene located on the long arm of chromosome 7. In 1985 the gene that caused this disease was localised to the long arm of chromosome 7, position 7q21-24 (66), with the sequence being fully identified in 1989 (67). This gene encodes the CFTR protein, which in its normal, wild-type form is made up of 1480 amino acids (68).

Over 2000 mutations within the CFTR gene have been described but less than 200 are known to be disease-causing (69); some mutations still produce a functional protein and are probably merely benign polymorphisms. Disease causing mutations can be divided into 6 classes according to the way in which the resulting protein is defective, described in Table 2.
<table>
<thead>
<tr>
<th>Mutation class</th>
<th>Basic defect</th>
<th>CFTR protein abnormality</th>
<th>Examples</th>
<th>Potential treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Non-sense mutations</td>
<td>Premature termination codon (PTC) leads to a truncated protein with no function.</td>
<td>G542X, W1282X</td>
<td>Drugs aiming to read-through the PTC are in development and clinical trials but to date results have been disappointing (70–72).</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Gly542X, Trp1282X</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Trafficking defect</td>
<td>Misfolding causes the protein to fail to traffic to the apical surface of the cell</td>
<td>ΔF508, N1303K</td>
<td>CFTR correctors have been developed and one, Lumacaftor stabilises the CFTR protein to allow transport to the cell membrane. Orkambi®; a combination of Lumacaftor and the CF potentiator ‘Ivacaftor (see below) has now been licensed in the USA. (73, 74)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phe508del, Asn1303Lys</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Gating defect</td>
<td>Normal quantities of CFTR protein reach the cell membrane but remain closed preventing normal function.</td>
<td>G551D</td>
<td>Ivacaftor; CFTR potentiator allowing opening the gate to improve function. Now licensed in USA and Europe those with all class 3 mutations and ≥2years of age s (75, 76)</td>
</tr>
<tr>
<td>IV</td>
<td>Decreased conductivity</td>
<td>Protein reaches the apical surface but conductance across the channel is poor due to the abnormal conformation</td>
<td>R117H</td>
<td>Ivacaftor; some evidence that function is improved by Ivacaftor in this class with improvement in sweat chloride and improved lung function in some adult patients, but not children(77). Approval has been granted in USA and being sought in Europe.</td>
</tr>
<tr>
<td>V</td>
<td>Splicing defect</td>
<td>Decreased amounts of CFTR reach the apical membrane. The CFTR that does, probably functions normally; hence, many patients with these mutations have a milder phenotype</td>
<td>38949+10kbC&gt;T</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>Decreased Half-life</td>
<td>CFTR is functional but half-life is decreased leading to overall decreased amount of CFTR present at the cell surface and may impair regulation of other channels</td>
<td>Q1412X, Gln1412X</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Summary of mutation classes
2.2.2 CFTR mutation classes

Class I mutations lead to a premature ‘stop’ in mRNA transcription and a short, non-functioning protein, which is immediately destroyed. Those of class II encode a structurally abnormal, misfolded, protein that is degraded by the endoplasmic reticulum before reaching the cell surface. Protein of classes III to VI reach the cell surface but fail to function appropriately: Class III mutations have decreased activation of the channel and remain closed and class IV mutations cause decreased conductance of ions across the channel. Class V mutations are usually splice site mutations which lead to a reduced amount of CFTR at the cell surface, so some function occurs but at a reduced level. Class VI mutations lead to a shortened half-life due to protein instability and may also impair the regulation of neighbouring channels by CFTR. Whilst these 6 classes have been well described it is not always clear-cut as to which class a mutation belongs to and classes are not mutually exclusive; for example F508del is described a class II mutation but also has class III and class VI properties.

2.2.3 CF pathophysiology

In normal health, the CFTR protein crosses the cytoplasm to the cell membrane where it functions, the best-described function being as a chloride channel, with inhibitory effects on its neighbouring epithelial sodium channel, ENaC. It is therefore critical in chloride and sodium transport across the cell membrane and as a consequence, regulates the hydration of the epithelial surface (water following sodium down its osmotic gradient). Bicarbonate secretion is also impaired and the abnormal pH of the cell surfaces is thought to cause
dysregulation of innate defence molecules within this environment. Thus, defective CFTR protein leads to absent or decreased chloride secretion, increased sodium and water absorption and airway surface liquid depletion. This results in the classical manifestations of CF: infection, inflammation and eventual bronchiectasis (Figure 2).

Figure 2: Flow chart to illustrate the downstream consequences of CFTR mutations.
The eventual outcome is on-going airway infection and inflammation leading to airway obstruction and ultimately respiratory failure (78) but the exact reason as to why increased infections and inflammation occur is not completely understood and studies provide conflicting results. Excessive inflammation and altered immune signalling occur, but whether this due to an innate immune defect, altered ion transport, the consequence of a dehydrated airway, defective mucociliary clearance or a combination of these remains debated.

2.2.4 High-salt model

One theory as to how the abnormal CFTR protein leads to abnormal airway function is the high salt hypothesis (79), although this is not currently a commonly held view. It was suggested that normal epithelial cells with normal CFTR produce a low salt solution environment optimising the efficacy of the activity of the antimicrobial peptides (80, 81). Some, although not all, antimicrobial peptides are salt sensitive and function less effectively, or not at all, in a high salt environment. Because the CF respiratory epithelium cells exhibit increased sodium chloride transport due to the defective CFTR, there is an assumption that the airway surface liquid will be hypertonic thereby rendering these peptides less active. This assumed hypertonicity of the airway surface liquid has been difficult to prove and in fact, most studies have found it to be isotonic (82).
2.2.5 Bicarbonate and pH

Defence molecules within the airway are affected by the acidity of the environment and so alterations in the composition of the airway surface liquid that alter pH may play a role the functioning of the immune system. Cell studies found that altering the pH of ASL from 8 to 6.8 decreased the ability of LL-37 to kill both *S. aureus* and *P. aeruginosa* and decreased the synergism between the antimicrobial peptides (11). The pH of nasal ASL has been shown to be more acidic in CF infants than in non-CF infants suggesting this may play a role in early immunity; this difference was not seen in older children and adults (83) making it unclear if it has relevance to human disease.

The CF pig develops lung disease very similar to that seen in humans, with inflammation, airway obstruction and mucous plugging. Whilst these features are not present at birth there is evidence of airway disease very early on (84) and evidence of an innate immune defect with reduced bacterial killing of *H. Influenza*, *P. aeruginosa* and *S. aureus* by airway surface liquid as compared to that from non-CF pigs (84). A series of experiments isolating different components immune function found that the reduced bacterial killing was due to altered functioning of the antimicrobial peptides, despite these being present at normal concentrations. The authors found that the ASL was more acidic in the CF pigs than the non-CF pigs. By decreasing the acidity of the ASL of the CF pigs, antimicrobial activity and thus bacterial killing was improved; and similarly, antimicrobial bacterial killing was reduced in the non-CF pigs by decreasing the environmental pH (85). This finding implies that there is normal production of the innate defence peptides, but their function is reduced within the environment of the CF airway, at least in the pig.
2.2.6 Defective mucociliary clearance

2.2.6.1 Overall view

Mucociliary clearance (MCC) appears to be the primary form of innate defence of the respiratory tract against bacteria, and effective MCC relies upon the cilia being intact and having normal structure and function, the depth and composition of the periciliary fluid layer to be optimal and mucous rheology to be within the normal range. Defective MCC can occur as a consequence of a decreased periciliary layer in which the cilia reside or due a primary defect in the action of MCC itself rather than a consequence of ASL dehydration. Although it is generally accepted that MCC is impaired, there is some disagreement amongst studies evaluating MCC in CF individuals with reports of increased, decreased and similar clearance to healthy individuals (82, 86). It has been suggested that differences in methodology may account for the differences described.

2.2.6.2 Low fluid leading to impaired MCC

A commonly held view is that effective MCC is impaired as a consequence of abnormal sodium, chloride and water transport arising from the defective function of the CFTR protein in the apical cell membrane (87). It has been shown that the height of the periciliary layer on the respiratory epithelial cells is dramatically reduced in CF and the effect of this is a predisposition to accumulation of debris from decreased pathogen clearance and the increased mucous layer provides excellent nutrition for invading organisms to thrive promoting bacterial growth (82, 88).

There is considerable evidence to support this theory including cell-culture studies, and animal and human studies. Cell culture experiments identified that 7μm is the optimal
height for cilia functioning and that in CF, the height of this layer is just 3 μm, with this fluid appearing to be present but trapped by collapsed cilia. In addition, such studies have found that mucus in CF is much denser with a much higher concentration of solids than the 2% seen in the mucus secreted by normal epithelial cells (87, 89). A study of MCC in CF airway cell cultures found that MCC decreased secondary to loss of periciliary volume and could be restored by replacement of water (86) suggesting depleted ASL the cause of the impaired MCC.

Whilst CF mice display little in the way of lung disease, likely due to the absence of significant CFTR in the airway of wild type mice with sodium and chloride secretion dependent upon other mechanisms, ENaC overexpressing mice do display lung disease akin to that seen in CF; decreased airway surface liquid, increased mucus concentration, mucus obstruction, goblet –cell hyperplasia and neutrophilic infiltration. This suggests that the low ASL volume is responsible for producing the much of the CF lung disease and illustrates the importance of ASL depletion in CF pathophysiology (87).

Findings of studies evaluating the effects on 7% hypertonic saline in CF patients support the airway hydration hypothesis in that the addition of the salt solution lead to improved MCC, a clinical improvement and reduced bacterial load, whereas one would expect a deterioration with the high-salt theory upon increasing the salt concentration further (90, 91).

MCC with decreased ASL appears to be slowed in-vivo, but static in-vitro, suggesting that other factors play a part in the human airway that do not translate to cell-culture studies. It has been demonstrated that sheer forces double the ATP release, which in turn increase
MCC and shear forces are increased by exercise, ventilation and motion (87). Many of these cell culture experiments were done in static cultures and further work (discussed below) suggests that a 2\textsuperscript{nd} hit is needed for these effects to be seen in-vivo.

2.2.6.3 MCC independent of airway surface liquid

An alternative view is that MCC is independent of ASL volume and this hypothesis will be discussed here in brief. It is uncertain whether the MCC is abnormal from birth, contributing to lung disease, or if it becomes abnormal due to the disease course, which then accelerates disease progression. CF mouse studies found tracheal MCC rates at birth to be half of that in wild type mice implying MCC is decreased prior to infection (92). In-vivo assessment of CF piglets was performed using radioactive particles to assess MCC at birth. The authors of this study concluded that MCC was normal at birth but became less effective with metacholine challenge, contrary to the expected response and as is seen in the non-CF piglets. In addition, the study found that MCC was impaired even when methods were used to preserve the periciliary layer, therefore concluding that reduced effectiveness of MCC was due to abnormalities of sub-mucous gland function and not to surface epithelial function (93).

2.2.6.4 Mucus rheology

Altered mucus rheology may also have an impact on MCC. In the CF piglet model, it was found that mucus rheology was abnormal even before it reached the airway, therefore precluding airway dehydration causing loss of mucus water as a cause of the dense mucus seen in CF (93). These results suggest that MCC is normal at birth but function is reduced in response to stimuli. The findings of this study were supported by another study evaluating
normal and CF-pig tracheas which found abnormally viscous mucous as an inherent defect and reduced MCC not related to periciliary layer (PCL) depth (94).

2.2.6.5 Second viral hit

It is hypothesised that MCC may initially be normal but a second hit from exposure to a virus can cause impaired cilia function. The presence of viruses within the airway in a clinical environment also appears to have a role to play in the differences seen between the in-vivo and in-vitro environments. Cell-culture experiments have shown that RSV infection decreases the PCL as a direct consequence (87, 95). This suggests that a viral hit to the CF airways leads to a depletion in the ASL predisposing to bacterial infection. It may therefore be that ASL is sufficient for MCC under normal circumstances but exposure to viruses alters this and leads to bacterial infections and the intermittent exacerbations seen in the clinical course of the disease.

2.2.7 Innate pro-inflammation and innate immune defect theory

2.2.7.1 Overall view

There are four proposed models as to how inflammation occurs within the CF airway, which are not mutually exclusive;

1. Abnormal signalling initiates inflammation in the absence of infection

2. Inflammation occurs only secondary to infection encountered in the airway

3. Inflammation occurs secondary to infection but in an excessive fashion
4. There is a failure to resolve inflammation that has occurred in response to infection.

Inflammation occurs as a protective mechanism as part of host defence and so can be argued that it a positive, not a negative factor within the airways. Certainly a degree of inflammation may be beneficial but once it becomes excessive or not switched off appropriately, this balance is tipped and it becomes detrimental with destructive effects of the integrity of the airway wall (96).

CFTR is located within the apical membrane of the surface epithelium, and as this is an area housing many innate defence molecules, the link between CF and innate immunity has been the subject of much research (97).

Altered signalling pathways with abnormal initiation of inflammation along with an exaggerated and prolonged inflammatory response (98, 99) have been described suggesting a predisposition towards an inflammatory state within the CF airway. No unifying pathway connecting these defective inflammatory response pathways to defective CFTR has been identified but the combination of these abnormalities may help to explain the inflammatory phenotype seen in CF.

TLR2 is the receptor most predominantly expressed on the cell surface in the airway; it is up-regulated by bacterial stimulation and this response is reduced in people with CF (97, 100) compared to those without CF suggesting a defect in the innate immune system and leading to reduced effectiveness of pathogen clearance.

It was thought that CFTR may have a role in clearance of *P. aeruginosa* and the defective channel altered this ability but more recent studies have shown that this organism is
predominantly found in the mucous layer and not directly at the cell surface making a direct role of CFTR in *P. aeruginosa* clearance less likely (82).

It is debated if neutrophil dysfunction in CF is due to chronic inflammation or to expression of CFTR in immune cells leading to impaired function (Table 3).

Recent studies of the CF ferret have found reduced antimicrobial activity against *P. aeruginosa*, reduced MCC by 7 days of age and also altered inflammatory cytokines and signalling pathways in sterile newborn lungs suggesting that more than one defect is responsible for the lung disease seen and that CF ferrets have defects in both innate immunity and in their inflammatory signalling (101).

It is likely that there is not one explanation for the CF lung disease seen in humans, but a combination of defects culminating in the phenotypic picture seen.
<table>
<thead>
<tr>
<th>Immune cell</th>
<th>Functional role</th>
<th>Impairment in CF lung disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils</td>
<td>Anti-bacterial and -fungal host defense</td>
<td>• Impaired chlorination/intracellular killing of phagocytosed bacteria</td>
</tr>
<tr>
<td></td>
<td>Cytokine/chemokine release</td>
<td>• Delayed apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Proteolytic and oxidative tissue damage</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cleavage of leukocyte surface receptors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• DNA release (necrosis/NETosis)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Increased elastase release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Enhanced chemotaxis</td>
</tr>
<tr>
<td>Macrophages/</td>
<td>Anti-bacterial and -fungal host defense</td>
<td>• Removal of apoptotic cells (efferocytosis)</td>
</tr>
<tr>
<td>monocytes</td>
<td></td>
<td>• Antigen-presentation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cytokine/chemokine release</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Immune regulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Increased secretion of pro-inflammatory cytokines</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Disturbed ceramide metabolism</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Dysregulated TLR4 trafficking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Downregulated autophagy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Macrophages: hyper-responsiveness towards LPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Monocytes: downregulation of TREM/hyporesponsiveness to LPS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Reduced expression of scavenger receptors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Cleavage of phosphatidylserine receptors/impaired efferocytosis</td>
</tr>
<tr>
<td>T cells</td>
<td>Anti-bacterial, -Fungal and -viral host defense</td>
<td>• CFTR-mediated Th2 shift</td>
</tr>
<tr>
<td></td>
<td>Antibody production</td>
<td>• P. aeruginosa-mediated Th2 shift</td>
</tr>
<tr>
<td></td>
<td>Neutrophil recruitment (Th17)</td>
<td>• Th17 activation</td>
</tr>
<tr>
<td></td>
<td>Allergic responses (ABPA, Th2)</td>
<td>• P. aeruginosa-mediated impairment of T cell proliferation</td>
</tr>
<tr>
<td></td>
<td>Immune regulation (Tregs)</td>
<td></td>
</tr>
</tbody>
</table>
Abnormal initiation of inflammation

It has been argued that this theory is supported by the presence of inflammation that has been reported in infants with CF, prior to detected infection or symptoms (56, 102-105). This finding is not universal, however, and other studies report that inflammation is absent if infection is not detected (106). Some soluble inflammatory markers, the cytokines IL-8 and TNF-α, have also been reported to be higher in bronchoalveolar lavage fluid (BALF) of infants with CF even in the absence of detected pathogens, and in the presence of pathogens the response was found to be exaggerated (103, 104, 107). However, although infection was not detected in the BALF samples, this was taken at a single time point and therefore earlier infection with persisting and prolonged inflammation could account for this. Alternatively, infection could be present but not detected as studies have found that whilst inflammation may be seen throughout airways, bacterial organisms may not be found in all lobes and if lavage taken from just one or two lobes as is standard practice (108), the presence of infection may be missed (109, 110). In addition, samples are taken whilst the person is on antibiotics in some cases in which case the bacteria may not grow on the cell culture plate even if present.

NFκB has a central role as a regulator of downstream inflammation including regulation of IL-8, GM-CSF, IL-1β and IL-6. Animal experiments and cell culture studies have shown that CF cells have an increase in reactive oxygen species, which have a role in NFκB activation, leading to an increase in the pro-inflammatory cytokines suggesting a non-infective inflammatory response (111, 112). Whilst NFκB appears to be normal in CF cells, this transcription factor is released from its precursor IκB during degradation. Degradation of IκB
occurs under the influence of IκBKinase (IKK), which has been found to be increased in CF cells.

2.2.7.3 Exaggerated inflammatory response

Recent work has suggested that the adaptive immune system may play a role in inflammation, with increased Th17 signalling and consequent increased expression of IL-17 being seen in patients with CF (113-116). IL-17 is thought to be important in the recruitment of neutrophils to the airways in response to infections, thereby increasing the neutrophilic inflammation within the CF airway. The inflammatory response that is seen may therefore be appropriately initiated but due in part to this increase in IL-17, the response heightened.

In-vitro studies using respiratory epithelial cells have show heightened inflammatory responses in the absence of CFTR and that the CFTR function correlates with basal and inducible inflammatory responses (117, 118). These cell studies also show that the inflammatory response is heightened in cells treated with chemical inhibitors of CFTR, and conversely a decreased inflammatory response is seen following treatment of the cells with CFTR potentiators – small molecules that have been developed to bind to CFTR at the cell surface and increase its gating potential.

2.2.7.4 Prolonged response/failure of resolution

Once the inflammatory signalling event is no longer present, resolution of inflammation must occur to prevent unnecessary on-going recruitment of inflammatory cells and without appropriate active mechanisms to do the inflammation persists leading to tissue destruction. Resolution of infection is now thought of as an active process, not merely the result of an absence of a pro-inflammatory drive. Neutrophils and macrophages express
CFTR giving rise to the suggestion that CF neutrophils have abnormal function and this has also been demonstrated in genetic studies evaluating the possibility of polymorphisms of CF modifier genes (119). They release less of the antimicrobial peptides LL-37 and lactoferrin and the proteinase MMP-9, which has role within the regulation of neutrophil activity. Correcting the CFTR protein defect corrects the expression of these components by neutrophils suggesting an innate immune defect that can be corrected (120).

IL-10 is generally considered to be an anti-inflammatory cytokine with a role in resolution of inflammation and which acts in part by decreased activation of NFκB through IκB preservation and has been found to be decreased in the CF airway (98, 99, 111). This reduction in IL-10 may be an important factor in the heightened and prolonged inflammatory response that has frequently been described. Lipoxin A4, which also has a role in resolution of inflammation decreasing IL-8 expression and arresting neutrophilic inflammation, is decreased in people with CF (121).

In-vitro cell culture studies have not conclusively shown an exaggerated inflammatory response and whether there is a constitutional CFTR related innate immune defect in remains unclear (122).

### 2.2.8 Antimicrobial peptides in the CF airway

Studies have reported that levels of BALF HBD-2 and LL37 are increased in CF as compared to healthy controls; levels increase in the presence of infection and HBD-2 is undetectable in health (123, 124). A weak association has been reported between these antimicrobial
peptides and MEF_{25} (maximal expiratory flow at 25% VC) but no other spirometric parameters. A lower MEF_{25} was related to increased levels of LL-37 but lower levels of HBD-2 (124). Cell studies however have found that HBD-2 mRNA expression is similar between CF and non-CF cells which may suggest that any increase in protein levels is due to the presence of infection rather than an innate alteration in signalling (125), or it may merely be that the protein has a shorter half-life in non-CF individuals or reduced mRNA translation.

LL-37 is found in high concentration with the CF airway, but it has been shown that this peptide binds to glycosaminoglycans (GAGs) (126), and is inactive in this bound form. Cell studies found that GAG-lysases cleave this bond, thereby releasing LL-37 and allowing microbial killing. A study evaluating the effect of hypertonic saline found that this was also capable of releasing LL-37 from GAGs (127). It has also been shown that LL-37 has significantly attenuated action against both *S. aureus* and *P. aeruginosa* with a lowered pH (11), as has been suggested may be the case in the CF airway, particularly in the CF infant (11). CF human infants and CF piglets have acidic ASL when examined in cultured cells, *in-vitro* experiments and *in-vivo* measurements, but the picture is considerably more variable in older children and adults (83, 85, 128-130) making this component more relevant to preschool children with CF.
2.3 CF airway infection

Although infection is frequently seen in infants and younger children with CF, the incidence of infection rises with age (13). It was traditionally thought that the bacterial organisms commonly encountered within the CF airway were *S. aureus*, *H. influenza*, *P. aeruginosa*, *B. cepacia complex*, *Stenotrophomonas maltophilia* and *Achromobactera xyloidans*.

Although still only used in the research setting, pathogens can now be detected using molecular techniques, rather than conventional microscopy and culture (131) and molecular techniques have allowed recognition that the range of bacteria is a great deal larger and more complex than previously thought, both in healthy and CF individuals (132, 133). The entire airway microbiome can now be explored and a diverse array of microbes is being detected using molecular assays (132, 133). A study of patients with CF exacerbations found that 17% of patients were negative for all bacterial organisms on conventional testing, but molecular testing revealed a diverse range of pathogens (132). The significance of the presence of many of these organisms is not known and research is currently being undertaken to understand this (Reviewed in (134)). In young children with CF, there is a complex microbiome with increasing diversity in the first two years of life (135), but as age increases diversity decreases and specific pathogen communities become apparent (136).

NTM are also now being identified more frequently within the CF airway (111) and can cause significant morbidity for some individuals. The two most commonly encountered disease causing NTMs are *Mycobacterium abscessus* and *Mycobacterium avium intracellulare*; these organisms do not always lead to lung disease but individual susceptibility appears important and severe disease is seen in some (particularly *M.*
with the need for intensive and prolonged treatments. Patient to patient cross-infection of NTMs, without direct contact but from sharing an environment but at different times, has been seen and knowledge about susceptibility, infection, disease spectrum and response to treatments is increasing.

2.4 Airway inflammation in cystic fibrosis

2.4.1 Inflammation in the airway of infants with CF

The lungs are thought to be normal at birth (84) but inflammation is seen in the airways from early on in infants with CF (105, 137) although as already discussed, the origin of the inflammation is debated. Until a curative therapy is found, reducing the degree of inflammation from birth is thought by most to be likely to improve the outlook. For this reason, it is an area of importance and several clinical observational studies have been conducted into this area. Armstrong et al found that almost 40% of infants diagnosed on NBS had infection in their airways by the age of 3 months (137) and one third of these infants had no symptoms despite having infection identified in their airways. Supporting the theory that inflammation is secondary to infection and not an innate defect, BALF of 10 infants with CF, none of whom had had antibiotics or had any respiratory symptoms at all was examined and no inflammation or increased pro-inflammatory cytokines were seen (106). However, other studies provide conflicting results and have reported inflammation in the airways of such infants, increased neutrophil count, IL-8, and neutrophil elastase even in the absence of infection (103-105), although it may be that infection was present but had been cleared ahead of the sampling whilst resulting inflammatory changes remained. It has
also been shown that although airways are structurally normal at birth, structural changes start at a relatively young age and occur in early childhood (138, 139). Structural changes have been seen both radiologically (140-144) and histologically (145) in children with CF. Increase in reticular membrane thickening (146) and smooth muscle hyperplasia and hypertrophy (147) have been reported and occur in early stage lung disease.

Further details of the airway in the newborn period can be seen in chapters 6 and 7.

2.4.2 Inflammation in the CF airway in established disease

Inflammatory cells, the proportion of neutrophils in BALF, IL-8 and neutrophil elastase have all been shown to be elevated in the CF airway compared to the healthy population (99, 106, 137, 148, 149). Several studies have reported an exaggerated inflammatory response in the presence of infection, and a deficiency in anti-inflammatory cytokines and altered lipoxin A₄:leukotriene B₄ ratio meaning inflammation is abnormally sustained (98, 121, 150). Most studies report an association between airway inflammation and pulmonary function (56, 151) although one study reports that pulmonary function was associated with infection but not inflammation (152).

2.4.3 Clinical sequelae of inflammation

CF is a multi-organ disease but premature death usually occurs as a result of lung disease (153).
The inflammation seen in the CF airway is predominantly neutrophilic (145, 154, 155) as with on-going or frequent bacterial stimulation, neutrophils are continuously recruited. Neutrophils release proteases including elastase, overwhelming the anti-proteases, and in excess, these proteases directly damage the airway wall by catabolism of elastin and structural proteins within the airway wall structure, leading to bronchiectasis (154). The degree of inflammation has been shown to correlate well with clinical lung disease severity with inflammatory markers, especially neutrophil elastase, increasing as FEV₁ decreases (156, 157). Whilst it seems logical that minimising airway inflammation would minimise lung damage, this was not supported by a recent trial evaluating the effect of a drug designed to suppress inflammation, BIL284 – an LTB₄ receptor antagonist (96). This randomised double blind placebo controlled trial was stopped early due to the increased incidence of adverse events in the treatment arm, 36% vs. 21% in adults and 30% vs., 23% in children and suggests caution in the use of anti-inflammatory agents as these may suppress a beneficial inflammatory response resulting in adverse outcomes.

2.4.4 Current CF management

Until very recently, all respiratory treatments were aimed at tackling the downstream consequences that occurred as a result of malfunctioning CFTR. Such treatments include: prevention/ suppression of infection by use of long-term oral or nebulised antibiotics; prompt and aggressive treatment of exacerbations with oral or intravenous antibiotics; exercise, physiotherapy and inhaled mucolytics to aid airway secretion clearance.
Despite airway inflammation being a major issue in the CF airway, anti-inflammatory agents are not often used in Europe. Systemic corticosteroids are beneficial in slowing lung function decline (158, 159) but the adverse events (160, 161) seen have prevented widespread use and a recent Cochrane review (159) advised that benefit should be weighed against the risk. Inhaled corticosteroids were evaluated with the hope of providing unequivocal benefits to pulmonary health whilst avoiding the significant adverse effects seen with systemic steroids. Tracheal xenografts implanted in mice showed decreased IL-6 and IL-8 secretion with inhaled fluticasone (162) and cell culture studies showed a reduction in these pro-inflammatory cytokines via the IκB pathway with fluticasone (163). Disappointingly though, this effect has not been seen in-vivo and no convincing clinical benefit on lung function or inflammatory markers has been observed (111).

The improvement in lung function with oral corticosteroids and no increase in infection burden supports that there is excessive inflammation relative to the infection and would suggest that anti-inflammatory medication would be of benefit to many patients with CF. Given the side –effects, only in patients with more severe disease would the benefit seem to out-weigh the risks.

Azithromycin is used in CF with undoubted benefit. The mechanism of action is not known. It is known to have anti-inflammatory properties, as well as antibacterial actions. One study has suggested that it inhibits neutrophilic recruitment and prevents an increase in pro-inflammatory cytokine levels (164). A recent study using CF bronchial epithelial cells found that azithromycin had a positive impact on cell chloride efflux and also had a weakly beneficial effect to the innate immune system by up-regulating the NOD-like receptors NLRC1 and NLRC2 (165). It also appears to have anti-pseudomonal antibacterial activity with
a greater effectiveness in the pseudomonal stationary phase and an impact on biofilm formation and quorum sensing (111); clinical studies found reduction in pulmonary exacerbations in both pseudomonas infected and pseudomonas naïve children (166). Azithromycin may therefore decrease inflammation by a combination of antimicrobial and immunomodulatory properties.

It has also been suggested that the mucolytic, nebulised rhDNAse, may have anti-inflammatory properties with a decrease in neutrophil elastase, neutrophils and IL-8 being seen in patients with DNAse use (167-169), although a causative association was not proven and may be a consequence of indirect actions. As described above, NFκB is integral in the pathway of pro-inflammatory cytokines and studies have suggested an increased activation of NFκB by CF airway secretions and mucous. This may explain the benefit of decreased inflammation as has been reported with improved airway clearance by the indirect means of a reduction in NFκB activation leading to a reduction in IL-8, IL-6, IL-1B and GMCSF (111). It may also have a role in releasing the antimicrobial peptide LL-37 bound in DNA/actin bundles in CF sputum (170) also contributing to the potentially beneficial anti-inflammatory actions seen.

In the USA ibuprofen is commonly used and the Cystic Fibrosis Foundation (CFF) recommend consideration of ibuprofen in patients with mild lung disease (171). A Cochrane review (172) concluded that high dose ibuprofen can slow disease progression of CF lung disease, especially in children and randomised controlled trials reported no significant increase in adverse events but concerns regarding the side effects have precluded its wide scale use in Europe. The exact action is not known but is thought to act via two pro-inflammatory transcription factors, NFκB and AP-1 (111) although a further study found no
impact of ibuprofen on IL-8 (173). In order to achieve the anti-inflammatory response the plasma levels have to be monitored closely and kept within a narrow range of 50-100 μg/ml with lower levels potentially having a pro-inflammatory effect by an increasing recruitment of neutrophils to the airway. Interestingly a recent paper has identified ibuprofen as a CFTR corrector improving CFTR trafficking, which may account for some of the clinical benefits seen (174).

Following the findings of preliminary studies evaluating sildenafil in CF which found decreased BALF inflammation (175), decreased IL-8 levels in 75% of people taking sildenafil and a reduction in neutrophil elastase in 67% (176) larger studies are planned. Following laboratory based studies (177, 178) a phase 2 study of alpha-1-antitrypsin evaluating the safety and efficacy as an anti-inflammatory agent in CF is also planned.

Standard treatments are aimed at managing down-stream effects of the disease whilst newer treatments are aimed at correcting the protein defect, and thus allowing sufficient chloride transport (179-181).

Advances in the technology of high-throughput screening have allowed up to 100,000 small molecules to be tested simultaneously, in an attempt to identify those with the potential to improve the function of the defective CFTR protein (182, 183). CFTR correctors are designed to correct trafficking of misfolded CFTR protein to the cell membrane, where it can function, albeit not at wild-type levels whilst CFTR potentiators target CFTR that is already at the cell surface (either naturally, as in the class III–VI mutations, or as the result of successful correction), improving its function. Ivacaftor is a small molecule therapy now approved for use in patients over the age of 2 with a gating mutation following randomised,
double-blind, placebo-controlled, multinational phase 3 studies that demonstrated significant improvements in lung function of around 17% of baseline, which were maintained out to 48 weeks along with significant decrease (c50%) in sweat chloride levels (75, 76, 184-188). It has also received approval for those over 18 years of age with the R117H mutation. A study of ivacaftor in pre-school children identified some concerns with elevated liver function tests (189) and further work is being done into this age group.

Phase 3 studies of the corrector VX-809 (Lumacaftor) alone and in combination with Ivacaftor have been completed and show only modest improvements in FEV₁ but significant reduction in exacerbations (73, 190, 191) and approval has now been granted in US and Europe. The huge clinical success of Ivacaftor has catalysed research in this area, with many different pharmaceutical companies now researching and developing corrector compounds but an ideal compound is yet to be found.

Gene therapy is also being explored as a therapeutic option for individuals with CF and would be applicable to all affected rather than genotype specific like the small molecule treatments. A recent 12-month study of nebulised gene therapy showed an overall modest improvement in FEV₁ of 3%, but bigger changes were seen in some individuals and it proved the principal of gene therapy and further work is being done in this field (192).

CF continues to carry a significant morbidity and treatment burden, despite which, life expectancy still falls far short from that of normal. Median age at death is currently 27 (193) but most children born today with CF are expected to survive to their fifth decade. Further understanding of the onset and progression of airway inflammation and factors influencing this may lead to improved treatments and subsequent decreased morbidity and mortality.
2.5 **Summary of infection and inflammation in the CF airway**

Infection and inflammation occur in early infancy with progression to structural and functional changes occurring well within the paediatric age group and by the teenage years, CF lung disease is well established, although spirometry may be preserved initially. A better understanding of the origin and cause of these early life events will clearly be advantageous in developing new treatment strategies with the aim of delaying the onset of chronic suppurative and destructive lung disease. It remains unclear as to whether all defects are secondary to early airway infection or whether an innate immune deficiency exists but regardless of cause, an exaggerated sustained and prolonged inflammatory response is seen. Recent evidence suggests that innate immunity may be modulated by other factors including vitamin D. Medications are now available that “correct” the abnormal CFTR protein. Once these drugs are available for newborn infants, correcting this protein from birth or soon after, this conundrum may be definitively answered. For the time being, further research into the mechanisms of inflammation and infection will lead to a greater understanding of the inflammatory processes allowing more appropriate and possibly new treatments to be developed in the aim of minimising and delaying lung damage.

2.6 **Non-CF chronic suppurative lung diseases**

Critical to any investigation is the choice of a control group. In order to ascertain whether any effects seen were specific to the effects of CFTR dysfunction, or a merely a non-specific consequence of airway inflammation, a control group of children with other respiratory illnesses that also cause neutrophilic airway inflammation was also studied.
2.6.1 Primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD) is also a genetic disease inherited as an autosomal recessive with the classic triad of symptoms consisting of situs inversus, chronic sinusitis and bronchiectasis. The true incidence is unknown but has been estimated to be between 1:4,100 and 1:40,000 births (194, 195). There are currently thought to be around 3000 people in the UK with PCD but the number who have been given a diagnosis is significantly lower than this (196). PCD is caused by an alteration to the function and usually also the ultrastructure of the cilia within the respiratory epithelium. The most common abnormality seen is absent dynein arms; other abnormalities include absent radial spokes and microtubular transposition. The cilia are static or dyskinetic, with an un-coordinated beat motion. The consequence is that inhaled matter is not removed from the airway. As the entire respiratory tract is involved, there are both lower and upper respiratory tract symptoms. Nasal congestion and rhinorrhea are common features, frequently with offensive mucopurulent nasal discharge. Nasal douching can be effective at reducing symptoms. Chronic otitis media is present in nearly all individuals to some degree and hearing aids may be required, although, as with all causes of chronic secretory otitis media, improvement in the teenage years is to be anticipated. Frequent audiology reviews should be performed in order to detect hearing loss. Unlike other causes of persistent chronic otitis media, tympanostomy is not recommended as this commonly results in copious mucopurulent discharge that can be quite distressing for the individual. Lower respiratory tract involvement occurs, with recurrent infection, a productive cough and bronchiectasis. Treatment is with antibiotics, inhaled mucolytics and chest physiotherapy. The arrangement of organs in utero is related to nodal ciliary function, and if nodal cilia are immotile (which is not the case in all ultrastructural causes of PCD, for example transposition defect, since
there are no central pairs in nodal cilia), situs is determined by chance (197). Situs inversus or heterotaxia is seen in approximately 40-60% of individuals with PCD (195). Nasal nitric oxide is decreased in PCD (198, 199) and this technique can be used as a screening investigation in a diagnostic work up for PCD (200). Definitive diagnosis is made by examining the structure and function of cilia from biopsy samples taken from brushings of nasal epithelium and the cilia examined under light microscopy. In PCD, ciliary beat frequency is usually reduced (196, 201) and an abnormal beat pattern is seen. The cilia can then be examined using electron microscopy where the ultrastructure is assessed (202).

Genetic mutations have been identified with 2 mutations (DNAI1 and DNAH5) being identified in about 20-30% of individuals with PCD. To date, over 30 genetic mutations have been identified (195) and account for approximately 65% of people with PCD. Prognosis is variable, and many individuals have normal life span (196) although morbidity can be high, especially in patients with a late diagnosis. It is unclear why this group of patients have a significantly better outcome than their CF counterparts despite the fact that children with PCD have increased level of the pro-inflammatory cytokine IL-8.

**Figure 3:** Electron microscope images of cilia showing a) normal cilia, b) outer dynein arm defect and c) inner dynein arm and microtubular disorganisation. (Pictures courtesy of Amelia Shoemark).
2.6.2 Non-CF bronchiectasis

Bronchiectasis can occur as a result of numerous insults to the airways. Causes include severe viral infections, inhaled foreign body, aspiration from gastro-oesophageal reflux disease (GORD), tracheo-oesophageal fistula or laryngeal cleft, immunodeficiency and in about up to 30% of paediatric cases the cause remains unknown (203). Diagnosis of bronchiectasis is made on high-resolution computerised tomography (HRCT) with the characteristic finding of a “signet ring” sign where the bronchus is dilated and larger in diameter than its adjacent blood vessel (204-206). Bronchiectasis can either be localised to one specific area, or more generalised disease can be seen. Investigation to try to identify a cause is necessary and CF and PCD should be excluded. Specific diagnoses including immunodeficiency, aspiration and GORD should be looked for in order to allow appropriate management of these conditions if found. In practice, treatment is similar to that of other chronic respiratory diseases, in the absence of any randomised controlled trials, and individuals may require antibiotics, mucolytics and chest physiotherapy, although much of this is extrapolated from CF as there is a lack of evidence in bronchiectasis. It is important not to extrapolate too much however, as pathophysiology is different and treatments may have different effects. A double-blind randomised placebo controlled trial of DNAse in patients with non-CF bronchiectasis found that the DNAse group had more hospital admission, more exacerbations, increased antibiotic and steroid use and a greater decrease in their FVC over the 24 weeks of the trial (207) and another of non-CF bronchiectasis patients including 4 with PCD found no benefit (208) of DNAse. This is the opposite of the results seen in DNAse in CF patients and highlights the importance of studies within disease populations rather than extrapolating data. Longitudinal follow up of children with non-CF
bronchiectasis found that whilst lung function stabilised it did not improve to normal levels highlighting the importance of prompt diagnosis and early treatment (209).
CHAPTER 3: VITAMIN D

3.1 Vitamin D synthesis

Vitamin D is a fat-soluble secosteroid, which is a steroid in which one bond of the chain is broken. There are five forms of vitamin D but the 2 major forms are ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃); the 2 together can be referred to as calciferol. From here on, reference to “vitamin D” means vitamin D₂ and vitamin D₃ together. The structural difference between these 2 forms is in the side chain with ergocalciferol having an additional double bond and a methyl group. Vitamin D can be obtained either from the diet or by the conversion of the steroid 7-dehydrocholesterol, present in the skin, to cholecalciferol by the exposure to ultra-violet B-light (UV-B). Vitamin D obtained through either route requires hydroxylation to its active form and this is done in 2 stages; firstly by hepatic enzymes, to calcidiol (25(OH)D) and secondly by renal hydroxylation to calcitriol (1,25(OH)₂D) (Figure 4 and Figure 5)(210, 211). Whilst it is 1,25(OH)₂D that is biologically active within the body, this molecule is biologically unstable with a very short half-life which makes measurement of this secosteroid challenging and therefore clinical measurement of vitamin D is a measurement of 25(OH)D. 1,25(OH)₂D binds to vitamin D receptors (VDR) which are located on both cell membrane and nucleus. Binding of 1,25(OH)₂D to VDR located on cellular membranes leads to intracellular signalling pathways whilst binding to nuclear receptor VDR has an impact on more than 900 genes and micro-RNA via a transcription complex. It therefore has the possibility of having numerous roles within the body.
3.2 Classical actions of vitamin D

Vitamin D has a significant role in calcium homeostasis and metabolism by promotion of intestinal absorption of calcium and promotion of bone resorption allowing the body to maintain adequate levels of calcium and phosphate for bone formation. Vitamin D
deficiency can lead low calcium deposition and decreased mineralisation of bones causing rickets in children and osteomalacia in adults.

### 3.3 Non-classical actions of vitamin D

Whilst it has long been established that vitamin D is required for good bone health recent evidence has pointed to a role of vitamin D beyond this, with vitamin D receptors being identified on many immune cells (5, 213, 214). It is now known that the final stage of activation, from 25(OH)D to 1,25(OH)₂D, by the enzyme 1-α-hydroxylase (CYP27b1), can occur in a number of cells and not just the kidney as was previously thought (212, 215). This enzyme has been found in keratinocytes, gastrointestinal cells, breast and prostate, and immune cells including macrophages, monocytes and dendritic cells (215).

1,25(OH)₂D crosses the cell membrane to enter the cytoplasm where it binds to VDR, which have been identified on many immune cells (5, 213, 214). The 1,25VitD/VDR complex then enters the cell nucleus and binds to the retinoid X receptor (RXR). The activated vitamin D subsequently binds to vitamin D response elements (VDRE) located on DNA, which have been identified on genes coding for the antimicrobial peptides LL-37 and HBD-2 (213, 216, 217).
3.4 The role of vitamin D in airway defence

That vitamin D is activated in immune cells, and that it promotes antimicrobial peptides gave rise to the hypothesis that vitamin D is important in host defence.

Vitamin D exhibits numerous actions within the immune system including both indirect antimicrobial and immunomodulatory actions. Several immune pathways are influenced by vitamin D via VDR modulation of gene expression with an overall effect of increasing mucosal defences and reducing excessive inflammation.

Vitamin D regulates the adaptive immune system to prevent an excessive response with potentially damaging inflammatory changes by influencing T-cell receptor signalling, inhibiting lymphocyte proliferation and Th1 and Th17 cytokines (218). Whilst the exact pathways are not fully understood, vitamin D appears to have an impact on numerous cytokines (Table 4) and these may be consequences of effects of vitamin D on up-stream protein expression rather than direct action (214, 219-221). Overall there is an anti-inflammatory effect by the decrease in pro-inflammatory cytokines and an increase in anti-inflammatory molecules. Animal studies have demonstrated a 40 % reduction in LPS induced neutrophil recruitment with vitamin D administration (222).

More relevant to this thesis, vitamin D also affects the innate immune system by increasing epithelial cell production of antimicrobial peptides including HBD-2 and LL-37 and stimulating the expression of these peptides from macrophages and neutrophils. As well as an increase in gene expression with vitamin D, inhibiting CYP27b1 led to a decrease in gene expression (215). Whilst it has been well demonstrated in-vitro that expression of LL-37 and HBD-2 is significantly up-regulated by vitamin D(213, 223), in-vivo data is lacking. One
### Table 4: Immunomodulatory effects of 1,25(OH)D$_2$

<table>
<thead>
<tr>
<th>The effect of vitamin D</th>
<th>Immunological molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibacterial effects</strong></td>
<td>Cathelicidin and HBD-2(223-225)</td>
</tr>
<tr>
<td>Increased</td>
<td>Nitric oxide(226)</td>
</tr>
<tr>
<td><strong>Anti-inflammatory effects</strong></td>
<td>IL-8(224)</td>
</tr>
<tr>
<td>Decrease in pro-inflammatory response</td>
<td>IL-1B(227, 228)</td>
</tr>
<tr>
<td></td>
<td>IL6(224, 228-230)</td>
</tr>
<tr>
<td></td>
<td>TNF-α(227-231)</td>
</tr>
<tr>
<td></td>
<td>IL-12(228, 230)</td>
</tr>
<tr>
<td></td>
<td>IFN-γ(232, 233)</td>
</tr>
<tr>
<td></td>
<td>IL-2(234, 235)</td>
</tr>
<tr>
<td></td>
<td>IL17(230, 236)</td>
</tr>
<tr>
<td></td>
<td>IL21(236)</td>
</tr>
<tr>
<td></td>
<td>T cell proliferation(234, 235)</td>
</tr>
<tr>
<td></td>
<td>B cells(237)</td>
</tr>
<tr>
<td></td>
<td>TLR-2 and TLR-4(238)</td>
</tr>
<tr>
<td>Increase in anti-inflammatory response</td>
<td>T regulatory cells(5, 239)</td>
</tr>
<tr>
<td></td>
<td>IL-10 (232, 239-241)</td>
</tr>
<tr>
<td></td>
<td>IL-4(232, 233, 240)</td>
</tr>
<tr>
<td></td>
<td>IL-5(232)</td>
</tr>
<tr>
<td></td>
<td>CD200 ligand(220)</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td>Matrix metalloproteinases(242)</td>
</tr>
<tr>
<td>Decreased</td>
<td>Matrix metalloproteinases(242)</td>
</tr>
</tbody>
</table>
prospective study looked at serum LL-37 levels in response to high doses of vitamin D and found no response but did see an increase in hCAP-18 mRNA expression in peripheral blood monocytes (225). This study evaluated the relationship of 25(OH)D, 1,25(OH)D and LL-37 in serum and found no relationship. The authors commented that it was this lack of relationship that supported their hypothesis that vitamin D only exerted its effects at the cellular level. They studied the effect of vitamin D at a cellular level, by examining peripheral blood cells taken from vitamin D deficient individuals, supplementing these individuals and again extracting blood. They found that monocyte activation of CYP27b1 was dependent on vitamin D and that expression of LL37 by monocytes was significantly higher after vitamin D supplementation.

LL-37 and to a lesser extent HBD-2 are induced by vitamin D in cultured bronchial epithelial cells (213). Vitamin D exerts its effects via vitamin D receptor and vitamin D response elements, which have been found to be located within the genes encoding these antimicrobial peptides(213). Wang et al evaluated the response of vitamin D on synthesis of these peptides in cultured normal epithelial cells and showed that vitamin D increased gene expression. Def-B expression was enhanced by vitamin D and in the presence of IL-1, known to be an inducer of def-B, vitamin D further increased expression. As well as gene expression, immunohistochemistry and western blotting confirmed that protein levels were also increased. The activity of the antimicrobial peptides against *E. coli* and *P. aeruginosa* was tested and an increased activity against these bacteria was seen in the presence of vitamin D.

A second study (1) studied the impact of vitamin D on wild-type and CF human epithelial cells using western blot and PCR to detect LL37 and HBD-2 mRNA and protein. The activity
of the antimicrobial peptides found within the airway surface fluid of these cells was also assessed by observing their efficacy against laboratory strain bacteria. LL37 mRNA was increased 10 fold by the addition of vitamin D in both normal (Figure 6a) and CF bronchial epithelial cells, and the activity of LL37 against bacterial organisms including *P. aeruginosa* was increased in the presence of vitamin D (Figure 6b). Unlike the first study, by Wand et al, there was no impact on HBD-2. IL-8 secretion by human macrophages and respiratory epithelial cells (224) is decreased by vitamin D, another possible downstream consequence of the vitamin. Vitamin D deficiency may therefore have numerous consequences on the immune system.
Figure 6: a) Increase in LL37 mRNA following treatment with 1,25(OH)₂D₃. b) A decrease in bacterial colonies was seen following exposure to 1,25(OH)₂D₃. Figures from (1).
3.5 Factors affecting Vitamin D levels

Whilst it is the inactive form of vitamin D generally measured, it is the active form (1,25)OHD2 that has the effects. The reason the inactive form is measured is that it has a longer half-life and is therefore easier to assay reliably. However, differences may exist in individuals ability to convert this to its active state meaning a high 25(OH)D2 may not equate to a high 1,25(OH)D2. The enzyme 1-alpha-hydroxylase, which is a P450 enzyme, carries out this conversion and results from experiments in mice show that gene expression of this enzyme is inhibited by VDR (243). Numerous polymorphisms in gene expression of VDR have been described and these in turn may have an impact of the gene expression of 1-alpha-hydroxylase and consequently the levels of 25 and 1-25(OH)D2. Other factors also play a part in the activation of this gene and dietary restriction of phosphate increases its activity but not associated with serum levels of phosphate or calcium suggesting another factor, possibly hormone related. This observation demonstrated the lack of full understanding of the inhibition and activation of the genetic expression of this converting enzyme. Mutations in this gene; at locus 12q13.1-913.3; have led to vitamin D independent rickets (244). Some drugs such as protease inhibitors have an inhibitory effect on 1-alpha-hydroxylase therefore potentially having the effect of increasing level of 25(OHD2 but decreasing the level of the active form 1,25(OH)D2. The presence of inflammation may promote activation of the enzyme via CYP27B1 increasing 1,25(OH)D2 temporarily (245), but as the half-life is short, just 4-6 hours, the effect of this is depletion of 25(OH)D2 levels and a decrease subsequently of 1,25(OH)D2 levels from lack of availability.

Another factor which may be of relevance is the level of vitamin D binding protein (VDBP) which is essential for transportation of 25(OH)D; approximately 99.5% of the body’s vitamin
D level is bound to VDBP (246). It is hypothesised that it is the free vitamin D that is available for action. Although the exact relationship between VDBP and vitamin D level is not known it is thought that circulating vitamin D binds to VDBP, lowering the vitamin D bioavailability (247). VDBP gene is highly polymorphic and different polymorphisms have differing affinities for vitamin D. Therefore genetic variations may also have a role to play in the amount of vitamin D bound to VBDP, which alter the available vitamin D for action (248) and thus will also have an impact on the actions of this molecule clinically. The level and genotype of VDBP will therefore have an effect on the amount of free vitamin D available and a patient who is vitamin D deficient may have adequate vitamin D activity if VDBP levels are low or there is a genetic variation giving low affinity.

3.6 Vitamin D reference ranges

Whilst it is apparent that vitamin D deficiency is linked with adverse events, it is not clear what constitutes deficiency. Unlike many normal ranges which are derived from the values observed in the normal population, i.e. height, weight (249) and lung function (250), vitamin D normal ranges are derived from clinical and biochemical parameters. Parathyroid hormone increases rapidly when 25(OH)D is lower than 50 nmol/L, and reaches a plateau when 25(OH)D is over 50 nmol/L (251, 252). The effect that 25(OH)D has on this hormone is taken into account when considering the ‘normal range’. The level that is required to see clinical benefit in the way of decreased fractures is also considered. As these factors are both open to different interpretations, the “normal” range is debatable and different cut-
offs for vitamin D deficiency have been suggested (253-257). The Institute of Medicine in
the USA suggests that a level of ≥ 50 nmol/L is adequate for good bone health in healthy
individuals and no additional benefit is gained by achieving levels higher than this (258). A
meta-analysis evaluating vitamin D and skeletal fractures concluded that a higher level of
vitamin D would be better and 25(OH)D should be maintained ≥ 60 nmol/L. The Institute of
Endocrinology concluded that vitamin D should only be measured in at risk individuals but
should be ≥ 75nmol/L, with deficiency being < 50 nmol/L and levels between 50-75 nmol/L
considered ‘insufficient’ (259). The US CF Foundation (260) and the Canadian Osteoporosis
scientific advisory committee agree (261) with these definitions. As there is no agreement
on what constitutes vitamin D deficiency, reported prevalence varies, but is quoted in the
literature as being between 20 and 100% in non-disease populations (259, 262, 263). The
Royal Brompton Hospital paediatric CF department considers levels over 75 nmol/L to be
optimal and levels over 50 nmol/L to be adequate. Levels below 50 nmol/L are inadequate
and patients are considered to be vitamin D deficient and prescribed additional
supplementation.

These “normal” values are based on optimal levels for bone health and it cannot be
assumed that for other functions of vitamin D including immunomodulatory levels would be
the same.
3.7 Vitamin D deficiency

Whatever definition is used, vitamin D deficiency is highly prevalent for a number of reasons. As the major source of vitamin D is derived from sunlight, a lack of this is a major contributing factor. In the northern hemisphere, the position of the sun during the winter months means that there is insufficient UVB light to derive vitamin D (264) and vitamin D is lower in the winter months than the summer. Moreover, during the months when sunlight is sufficient for conversion of 7-cholesterol to 25(OH)D to occur, the widespread use of sunscreen reduces UVB penetration and impairs vitamin D synthesis (265). For this reason, vitamin D deficiency is commonly seen in the normal population year round. These factors are compounded in certain ethnic groups by the practice of wearing skin covering clothing.

3.8 Vitamin D deficiency in CF

Vitamin D deficiency is particularly prevalent in the CF population being seen in up to 90% of individuals (266-269). There are a number of reasons why people with CF are at risk including generalised malabsorption of fat soluble vitamins, avoidance on sunlight exposure and impaired hepatic hydroxylation (267). In addition, vitamin D derived from sunlight may be stored in subcutaneous fat for the winter months but in CF patients tend to have less fat and subsequent less storage.

CF patients have also been shown to have reduced VDBP levels (270, 271). The reason for this is unclear but it has been suggested that it could be to impaired glycosylation of this
protein (270). Most vitamin D is carried by VDBP which may impact the availability of vitamin D for action but the effects of VDBP on vitamin D levels are not entirely clear.

Vitamin D supplementation is recommended for all patients with cystic fibrosis and studies have shown that adequate levels can be achieved but in some cases very high doses of up to 50,000 IU weekly was used to achieve this (267). The CF trust advises vitamin D dose of 1000-2000 IU daily for infants and 1000-5000 in others (272).

3.9 Vitamin D in respiratory health (non-CF)

Vitamin D has been implicated as having a role in a variety of respiratory diseases. It has been used empirically as a treatment for respiratory disease for many years although it was not known why at the time. As far back as the 1800s, sunlight was used as a treatment for TB (273) but with the introduction of antibiotics, this approach fell out of favour. As part of the body’s defence mechanisms, mycobacteria are ingested by macrophages and LL-37 induced killing can then destroy these macrophage-mycobacterium complexes. In-vitro studies have shown that killing does not occur in the absence of vitamin D but can be restored by the addition of vitamin D (223). Moreover, clinical studies have found greater levels of severity and mortality in tuberculosis (TB) patients with vitamin D deficiency (253, 274). Other studies have reported associations between low vitamin D and respiratory infections, including influenza and lower respiratory tract infections (254-257), especially in the asthmatic and COPD populations (275). It has also been shown that in the healthy
population, better pulmonary function is seen in people with higher levels of vitamin D (276) – although the clinical significance of these small differences has not been established. However, not all studies report an association between vitamin D and respiratory infection. Two studies of young Canadian children found no relationship between vitamin D and the incidence of bronchiolitis (277, 278) although in one, low vitamin D was associated with an increased risk of admission to intensive care (278). Inflammatory markers have also been shown to be related to vitamin D, with vitamin D-deficient bronchiectasis patients having higher levels of pro-inflammatory cytokines than those vitamin D sufficient (227). A lower vitamin D was also observed in patients chronically infected with bacteria in this group of patients.

Given the mounting evidence suggesting a role for vitamin D in lung health, randomised double blind, placebo controlled studies have been conducted to assess if vitamin D supplementation prevents lower respiratory infection. The largest randomised control trial (RCT) to date included just over 3000 children randomised to receive either 3 monthly high dose vitamin D or placebo; the investigators found no difference in incidence of pneumonia between the 2 groups, although a higher vitamin D level was achieved in the vitamin D group (p<0.01) (279). However, a meta-analysis of 5 studies found vitamin D to be associated with fewer infections in children but not adults (280), whilst a meta-analysis involving 11 studies (including all 5 studies in the previous meta-analysis) reported that vitamin D had a protective effect (OR 0.64, 95% CI 0.49-0.84) against respiratory infection (281). This meta-analysis found that daily dosing was more effective than less frequent bolus dosing. Even in TB, where observational studies have convincingly shown benefit of adequate vitamin D levels, studies evaluating vitamin D against placebo as a treatment have
reported mixed results; some have demonstrated no clinical improvement (282, 283) whilst a more recent study (284) reported both radiological and clinical improvement. Results of studies looking for a relationship between vitamin D and respiratory infection are mixed and the question of whether vitamin D deficiency plays a role in respiratory disease remains controversial but there is mounting evidence that adequate levels of vitamin D may be important in TB (253, 274), influenza and other lower respiratory tract infections (254-257), as well as asthma (231, 285).

3.10 The role of Vitamin D in CF lung disease

Evidence from studies evaluating the role of vitamin D in respiratory infections and respiratory health in CF is limited and results are mixed. The largest study to date, involving 800 individuals with CF, found that vitamin D had a positive but very weak correlation with FEV₁ and a negative correlation with IgG (286) (used a marker of lung inflammation and previously reported to correlate negatively with FEV₁ (287) (Figure 7). However the results of several smaller studies are conflicting (268, 288-291) and this effect has not been observed in children with CF (266). One small pilot study found increased survival, increased hospital free days and decrease in serum TNF-α and IL-6 following a single bolus dose of vitamin D (292). A systematic review (293) concluded that there is a weak positive correlation between vitamin D and spirometry. Given the research interest in vitamin D within the medical world at present, the lack of data within CF, and especially within CF airway inflammation is surprising.
Figure 7: Scandanavian CF nutritional study outcomes
Figure a shows the vitamin D level against the IgG, which is used as a proxy for inflammation and shows a weak relationship with \( r^2 = 0.376, \ p < 0.001 \). Figure b shows the relationship between vitamin D and FEV1 in this cohort (\( R^2 = 0.308; \ P=0.025 \)). Figures from (286).

---

**Figure a**

Partial residual plot

![Partial residual plot](image1)

**Figure b**

Partial residual plot

![Partial residual plot](image2)
CHAPTER 4: Hypothesis, aims and objectives

4.1 Hypotheses

1. The earlier institution of improved and more aggressive treatments strategies possible in infants diagnosed with CF by NBS leads to a healthy airway during the first year of life

2. Vitamin D has immunomodulatory effects via antimicrobial peptide pathways; consequently vitamin D deficiency will lead to greater risk of bacterial infection and subsequent inflammation and worse clinical outcomes in CF throughout childhood

4.2 Aims

a. To describe the spectrum of airways disease in a large cohort of young infants with CF diagnosed on NBS by evaluating the relationships between infection and inflammation and structural and functional changes cross-sectionally and longitudinally over the first year of life

b. To describe vitamin D levels in children with CF of all ages comparing them to two control groups: chronic airway disease and healthy children and relate them to airway structure, function and infection and inflammation status
4.3 Objectives

i. Gather samples of BALF from children undergoing routine fibreoptic bronchoscopy (FOB) at 3 months of age and process samples according to set protocol, or use previously stored samples and measure cellular and soluble inflammatory markers and utilise microbiological culture results from clinically run tests.

ii. Measure serum levels of 25(OH)D by collecting serum samples from children undergoing general anaesthesia (GA) for FOB procedure, using previously stored samples or by obtaining results from the electronic patient records for levels measured for clinical evaluation and compare levels in 3 discrete populations; CF, non-CF CSLD and healthy controls.

iii. Using collected BALF samples, measure the levels of the antimicrobial peptides LL-37 and HBD-2, relate them to Vitamin D status, and establish if there is a relationship between these peptides and the presence of infection and levels of soluble and cellular inflammation.

iv. Use longitudinal outcome data available explore the relationship between vitamin D at 3 months and airway health at 12 months.
PART B: METHODS
5  CHAPTER 5: Patients and Methods

This chapter describes methods common to all parts of this research. Details regarding methods specific to particular investigations can be found in the relevant chapters.

5.1  Patients

5.1.1  Patient selection

Children aged <17 years at the Royal Brompton hospital, who fulfilled criteria for one of the following groups were included. If patients fulfilled the criteria on more than one occasion, the first occasion was selected and each individual patient included only once.

5.1.2  Group 1: Cystic fibrosis

Subjects had a diagnosis of CF (42, 294), and had undergone examination of their airways by fibreoptic bronchoscopy.

Group 1a) Infants diagnosed on newborn screening and undergoing routine FOB at approximately 3 months of age.

Group 1b) Children who had undergone FOB previously for exacerbations or if having GA for another reason and had stored serum AND
BALF available, OR children recruited prospectively whose parents agreed to these samples being used for research.

5.1.3 Group 2: Non-CF chronic suppurative lung disease (CSDL)

Children recruited to this group had either primary ciliary dyskinesia (PCD) diagnosed on conventional criteria (295) or non-CF bronchiectasis (diagnosed on HRCT by consultant radiologists independent of this study).

- **Group 2a)** Children who had undergone FOB previously and had stored serum AND BALF available, OR children recruited prior to the FOB and these samples collected.

- **Group 2b)** Non-bronchoscopic cohort comprising children with a measurement of serum vitamin D and spirometry (within 4 days of the vitamin D sample) results available from clinical testing.

5.1.4 Group 3: Healthy Controls

Other children undergoing FOB were included as ‘healthy’ controls (HC) only if they had macroscopically normal airways, no bacteria, fungi or viruses identified and a BALF neutrophil and eosinophil differential of ≤4% and <0.5% respectively.
5.2 Fibreoptic bronchoscopy

5.2.1 Royal Brompton Hospital bronchoscopy programme

In the Royal Brompton Hospital (RBH) paediatric respiratory department, it is routine practice to perform a surveillance FOB under general anaesthetic at around 3 months of age. This practice was started in 2003 after evidence began emerging from the London Cystic Fibrosis collaboration (LCFC) of airflow limitation at an early age that had not normalised by 1 year of age ((138, 296, 297), even in those with apparently no respiratory disease. In the first 2 years of doing this, infection was detected in approximately one quarter of infants (298, 299), underscoring the importance of this practice. This rate of infection was also seen in the first 2 years of NBS infant data (299). Such practice is not routine amongst CF centres and this practice has been extensively analysed and it has been found to be a safe procedure, which does not cause unacceptable clinical side effects.

5.2.2 Indications for bronchoscopy

All FOBs were carried out for clinical indications. The infants having 3-month surveillance FOB made up part of the CF cohort; the remainder of the CF group and the PCD and bronchiectasis patients were undergoing the procedure because of unexplained decline in respiratory status or clinical symptoms and, in the majority, an inability to expectorate sputum for culture. Healthy controls were bronchoscoped for other reasons such as upper airway examination or a history of haemoptysis. No child underwent FOB purely for research purposes; all sampling was opportunistic.
5.2.3 Consent

Written informed consent was obtained from parents/guardians of children undergoing clinically indicated FOB. Specific consent (and assent where age-appropriate) was obtained to retain samples surplus to clinical needs, and obtain additional samples for research.

5.2.4 Safety considerations

When undertaking sample collection from bronchoscopy for research purposes there are important safety aspects to consider. Previous researchers within this group have explored the safety aspects in detail (298, 300-302).

The children within the study underwent FOB as part of clinical management and so the safety of the actual bronchoscopy is not a specific research concern, except insofar as a little extra time is required to obtain research samples, (303) but a consideration of the clinical management decisions. The procedure is safe with the majority of side effects being minor (transient fever, short term increased cough, sore throat). Additional samples e.g. addition blood sampling or performing endobronchial biopsies may be taken purely for research purposes and therefore it is important to consider the safety of these separately; (a) the clinical effects of study sampling; (b) the additional time required for sampling; and (c) ensuring the samples obtained are of sufficient quality to address the research questions.

We have published data on the on the safety and quality of research samples from bronchoscopy in the under 5 year olds (300); 33 patients underwent FOB with endobronchial biopsy matched with 33 controls in whom no biopsy was taken. The groups were matched for age, weight and bronchoscopist and were performed within the same
time period. Most procedures were done for investigation of cough, recurrent lower respiratory tract infection or wheeze. Two patients who were not biopsied had CF; there were no patients with CF in the biopsy group.

There was no significant difference between groups in the number, type, or severity of complications occurring during or after the procedure. Biopsies from 30 of the 33 subjects could be assessed; reticular basement membrane was identified in all 30; inflammation could be assessed in 26 and areas of smooth muscle were present in 23. A further study evaluated the safety of research bronchoscopies, specifically endobronchial biopsies, in children with CF compared with non-CF children (301). 42 children underwent 45 FOB and endobronchial biopsies and a comparison was made to a control group of 39 children with other respiratory conditions also undergoing FOB and endobronchial biopsies. 13% of CF children had minor complications during the procedure compared with 18% in the control group. There was one significant event within the control group when a patient suffered transient loss of pulse following a cardiac catheter done at the same time, but this was not thought to be related to the bronchoscopy.

There was no significant difference in the post-operative complication rate with post procedural fever being seen in 2 patients in the CF group (4.4%) and 3 patients (7.6%) in the control group, 1 CF patient (2.2%) requiring overnight oxygen and 1 patient (2.6%) in the control group describing pleuritic chest pain.

The timing of procedures has also been evaluated to assess if the additional time taken for research sampling was acceptable. This study was done covertly without the bronchoscopists being aware of the timing. The median (range) duration (min) was 2.5 (1.0-
8.2) for airway inspection, 2.8 (1.7-9.4) for performing bronchoalveolar lavage, 5.3 (2.5-16.6) for biopsy sampling, 2.4 (1.5-6.6) for teaching and 4.1 (0.8-18.5) for other interventions. Three adequate biopsies were obtained in 33 (83%) children it took a median of just over 5 min to obtain three endobronchial biopsies, which was considered an acceptable increase in the duration of fibreoptic bronchoscopy for the purpose of research (303). These three studies concluded that FOB and endobronchial biopsy was safe, requires minimal additional time and produces samples that are of acceptable quality for research. In addition, an independent consultant anaesthetist is present and acts as a guarantor of the child’s safety, with authority to stop research sampling at any time.

5.2.5 Bronchoscopy Protocol

The procedure was carried out under general anaesthetic as is usual practice within the centre and the specific anaesthetic technique was determined by the consultant anaesthetist. Most children have anaesthetic induction by inhaled sevofluorane via face mask, with the remaining children receiving intravenous propofol. Anaesthetic maintenance is with sevofluorene and intravenous propofol if needed. The airway was most commonly maintained by facemask and manual chin lift. Laryngeal mask airway (LMA) and endotracheal tube were used if deemed appropriate by the anaesthetic consultant.

2.8 internal diameter Olympus BF-XP40, 3.6 mm BF-3C20 or 3C40, 4.0 mm BF-MP60 and 4.6mm BF-P2OD bronchoscopes were used, the choice being dictated by the size of the child. An initial inspection of upper and lower airways was made prior to samples being
obtained. Where possible, no suctioning was carried out in the upper airway to prevent contamination of samples with upper airway flora as far as possible.

5.3 Samples collected at bronchoscopy

5.3.1 BALF collection and processing

BALF samples were obtained by instilling 3 aliquots of 1ml/kg, up to a maximum volume of 40ml, 0.9% saline at room temperature to the right middle lobe and lingula, or the most affected lobe(s). The aliquots were pooled. 1ml aliquots were sent to clinical laboratories for microbiology analysis (304), virology (immunofluorescence and PCR) and cytology for differential cell count. Cellular differential was performed following cytopinning for 3 minutes at 200 g, air drying then fixation with methanol and May-Grünwald-Geimsa staining. The research aliquot was put immediately on to ice. A 50 μl aliquot of whole BALF was mixed 1:1 with trypan blue (Sigma-Aldrich, USA) and a total cell count performed using a dual chamber Neubauer haemocytometer (Assisten, Sondheim, Germany). The remaining BALF was centrifuged at 4°C, 2000 g for 10 minutes and the supernatant stored in aliquots at -80°C. As per current research governance stipulations, samples were coded and individual aliquots item tracked.
5.3.2 Sample suitability

Not all samples were suitable for cellular differential counts due to small volumes, cell breakdown or excess blood within the sample; hence these data are not available for all patients. In addition, there are some missing data from archived material. Wherever both neutrophil cell differential and total cell count were available, a BALF neutrophil cell count was calculated.

5.3.3 Blood samples

Blood samples were obtained through venepuncture whilst under general anaesthetic. In nearly all cases venepuncture was clinically indicated and most had sampling through the line which was placed for anaesthetic reasons; an addition aliquot of up to 3 ml of blood taken in lithium heparin vials for research purposes. These samples were centrifuged at 4°C, 2200 g for 10 minutes and the serum carefully pipetted and stored in aliquots of between 125 and 500 μl at -80°C.

5.4 Clinical patient data

Clinical data were obtained at the time of bronchoscopy and recorded in a research database (Access 2000) alongside data from electronic patient records and the clinical lung
function database. For CF patients, data from annual assessment was also obtained from the national CF registry, “Port CF”. Data recorded were:

- Weight and height
- Pancreatic status
- Spirometric values
- Presence /absence CF related diabetes
- Use of intravenous antibiotics (IVAB)
- CRP
- Microbiology

### 5.5 Lung function

Spirometry was performed according to ATS/ERS standards using Vitalograph (Buckingham, England) alpha touch spirometer and expressed as percent predicted using the Rosenthal reference equations (305), in all children old enough to complete the manoeuvre. Three manoeuvres were performed and the best FEV₁ and FVC taken (306); these do not necessarily have to be from the same manoeuvre.

### 5.6 Vitamin D measurement

#### 5.6.1 Vitamin D assay

Prior to June 2010, vitamin D assays performed by the Biochemistry department at Royal Brompton and Harefield NHS trust were done using immunoassay. Thereafter, samples
have been analysed using mass spectrometry with high performance liquid chromatography (HPLC). When the analytical method was changed, the biochemistry department ran approximately 100 samples concurrently with both techniques and found a good correlation with minimal inter-assay variability. However, to maximise accuracy, vitamin D levels performed before June 2010 were not included in this study. If such patients had a stored serum sample available, a repeat assay was performed using the newer technique, but if no serum was available the patient was excluded from this study. All samples were analysed in the same laboratory and by excluding samples prior to June 2010, all samples were tested using the same technique and on the same apparatus. Included samples were processed in one of 2 ways:

Method 1: assay performed at time of FOB on” fresh” samples (“clinical”)

Method 2: assay performed on frozen / thawed samples. Some were performed as part of the medical student pilot project (“pilot”) and some as part of this research study (“research”).

(Further explained in flow diagram in Figure 11.

5.6.2 Comparing vitamin D results when performed using different methods

14 samples were analysed using both of these ways (clinical and research) and these were compared to assess agreement. (The pilot study samples predated the change to HPLC so no clinical results are available but the stored samples had vitamin D analysis using HPLC and so are included in the overall research but not this section of comparing clinical and research
results.) There was no statistical difference seen between the paired values of “clinical” result and “research” result (median (95% CI) 62 (37 – 88) vs. 50 (35 – 73) nmol/L, p=0.2 paired t-test). However, as results did vary, this was explored further. I considered the following possibilities: a) differences in equipment used for the assay, b) different personnel performing the assay, c) the stability of Vitamin D during storage. Of note, as mentioned previously, the research batches were analysed on the same machine as clinical samples, and also by the same personnel. The assays were performed by the Biochemistry staff, and not by the researchers.

5.6.3 Assessing stability of vitamin D over time

Firstly, I determined whether vitamin D had denatured over time, as although the clinical samples are assayed within 7 days of sample collection, the rest were performed in 2 batches and some samples analysed as many as 10 years after collection. I therefore looked to see if the oldest samples had lower levels of vitamin D than more recent samples. Figure 8 shows vitamin D by sample number for all groups, with sample 1 being the oldest sample (from 2002) and sample 680 being the newest (2011). The “pilot” assays were performed in 2010, and “research” assays in 2012 on samples obtained between 2002 and 2012. If degradation had occurred, the later groups would be expected to have lower values than those analysed at the time. There is no decrease over time and the group mean and spread was unchanged (Figure 8).
The 5 samples assayed in both the pilot study and the research study show very good agreement on inspection, despite being done almost 2 years apart (Figure 9) the numbers are too low for a statistical test. This again suggests that vitamin D is not subject to degradation over time in frozen samples.

Figure 8: 25(OH)D2 levels over time. There is no decrease in vitamin D levels when assayed at a later date (blue and red symbols) compared with results obtained from serum assayed at the time of collection (green symbols).

Figure 9: 25(OH)D results performed on separate occasions. The pilot and research study results are very similar despite being performed 2 years apart.
Figure 10: Bland Altman plot of agreement of 25(OH)D2 levels

Bland-Altman agreement of the 25(OH)D level as measured by clinical method and research method. There is a systematic difference seen with a mean difference of 16.6 in the direction of the clinical assay being higher than research assay.

Figure 10 shows agreement between paired clinical and research samples. Clinical samples are processed on recently taken “fresh” samples, whilst research samples are processed, frozen, stored and thawed at a later date for the assay. Positive values represent a higher value seen in clinical result than research samples. Overall, values are higher in the samples analysed at the time than samples analysed at a later date on stored aliquots. However, as shown above this does not appear to be explained by an effect of time. An effect of the freeze/thaw cycle was considered but such an effect, if present, is not uniform as 2 of the 14 samples are lower in the clinical sample than that tested at a later date on frozen/thawed sample.

The result of one paired sample is grossly different and by looking at other blood tests for vitamin D performed clinically on this patient it appears that the “clinical” result is the
correct result and is the one used here (Table 5). The study sample aliquot was small, and upon checking, there was no sample at all left in the vial, so the inaccuracy could perhaps be due to a small sample, possibly with fibrinous material included within it.

The apparent systematic difference was not taken into account in further analyses due to the small sample size giving rise to a very large 95% CI which crossed zero and therefore accuracy of such an adjustment could not be certain and may mislead results.
A large discrepancy between clinical and study result was seen for patient 640 despite the sample being taken on the same day. Comparing the results with other clinical blood tests, it is clear that the clinical result is the correct result. The error in the research result is thought to be due to too small sample. Of note, the patient stopped his vitamin D supplements in November 2011.

### Table 5: difference between clinical and research results.

<table>
<thead>
<tr>
<th>Date</th>
<th>Days between collection and assay</th>
<th>Clinical or research aliquot</th>
<th>25(OH)D (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/05/2011</td>
<td>2</td>
<td>clinical</td>
<td>176</td>
</tr>
<tr>
<td>30/09/2011</td>
<td>5</td>
<td>clinical</td>
<td>191</td>
</tr>
<tr>
<td>30/09/2011</td>
<td>174</td>
<td>research</td>
<td>93</td>
</tr>
<tr>
<td>14/05/2012</td>
<td>2</td>
<td>clinical</td>
<td>118</td>
</tr>
</tbody>
</table>

5.6.4 Conclusions of establishing methodology for vitamin D assay

Overall, there is very good correlation between the values obtained from clinical and research samples but not as good agreement as expected given the fact that the samples were taken at the same time, from the same patient and analysed in the same way, in the same laboratory. Accuracy of the test is said to be within 1%. A decision was made to include clinical, research and pilot study samples. If more than 1 result was available, the clinical result was prioritised for inclusion as these results were obtained from samples processed immediately reducing potential factors, followed as needed by the research result as this my own study and samples retrieved and observed by me, then pilot study result (Figure 11).

In summary, the results include 71 research samples, 34 pilot and 37 clinical values.
* eligible patients were patients with a diagnosis of CF, PCD, bronchiectasis or healthy control and had BALF available

** new method vitamin D result not available; patients had either not had vitamin D level measured or this was done prior to June 2010

Figure 11: Consort diagram to show determination of vitamin D samples used in the study
5.6.5 Definition of vitamin D deficiency

Data were explored using vitamin D as both continuous and categorical data. For the latter, patients were grouped into those sufficient (50 and over) and those insufficient (less than 50). The cut-off values used here were chosen as they are the most frequently used cut-off values in clinical practice (273-276). Based on values used in other studies (227, 266, 277-279) and to ensure that signals were not being missed, post-hoc analyses were also performed using alternative cut-off values (Table 6).

Table 6: various cut-off values used for vitamin D deficiency
Post hoc analyses were performed using different cut-off values for vitamin D sufficiency and insufficiency.

<table>
<thead>
<tr>
<th>Original cut-off values</th>
<th>Insufficient</th>
<th>Relatively sufficient</th>
<th>Sufficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;50 nmol/L</td>
<td>≥50 nmol/L</td>
<td></td>
</tr>
<tr>
<td>Alternative 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternative 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alternative 3</td>
<td>Deficient</td>
<td>Insufficient 50–74 nmol/L</td>
<td>Sufficient ≥75 nmol/L</td>
</tr>
<tr>
<td></td>
<td>&lt;25 nmol/L</td>
<td>25–74 nmol/L</td>
<td>≥75 nmol/L</td>
</tr>
<tr>
<td>Alternative 4</td>
<td>Deficient</td>
<td>Insufficient 50–74 nmol/L</td>
<td>Sufficient ≥75 nmol/L</td>
</tr>
<tr>
<td></td>
<td>&lt;25 nmol/L</td>
<td>25–49 nmol/L</td>
<td>≥75 nmol/L</td>
</tr>
</tbody>
</table>

(46) (47) (18;48;49) Combin ation of above
5.7 Cytokine analysis

5.7.1 Choice of assay

Cytokine analysis was initially planned to be performed using Luminex (Luminex corporation, Texas, USA) with 17 cytokines chosen to be measured; GRO-α, G-CSF, GM-CSF, IL-1β, IL-2, IL4, IL5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, MCP-1, MIP1-β and TNF-α.

One run was completed but many samples had error readings as insufficient beads were read, perhaps due to bead clumping or to difficulties encountered with the filtration. For optimal results >100 beads should be read in each assay, but for a more pragmatic approach, 50 beads can be taken as acceptable. Even using this lower bead count 43/80 (54%) were insufficient meaning that many readings obtained in these assays were too inaccurate to use. None of the 80 samples had >100 beads read in all 17 assays.

The second run encountered instrument reader equipment difficulties making the results potentially unreliable, and the earlier problem with insufficient bead count was see again in this run. 25 samples were analysed before the machine stopped due to technical difficulties; of these 25 samples 13 were excluded for insufficient bead readings. The technical difficulties with the machine were overcome and the entire plate with all 80 samples on measured. Just 24/80 (30%) samples could be used due to insufficient bead counting. From part of this plate being read twice, 7 samples had sufficient quality readings on both readings to use and so these 7 samples had their 17 cytokine assays compared.
Good agreement was seen in the few samples with results available from both readings in this second run and further analysis was then planned comparing samples assayed in both runs. 29 samples from the 1st run were repeated but just 3/29 had valid results and none of these 3 had valid results from run 1; comparison of the 2 runs could therefore not be carried out.

Due to problems encountered on both runs, culminating in little usable data, all results were discarded and further consideration given to the techniques available for cytokine measurements. A decision was made to use meso scale discovery (MSD) for measurements of cytokines.

IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, GM-CSF and IL-17a were measured using a combination of single and multiplex cytokine assays (Meso Scale Discovery, Rockville, USA) on BALF.

5.7.2 Choice of cytokine

The pro-inflammatory cytokines IL-8, IL-6, IL-1β, TNF-α are elevated in the CF airway (307) and there are reduced levels of what has traditionally thought be an anti-inflammatory cytokine, IL-10 (99), although mixed functions with pro-and anti-inflammatory properties have been reported (308, 309).

IL-8, produced primarily by activated macrophages, is a potent neutrophil attractor stimulated by certain bacterial pathogens and by activation of the TLRs (310).
IL-6 is another pro-inflammatory molecule secreted by monocytes and macrophages in response to infection; it is involved in the B-cell response signalling and aids the differentiation of T-helper cells, which in turn secrete IL-17 (311).

IL-1β induces production of adhesion molecules that enable leukocytes to migrate into inflamed tissue and is secreted by macrophages, monocytes, dendritic cells and certain epithelial cells (312).

TNF-α, produced by activated macrophages triggers a wide range of signalling pathways including apoptosis, cytokine production and chemoattraction (313). IL-17 was included due to the interest of the department in this pro-inflammatory cytokine, which induces the production of many other cytokines with pro-inflammatory effects; C-CSF, GM-CSF, Gro-α, TGF-β and MCP-1 as well as the previously mentioned pro-inflammatory cytokines (113).

5.7.3 MSD assay

In order to measure the cytokines, an antibody is coated onto a well, or for the multiplex assays, a range of antibodies are coated in a specific pattern (Figure 12). The samples are prepared by the addition of a solution containing detection antibodies (anti-IL-6, anti-IL-8 etc.) each of which is labelled with an electrochemiluminescent compound. During incubation periods, binding occurs and the final solutions are used for the readings. The MSD instrument measures the quantity of emitted light, which provides a quantitative measurement of the desired compound.
BALF samples were processed as per the assay protocol with a few minor changes (detailed here), as the protocol was not written for BALF. 12 μl 10% BSA solution was added to each of the samples (108μl) to achieve a concentration of 1% BSA within each sample. The standards were made up in 1% BSA and not the diluent as stated in the protocol to allow for increased protein content within BALF, as was suggested in the manufactures’ product information and by correspondence with others within the lab experienced in working with this material.

5.7.4 MSD detection ranges and standard curves

Detection limits are set by the MSD software depending on results of the standards; the lower limit of detection (LLD) is 2.5 standard deviations above the zero calibration and therefore varies from plate to plate. For simplicity, the highest of the values for each
individual cytokine from the 3 plates was used. The highest value was used to ensure that samples below the level of detection were not included as this would have meant the inclusion of unvalidated results.

The upper limit of detection (ULD) is set at 2500 pg/ml but extrapolated values are obtained beyond this. For samples that were above the ULD analyses were performed using the extrapolated value and again using just those within the detection range parameters and no notable differences were seen. A decision was made to use the extrapolated values.

5.8 HBD-2 ELISA

5.8.1 HBD-2 measurement

All 144 patients included in the vitamin D part of the study had HBD-2 measured as BAL availability was part of the criteria for inclusion into that study. Some results were above/below level of detection; therefore results are available for 128/144 patients. Of the 77 infants in the NBS cohort, just 14 had HBD-2 results measured and within range.

HBD-2 was measured in BALF samples using ELISA (Phoenix pharmaceuticals, USA). This was performed as per manufacturers’ instructions the lower and upper limits of detection of HBD-2 being 7.8 and 500 pg/ml respectively (314). Samples were diluted 1:1 with assay buffer, therefore after correcting for this dilution the detectable range was 15.6 pg/ml – 1000 pg/ml. A standard curve for this can be seen in Figure 13.
5.8.2 Establishing reproducibility

Intra and inter-run reproducibility and accuracy were considered.

5.8.2.1 Intra-assay reproducibility

Firstly, intra-run reproducibility was assessed. Most assays could not be performed in duplicate due to financial resources, but good agreement was observed in those that were done in duplicate in all but one (Figure 14). This sample was not intended to be a duplicate but was used to fill an empty well left at the end. Neat sample was used, and the last portion of the aliquot used up and this sample gave an undetectable reading, below the level of detection. Given the good agreement of the rest of the samples, it is likely that by using this end of vial sample, fibrinous material was obtained; therefore this result was discarded as spurious. Overall, 14 samples were measured in duplicate and paired values very similar (CoV median (IQR) 4% (0.3-10%). The remainder were run in singlicate.
Next, the reproducibility of samples between runs was compared. The ELISA was run on 4 separate occasions, and to ensure comparability between different runs, some samples were repeated on different runs. Overall, 20 samples were analysed on 2 different runs (Figure 15) with large variation between runs; CoV of 21% (IQR 9.9 – 52.2). This may be due to variation in technique. Whilst there is variation in the values, the ranking order was not significantly different (p = 0.16). The absolute values may therefore not be totally reliable but as the ranking order is not significantly different, this difference in absolute values does not affect the conclusions.
Figure 15: HBD-2 levels (pg/ml) of samples assayed on more than one run.
Whilst there is clearly a variation in the numerical value of results, the ranking order did not change significantly. No significant difference in results between runs was seen. (p=0.16, wilcoxon matched pair test).

5.8.2.3 “Fresh” vs “old” samples

In order to try to explain the differences seen in some samples between runs, the possibility of different results being obtained from samples thawed for the first time (“fresh”) and samples previously thawed and refrozen (“old”) was explored. It was hypothesized that any difference seen could be due to instability of the peptide in the thaw/freeze cycle and
therefore results would be lower from “old” sample than from “fresh” samples. On the 4th run, paired “fresh” and “old” samples were compared

Table 7: Results obtained from “fresh” and “old” samples.
There was no statistically significant difference between the results obtained on these samples (p=0.13) but this may be due to small sample size. Although there are not enough samples for accurate comparisons to be made but the results of this small experiment do suggest that the protein does not denature during the freezing process.

<table>
<thead>
<tr>
<th>Study no.</th>
<th>HBD-2 (pg/ml) “fresh” samples</th>
<th>HBD-2 (pg/ml) “old” samples</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>38.7</td>
<td>41.8</td>
<td>+3.2</td>
</tr>
<tr>
<td>150</td>
<td>71.4</td>
<td>87.5</td>
<td>+16.1</td>
</tr>
<tr>
<td>600</td>
<td>&lt;15.6</td>
<td>&lt;15.6</td>
<td>0</td>
</tr>
<tr>
<td>620</td>
<td>&lt;15.6</td>
<td>31.4</td>
<td>+15.8</td>
</tr>
<tr>
<td>620</td>
<td>&lt;15.6</td>
<td>51.6</td>
<td>+36</td>
</tr>
</tbody>
</table>

The difference between pairs was not statistically significant (p = 0.13); this may be due to small numbers but the results do suggest that no degradation of the peptide has occurred with the thawing/refreezing process. Values are all therefore included in the analysis for this study, from fresh and from previously thawed samples.

5.8.2.4 Dilution experiments
On the 1st run, some results were above and some were below the level of detection. 16% of the serum samples were above the upper level of detection, and the lowest sample concentration was 137 pg/ml, with the kit being able to detect values as low 7.5 pg/ml. Therefore in the next run the samples were further diluted to 1:3 in order to capture more sample results within the range of the ELISA kit. It was therefore very surprising that 22% of
these samples were above the upper level of detection, which was now 2000 pg/ml, compared to 1000 pg/ml on the previous run. The assay had not changed, but once the dilution factor was accounted for, readings up to 2000 pg/ml were now possible. Table 8 and Figure 16 show the medians and ranges of samples assayed in the different runs.

No significant difference was observed in the results of the BALF samples processed in the same way from the two runs and therefore it appeared to be an issue with the samples not with the ELISA run. I hypothesised that this difference seen was due to an effect of dilution and to test this hypothesis dilution curves were planned for the next run.

Dilution curves were performed on two BALF samples and two serum samples, but unfortunately the ELISA kit “stop solution” leaked in transit meaning insufficient volume was available for all ELISA wells and so results were only obtained for the BALF samples. No dilution curves could be obtained for the serum samples. Limited resources prevented more dilution curves being undertaken. It was expected that the read concentrations of different dilutions of the same sample would be different, but once adjusted for dilution, the concentrations would be the same, allowing for the margin of error within the assay. However, this was not the case and the readings were not the same but rather, increased in a linear fashion as shown in figure 8. The same phenomenon was seen in both samples on which the dilution curve was performed (Figure 17).
Table 8: The results of the serum samples assayed

The samples in run 1 were diluted 1:1 and run 2 were 1:3. The 2nd run was therefore able to capture a much higher level of HBD-2 within the sample. However, despite the upper limit of detection being 2000 pg/ml rather than 1000 pg/ml, 22% of samples were above the upper limit of detection. The median of the samples on run 2 was significantly higher than run 1 (p<0.001).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Min HBD-2 (pg/ml)</th>
<th>Max HBD-2 (pg/ml)</th>
<th>Median HBD-2 (pg/ml)</th>
<th>n below detection (%)</th>
<th>n above detection (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>25</td>
<td>137.5</td>
<td>941.6</td>
<td>800.8</td>
<td>0</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>(1:1 dilution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Run 2</td>
<td>36</td>
<td>21.2</td>
<td>1783.3</td>
<td>1270</td>
<td>0</td>
<td>8 (22%)</td>
</tr>
<tr>
<td>(1:3 dilution)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 16: Comparison of serum samples assayed.

Samples diluted 1:3 had significantly higher HBD-2 levels after correction than samples diluted 1:1. This shows that distribution of the molecule is not linear and that assays performed at different dilutions cannot be compared.
Figure 17: Dilution curves of 2 samples with concentrations before and after adjustment for the dilution.

Increased levels of HBD-2 are detected in more dilute samples, this is thought to be due to an effect of altering the protein content within the sample.
The results were reviewed along with the method used in the ELISA. The dilution was performed using assay buffer, which altered the protein content within the sample. The likeliest explanation for this effect of increasing concentration with more dilute samples was having proportionally less protein within the sample affecting the ELISA. This explains why the samples as a group, were so much higher when diluted 1:3 rather than 1:1. This experiment was clearly very useful and showed that differing dilutions cannot be compared. As most samples were assayed using a 1:1 dilution, these samples were used in the analysis and unfortunately, on the basis of this finding, I elected to exclude all other results. Samples performed using other dilutions were excluded from the analyses and re-run where available.

5.8.2.5 Outcome of HBD2 methodology experiments

- Samples with 1:1 dilution only were used in the analysis
- Both fresh and previously thawed samples were included.
- For samples included on more than one run the mean was used.

5.9 LL-37 ELISA

5.9.1 LL-37 measurement

All 144 patients included in the vitamin D part of the study had LL-37 measured as BAL availability was part of the criteria for inclusion into that study. Some results were above/below level of detection; therefore results are available for 134/144 patients. Of the 77 infants in the NBS cohort, just 14 had LL-37 results measured and within range.
LL-37 was measured in BALF using ELISA (Hyocult biotech, Uden, The Netherlands). Samples were undiluted and the ELISA performed as per manufacturer’s instructions (315). The assay allowed measurement over the range 0.1 to 100 ng/ml.

### 5.9.2 Establishing reproducibility

LL-37 results were all performed on the same run so no inter-assay analyses were required. The standard curve (Figure 18) obtained for the ELISA does not show a linear relationship between concentration and optical density and therefore a logarithmic equation to extrapolate unknowns was used.

![Figure 18: Standard curves of LL-37 assay](image)

The curve shows a good line fit between measured absorption and known concentrations.

Due to cost and availability of materials, only 2 samples were run in duplicate. Good agreement was seen in these samples (Figure 19a). As only 2 samples were run as duplicate,
in order to further assess reproducibility of the ELISA, agreement between the 14 standards, run in duplicate, was considered and found to be very good (Figure 19b) with CoV of 0.89% (IQR 0.3 – 3.5%).

The assay was performed on 159 different samples, and the results ranged from less than 0.1 - 30.0 ng/ml; 2 samples fell below the lower limit of detection and so the minimum range detected, 0.1 ng/ml, was used for the analysis of these samples.

Figure 19: Agreement between a) duplicate samples, b) standards and c) bland-altman bias plot of standards
Good agreement is seen amongst duplicate samples/standards; CoV 0.89% (IQR 0.3 – 3.5%); standards bias 0.01, limits of agreement -0.06 to 0.08.
5.10 Microbiology of BALF samples

Microbiology results were obtained from the clinical laboratory which follows published standards for the processing of CF samples (304). Patients were divided into culture positive and culture negative groups; molecular techniques were not utilised. The culture positive group was comprised of those with *any* bacterial or fungal growth in their BALF sample and the negative group had sterile BALF. As the clinical laboratory changed its protocol from immunofluorescence to PCR during the study period, data on viral infections have not been analysed.

5.11 Statistical analyses

The sample size is opportunistic, as there were no data to inform a power calculation. Data are presented as median and 95% confidence intervals of the median. Non-parametric analysis was used: Mann Whitney for 2 groups, Kruskal-Wallis with Dunn’s correction for multiple groups, and logistic regression for binary outcomes. In view of the multiple comparisons undertaken, the null hypothesis was rejected at $p<0.01$. SPSS v21 (IBM Corporation, New York) and Graphpad prism v6 (Graphpad software, San Diego) were used.
PART C: STUDY SPECIFIC
INTRODUCTION, METHODS,
RESULTS AND DISCUSSION
6 Chapter 6: The role of Vitamin D in infection and inflammation in the airways of infants diagnosed on new born screening

6.1 Introduction

The purpose of this study was to explore the infection and inflammation present in a large cohort of infants diagnosed on newborn screening and to examine the relationship between these findings and vitamin D levels, to try to determine if Vitamin D was an immunomodulator in this age group. As vitamin D is thought to exhibit immune modulation via the innate immune pathway, of major importance in early life, I hypothesised that there may be a greater effect of immune modulation seen in young infants than older children.

The hypothesised mechanism of action was a direct impact of vitamin D on the antimicrobial peptides LL-37 and HBD-2 with subsequent effect on infection and the inflammatory cascade.

6.1.1 Early lung disease outcome measures

Once children are diagnosed with CF, treatments are started with the aim of improving their prognosis and their long-term lung health. The onset of lung disease is seen early in CF (as described in section 2.4.1) and if not treated aggressively leads to more rapid decline in respiratory function. In its early stages, CF lung disease may be silent with few or no clinical symptoms meaning that history and examination are not sufficient for accurate early detection. Detection of early lung disease is important and other markers are therefore
needed but are not without their challenges. The ideal scenario would be to assess function, structure, microbiology and inflammation of the airways, but procedures to gain this information involve more co-operation than is often possible in such young children, and are invasive and many require sedation or general anaesthesia; in addition there is the fact that young children are vulnerable to the side effects of radiation so this exposure would ideally be limited. Due to these challenges, collecting data can be difficult and confidence is required, as much as is possible, that such tests will be beneficial and it is worthwhile subjecting the child to such procedures. Functional and structural changes are discussed in more detail in chapter 7.

6.1.2 Inflammatory changes

Detecting airway inflammation usually requires direct airway sampling, which is invasive and expensive. Studies of infants with CF have found airway inflammatory changes in addition to structural changes, and this was associated with worse lung function (56, 316). A study of 127 infants with CF diagnosed on newborn screening found that elevated neutrophil elastase at 12 months of age was associated with CT evidence of bronchiectatic change at 12 months and at 3 years of age (157).

A study to evaluate BALF directed therapy versus standard treatment was carried out and no differences in the outcomes of *P. aeruginosa* infection or HRCT chest score at age 5 were seen (317). Increased BALF cell and neutrophil counts were seen in those who had evidence of bronchiectasis on their CT scan but not in those with just airway wall thickening or air trapping (316).
6.2 Hypotheses

The earlier institution of improved and more aggressive treatments strategies possible in infants diagnosed with CF by NBS leads to a healthy airway during the first year of life.

Vitamin D has immunomodulatory effects via antimicrobial peptide pathways; consequently vitamin D deficiency will lead to greater risk of bacterial infection and subsequent inflammation and worse clinical outcomes in CF throughout childhood.

6.3 Methods

In order to assess the health of the airways of infants diagnosed on newborn screening, and therefore diagnosed early with early onset of treatment, the airways were examined as previously described (section 5.2). Comparisons were made with 2 ‘control’ groups; a disease control group and a healthy control group. The disease control group consisted of children over the age of 2 years with an established diagnosis of CF who had undergone clinically-indicated bronchoscopy and had cellular inflammatory data from the BALF available (group 1b). The age of 2 years was chosen so that there was a clear distinction between this and the NBS group and no potential overlap, whilst still including young children. The healthy controls had no lower respiratory symptoms and normal lower airways on microscopic and macroscopic examination; this group is the same as the control group used in other sections of this thesis and are not specifically recruited to act as a comparison for new born infants (group 3).
Statistical tests unless stated otherwise in this chapter are Mann-Whitney for comparison of 2 groups and Kruskal-Wallis with Dunn’s correction for multiple groups. As before, due to multiple comparisons a priori p value was set at < 0.01.

### 6.4 Results

#### 6.4.1 Subjects

77 infants who were referred to the RBH following positive NBS were confirmed as having CF during this study period. Four of these infants were not included in the study; 1 patient moved away from the area shortly after diagnosis, 1 patient did not have FOB performed by parental choice and 2 patients were excluded for significant co-morbidity (neurometabolic disorder and mitochondrial disorder) making them unrepresentative of the usual CF population. Four additional patients were diagnosed following a positive NBS elsewhere and were referred to our centre within the first few weeks of life and these 4 patients are included in the study. There were therefore a total of 77 patients recruited to the study with 73 diagnosed at RBH and 4 diagnosed elsewhere. 62/77 of these patients had samples suitable for inflammatory cell analysis. Data was obtained from 75 paediatric patients with established CF and 6 healthy controls, all of whom underwent FOB. Demographic details of all 3 study groups, plus the NBS sub-group with inflammatory data available, can be seen in Table 9.
The median age of infants undergoing FOB was 3.5 months, with a range of 2 to 9 months and 40/77 (52%) were male. As the group being studied was infants they were younger by definition than the disease control group. Unfortunately, samples were not available from healthy infants so the healthy controls are also older.
<table>
<thead>
<tr>
<th></th>
<th>NBS infants - all</th>
<th>NBS infants – inflammatory analysis group</th>
<th>Established CF</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>77</td>
<td>68</td>
<td>75</td>
<td>6</td>
</tr>
<tr>
<td>Median age in months (range)</td>
<td>3.5 (2–7)</td>
<td>3.5 (2–7)</td>
<td>112 (28–211)</td>
<td>149 (126–185)</td>
</tr>
<tr>
<td>BMI z-score median (95% CI)</td>
<td>-0.87 (-1.42 to -0.57)</td>
<td>-0.93 (-1.48 to -0.57)</td>
<td>-0.28 (-0.56 to -0.19)</td>
<td>1.11 (0.30 to 2.74)</td>
</tr>
<tr>
<td>Weight z-score median (95% CI)</td>
<td>-0.65 (-1.26 to -0.20)</td>
<td>-0.60 (-1.01 to -0.06)</td>
<td>-0.44 (-0.63 to -0.17)</td>
<td>1.11 (0.25 to 2.84)</td>
</tr>
<tr>
<td>Height z-score median (95% CI)</td>
<td>-0.07 (-1.18 to 0.82)</td>
<td>-0.05 (-0.99 to 0.89)</td>
<td>-0.48 (-0.74 to -0.18)</td>
<td>0.23 (-0.08 to 1.86)</td>
</tr>
<tr>
<td>Male</td>
<td>40 (52%)</td>
<td>37 (54%)</td>
<td>27 (36%)</td>
<td>2 (33%)</td>
</tr>
<tr>
<td>Phe508del*</td>
<td>56/61 (92%)</td>
<td>44/50 (90%)</td>
<td>61/75 (91%)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

- At least one allele
6.4.2 Nutritional status of NBS CF infants

86% of the infants were pancreatic insufficient as shown by a measurement of faecal human pancreatic elastase of less than 15 μg/g and were receiving pancreatic enzyme replacement therapy. Despite these infants being diagnosed on newborn screening and pancreatic supplements started from a very early age, nutritional state was poorer than that seen in the general population with a median z-score (95% CI) BMI of -0.87 (-1.42 to -0.57). Median (95% CI) height was -0.07 (-1.18 to 0.82) and weight -0.65 (-1.26 to -0.20) z-scores.

6.4.3 Clinical symptoms

Data regarding the presence or absence of clinical respiratory symptoms at the time of bronchoscopy are not available for all patients. Of the 38 infants in whom this data available, 13 (34%) reported clinical respiratory symptoms at the time of the bronchoscopy. More patients with bacteria isolated in their airways had symptoms compared with those with no infection identified although this was not statistically significant (p = 0.08), most likely due to the small numbers. 54% (7/13) culture positive patients reported respiratory symptoms and 24% (6/25) culture negative patients reported symptoms.

6.4.4 Infection

23 infants (30%) isolated 26 bacterial organisms from their BALF on routine culture. There was no difference in age between those who had positive bacterial cultures and those who did not. When comparing this to the non-CF CSLD control group and those with established
CF disease, slightly more of the children with established CF, 30/75 (40%) isolated bacteria on their BALF but this was not statistically significant (Fishers exact test p = 0.2). The specific bacteria that were isolated at bronchoscopy can be seen in Figure 20.

6.4.5 Inflammation

The median BALF absolute cell count of the NBS group was higher than the healthy control group in this study but not statistically significantly so on 3-way comparison (p = 0.18) (Figure 21a). The children in the established CF cohort had a higher BALF absolute cell count than either of the other groups (p < 0.001). A similar picture was seen with the BALF neutrophil differential (Figure 21b). Neutrophilic inflammation was seen in both the NBS and the established CF group with significantly more inflammation in the established CF group (p<0.0001). The median (95% CI) neutrophil differential for NBS infants and established CF were 9.3% (6 -15%) and 38.7% (34.6 - 49.7%) respectively. A much lower neutrophil differential was seen in the healthy control group, median 1.4%, (95% CI 0.3 to 2.7 %), which is of course inevitable from the pre-determined patient selection for this group. The established CF group had a statistically significantly higher neutrophil differential than the other 2 groups. Although there was neutrophilic inflammation in the NBS cohort compared with the healthy group, on 3-way comparison this was not statistically significant (p = 0.07).
Figure 20: Pie chart of bacterial organisms isolated on BALF in NBS CF infants

P. aerug = Pseudomonas aeruginosa
S.aureus = Staphylococcus aureus
MRSA = Methicillin resistant staphylococcus aureus
S.Malt = Stenotrophomonas maltophilia
S.Pneumo = Streptococcus pneumoniae
M. catt = Moraxella catarrhalis
H.Inf = Haemophilus influenzae
Figure 21: BALF cell counts of the 3 patient groups.

a) The children in the established CF cohort had a higher BALF absolute cell count (median (95% CI) 977 (643 to 1620) x10³), than either of the other groups (p < 0.001). There was no statistical difference between the median (95% CI) cell count of the infants in the NBS group (355 (205 to 568) x10³) and the healthy control group (143 (70 to 270) x10³) (p = 0.18).

b) Figure shows the neutrophil differential of BALF of the 3 patient groups. The median (95% CI) of neutrophil differential for NBS infants and established CF were 9.3% (6 to 15%) and 38.7% (34.6 to 49.7%) respectively. There was a much lower neutrophil differential in the healthy control group with a median of 1.4%, (95% CI 0.3 to 2.7 %). Shaded area represents generally accepted upper limit normal.
6.4.6 Relationship between infection and inflammation

Comparison was then made of infants with positive bacterial culture and those with sterile BALF. Infants with bacteria identified had a trend towards a higher neutrophil count ($p=0.04$) (Figure 22) and more symptoms ($p = 0.08$). There were insufficient numbers of children isolating *MRSA*, *Staphylococcal aureus* and *P. aeruginosa* to make useful comparisons of any different effects of these micro-organisms.

![Figure 22: Inflammation according to microbiologic status](image)

The different coloured dots represent the individuals with the organisms generally considered to be the most significant. The child with *MRSA* (red dot) had one of the greatest levels of inflammation seen in this cohort, but the 3 with *S. aureus* (blue dots) are divided, with 2 having relatively normal neutrophil differential counts whilst the other had a significant neutrophilia. One of the patients with *P. aeruginosa* isolated, (purple dot), also had evidence of neutrophilic inflammation.
Table 10: Comparison of infants with culture +ve and culture –ve BALF

<table>
<thead>
<tr>
<th></th>
<th>NBS - all</th>
<th>NBS – bacteria +ve</th>
<th>NBS – bacteria -ve</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>77</td>
<td>23</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Age (months)</td>
<td>3.5 (2-9)</td>
<td>3.4 (2-6)</td>
<td>3.6 (2-9)</td>
<td>0.15</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>61.0</td>
<td>59.95 (56.2 – 61.8)</td>
<td>61.0 (57.3 – 63.5)</td>
<td>0.28</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>5.76</td>
<td>5.38 (4.46 – 6.16)</td>
<td>5.8 (5.48 – 6.3)</td>
<td>0.11</td>
</tr>
<tr>
<td>Respiratory symptoms at FOB</td>
<td>13/38 (34%)</td>
<td>7/13 (54%)</td>
<td>6/25 (24%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Previous P. aeruginosa</td>
<td>4/73 (5%)</td>
<td>1/23 (4%)</td>
<td>3/50 (6%)</td>
<td>1.00</td>
</tr>
<tr>
<td>BALF total cell count (x 10³)</td>
<td>355 (205 – 568)</td>
<td>574 (178 – 1047)</td>
<td>343 (193 – 510)</td>
<td>0.16</td>
</tr>
<tr>
<td>BALF neutrophil differential (%)</td>
<td>9.3 (6 – 15)</td>
<td>15 (6 – 43)</td>
<td>8.4 (3 – 13)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Priori p value p < 0.01

6.4.7 Antimicrobial peptides

Antimicrobial peptide levels were only available for 13 (17%) of this cohort; 5 of these infants had bacteria isolated in their BALF and 7 did not. Even with these small numbers there was a trend towards more LL-37 in infected BALF (p = 0.06) but no difference in BALF HBD-2 according to infection state (p=0.1). No correlation was seen between the antimicrobial peptides and cellular or soluble markers of inflammation.

6.4.8 Vitamin D in relation to infection and inflammation within the airways

6.4.8.1 Vitamin D levels

48/77 of the NBS cohort had vitamin D measured at the time of the bronchoscopy. Median (95% CI) vitamin D was 77 (72: 84) nmol/L and only 4 babies were deficient (< 50 nmol/L).
6.4.8.2 Vitamin D and Antimicrobial peptides

Contrary to my hypothesis, no relationship between vitamin D and the antimicrobial peptides HBD-2 and LL-37 was seen (Table 11).

### Table 11: Correlation of vitamin D with antimicrobial peptides

<table>
<thead>
<tr>
<th>Inflammatory marker</th>
<th>Spearman r</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF LL-37 ng/ml</td>
<td>0.03</td>
<td>0.93</td>
</tr>
<tr>
<td>BALF HBD-2 (pg/ml)</td>
<td>0.20</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Linear correlation statistical test

6.4.8.3 Vitamin D and Inflammatory markers

No relationship was seen between vitamin D and any of the cellular or soluble inflammatory markers.

6.4.8.4 Vitamin D and microbiology

Vitamin D levels were similar in infants who isolated bacteria in their BALF and those who did not (p = 0.85). Median (95% CI) vitamin D was 77 (54: 99) nmol/l in the positive group and 78 (72: 85) nmol/L in the negative group (Figure 23).
6.5 Discussion

6.5.1 Statement and interpretation of principal findings

I hypothesised that earlier, improved and more aggressive treatments made possible through NBS programmes would lead to better airway health in the first year of life and also that vitamin D has immunomodulatory effects with vitamin D deficiency leading to worse airway health with more infection and inflammation via the antimicrobial peptide pathway.

Contrary to this hypothesis, no relationship was seen between vitamin D and cellular or soluble inflammation in this cohort of NBS diagnosed infants with CF. Additionally, no relationship was identified with vitamin D and the antimicrobial peptides LL-37 and HBD-2, which were hypothesised to be the mechanism of action.
LL-37 and HBD-2 have antimicrobial activity, and given their lack of relationship with vitamin D it was not surprising that following on from this, there was no difference in vitamin D level of those with infection in their BALF and those without. This suggests that not only is vitamin D not impacting on infection via antimicrobial pathway, but that no other Vitamin-D dependant pathway is involved instead. However, an alternate explanation may be that the study was underpowered to show this.

A large proportion of the NBS infants in this study had evidence of infection and inflammation within their airways despite being diagnosed on routine screening and having treatment implemented within the first few weeks of life. Almost 1/3 had bacterial growth on their BALF and some infants had neutrophilic inflammation with levels of up to 75%. Unsurprisingly, more inflammation was seen in those with bacterial growth but this was not statistically significant which may be due to the small number or perhaps due to sampling errors for infection. Some infants without any bacterial infection detected also had increased neutrophilic inflammation.

Data were not available for all infants for all parameters. The low numbers of available antimicrobial peptides, vitamin D and cytokines mean that limited conclusions can be made and may contribute to an underpowering effect and if all data had been available it is possible that outcomes would be different.
6.5.2 Strengths of the study

A large number of infants were included in the study, which is a major strength. In addition, the study is able to compare clinical data with invasive markers of inflammation. By collecting information from bronchoscopy and BALF, the inflammatory status of the airway could be assessed and correlated with other invasive sampling results such as bacterial growth, but also viewed in relation to clinical symptoms experienced by the infants.

6.5.3 Limitations of the study

Due to the fact I used clinically collected data rather than proforma driven data, on occasions, the outcome of interest was missing. This was the case regarding data on whether the infants were symptomatic or asymptomatic at the time of their bronchoscopy. It is important in such situations to be aware of potential reporting bias. I suspect the percentages reported here are significant overestimates of the true figures due to reporting bias as the presence of symptoms is far more likely to be documented than the absence of symptoms. However information can still be gained from the data that are available. At least 2/3 of the infants were asymptomatic at the time of the procedure. Almost half of those with bacteria isolated in their airways had no respiratory symptoms at all at the time of the bronchoscopy which supports earlier findings that infection is seen at an early age even in asymptomatic infants.

It is impossible ethically to obtain samples from truly healthy infants. The control group is significantly older than this group of NBS CF infants reflecting this. Unfortunately samples
were not available on age matched healthy individuals. This is an issue in all studies of this nature.

Neutrophil elastase was not measured in the present study and with retrospect would have made a good addition to the current findings.

6.5.4 Findings in relation to other studies

BALF neutrophil differential count was higher in infants with CF than the healthy controls; although a criterion for entry into the control group was neutrophil differential less than 4% it can still be seen that even at this early age the airways of infants with CF are abnormally inflamed compared with healthy individuals. When evaluating airway inflammation in infants, it is difficult to say what is normal due to limited data on absolute cell counts and neutrophil differentials in healthy infants. Tessier et al (318) obtained samples from 16 healthy children, of whom 8 were aged under 12 months. Of these 8 infants, the median total cell count was $350 \times 10^3$, (range $180-680 \times 10^3$) and mean neutrophil differential 1.5%, (range 0-3%). Another group (319) found the median cell count and neutrophil differential to be much higher in their population. Of the 9 infants within their study, the median cell count was $400 \times 10^3$ (range $200-1300 \times 10^3$) and the neutrophil differential 7% (range 2-17%).

The median total cell count of the CF NBS population in my study was higher than that of normal healthy infants regardless of which of these two ‘normal’ value cut-offs are chosen for comparison. Even taking the higher suggested value discussed above, 19/49 (39%) of the CF NBS infants had evidence of neutrophilic inflammation with values above the 95th
confidence interval (11.6%). In addition, there is a strong skew towards greater level of inflammation in this cohort, with some significantly raised values. There were no other striking features about these infants compared to the others; they were no older, they were a mixture of genotypes, pancreatic status and sex in similar proportion to the entire groups and one of these infants had no bacteria isolated on BALF.

Similarly to previous studies, there was an increase in inflammatory markers within this population (103) and a trend towards an increase in those with bacterial infection compared to those with sterile BALF (320). Brennan et al, as part of the Australian Respiratory Early Surveillance Team for CF (AREST CF) study evaluated airway inflammation of 24 children and found a neutrophil differential of 52% in those with infected BALF compared with 17% in those without infection. The total cell count was also increased; $1290 \times 10^3$ vs. $340 \times 10^3$. The neutrophilic inflammation seen in this population was greater than the cohort in my study, which likely reflects the older age of these patients, a median of 19 months compared with 3.4 months in my study population. Another study by the same group explored airway inflammation shortly after diagnosis (321). Infants were diagnosed on NBS at a median age of 28 days and had FOB and BAL carried out at a median age of 3.6 months. In agreement with the present study, the authors found evidence of airway inflammation with a BAL total cell count of $242 \times 10^3$, ($355 \times 10^3$ in my study); and neutrophil differential of 18.7% (IQR 9.4 to 35.7%) (9.3% (IQR 3.2 to 20%) in my study). Similarly, increased neutrophil differential count changes were seen in the presence of bacterial infection.

To my knowledge this is the largest cohort of infants with CF diagnosed on NBS to have BALF examined for infection and inflammatory changes. Almost one third of infants isolated at least one bacterial organism and there is evidence of airway inflammation by 3.5 months of
age. Levels of vitamin D did not have any association with the presence of infection or any of the measured markers of inflammation. The results suggest that abnormal lung function at 3 months as found in other studies, may be due to the presence of airway inflammation suggesting early treatment indicated, even in the absence of symptoms. The cause of the inflammation needs to be determined in order for this to be treated. Patients with inflammation and no detected infection were not targeted for treatment other than their routine antibiotic prophylaxis (twice daily oral flucloxacillin from diagnosis) and perhaps if they had been treated with more infection cover less inflammation may have been seen.

6.6 Conclusions

Disappointingly neutrophilic airway inflammation and bacterial infection were present in the airways of infants diagnosed on NBS despite early treatment. This may be because the airway is abnormal at birth as suggested by the CF pig, or may be due to early bacterial exposure.

The evidence here does not support a role of vitamin D in infection and airway inflammation of this cohort of young infants. This is a young group of patients, possessing less chronic inflammation and likely proteolytic activity within the airway than those with more established CF lung disease and therefore the chance that a significant relationship is being masked by degradation is likely reduced.
I aimed to evaluate the difference between patients who were vitamin D sufficient and those with vitamin D deficiency, but only 4 patients in this group had deficient levels. I therefore decided to explore this further in older patients to assess the relevance of vitamin D over a wider range of vitamin D values and to ascertain its role in more established disease (chapter 8).

I also wanted to explore the impact that vitamin D level may have longitudinally, so before going on to explore an expanded CF cohort, addressed the question of whether vitamin D at 3 months was associated with longitudinal outcome measures, or whether it could be viewed as a biomarker of disease severity predicted future outcomes (chapter 7).
7 Chapter 7: Longitudinal study of newborn screened infants; infection, inflammation, structural and functional change and serum vitamin D levels

7.1 Introduction

As has already been discussed and demonstrated in my data in Chapter 6, infection and inflammation are seen early on in children with CF and these are associated with long-term adverse structural and functional changes.

This chapter explores the impact on inflammation during that first year of life, and the structural and functional changes associated with this. It also looks at the impact of vitamin D to ascertain if there is a clinical relevance of this; although the results have shown that it is not of relevance to outcomes in the cross-sectional study, this study explores its predictive value. If vitamin D was found to be a better biomarker for adverse outcomes this could define a group in whom intensification of conventional therapy, or early introduction of novel molecular therapies, could be justified.

7.1.1 Early functional changes

The LCFC comprises 6 paediatric CF centres within London; Royal Brompton Hospital, Great Ormond Street Hospital (GOSH), The Royal London Hospital, Kings College Hospital, St Helier Hospital and Lewisham University Hospital. Infants from each of these 6 centres were
recruited to a longitudinal study to evaluate their lung health. Importantly, this longitudinal study also recruited contemporaneous healthy controls.

The Collaboration began prior to newborn screening being implemented in the region and the initial studies were conducted on children diagnosed clinically. They reported that, at diagnosis, infants with CF (even with no respiratory symptoms) had airflow obstruction measured by the raised lung volume rapid thoracoabdominal compression technique (RVRTC) compared with healthy controls (296, 297) and that this abnormality persisted on follow-up testing at both 6 months post-diagnosis (138) and 3-5 years of age (139) despite appropriate treatments.

Following the introduction of NBS a new cohort of infants was recruited. Despite early identification of these infants and early initiation of treatment, lung function was also abnormal when tested at a median of 3 months of age. By this age, hyperinflation, diminished airway function (FEV0.5) and increased ventilation inhomogeneity (lung clearance index (LCI)) was already present in over 1/3 of infants and values were significantly worse than healthy age-matched controls (55). Surprisingly, by the age of 12 months, these infants had stable or improved lung function (322). Improvements have been maintained out to 2 years (322, 323). Previous work at my centre found that abnormal airway obstruction occurred within the first year and was independent of infection, inflammation or symptoms in clinically diagnosed children (145). The AREST CF study is a longitudinal study evaluating inflammatory, functional and structural change in the airways of CF children diagnosed on newborn screening and this study did not find evidence of airflow limitation in their infants with CF, although a decline was seen in the first 2 years of life (56). Pre-school results
however, do suggest that this significant deterioration in lung function is in fact not real (324) so this deterioration in lung function in the early years should be viewed with caution.

Multiple-breath washout (MBW) tests have been carried out in young children with CF and the outcome measure, LCI, found to be a sensitive marker of early lung disease. The LCFC evaluated the LCI of 48 children with CF, diagnosed clinically, and 45 healthy controls. LCI was abnormal in almost three-quarters (73%) of pre-school children and of these the majority had persisting abnormality with elevated LCI also being present at school age. The results suggest that LCI may be a good marker to detect early lung disease (325). MBW has been shown to be a more sensitive measure of early lung disease than spirometry or plethysmography (326) and superior to measurement of cough frequency and nocturnal oxygen saturations which found no difference between CF pre-school children (n=20) and healthy age matched controls (n=30) (327). In older children LCI is more sensitive than spirometry but in infants this is less clear and there appear to be some differences in physiology of tests in this age group; LCI and RVRTC are complimentary, with each detecting abnormalities not detected by the other (328).

Where airway function is decreased, whether this is due to inflammation, infection or an innate abnormality remains unclear. The CF pig suggest that airway cartilages are abnormal at birth, but the improvement seen in the LCFC NBS infants over the first year suggests a shorter term abnormality that can be improved with treatment, rather than an abnormality pre-dating birth and exposure to a non-sterile world.
7.1.2 Early Structural changes

Structural changes found on thoracic HRCT scans, in the form dilated airways, airway wall thickening, air trapping and large and small mucous plugs have been demonstrated to occur very early in life (329-331), including in asymptomatic infants. This finding has been said to suggest that structural changes as seen on HRCT scan may be an early marker of lung disease. In the AREST-CF study, structural changes were seen in 80% of infants at the age of 3 months following diagnosis on newborn screening (321). This study concluded that the structural changes occur early, within the first few years of life, and are related to increased infection and increased inflammation (316). Longitudinal CT scans showed progression of lung disease in most cases, although apparent resolution of abnormalities was seen in 26% (140) to 40% (157) questioning the accuracy of the interpretation of the initial scan result. An alternative explanation for this is a potential over-interpretation of small changes on the initial scans. It may be that what is thought to be irreversible lung damage is in fact reversible pathology. Bronchiectasis is defined as the bronchial diameter being greater than its corresponding artery but airway dilatation maybe due to mucus plugging and airway wall thickening due to transient airway oedema, both reversible with treatment rather than the irreversible pathological process of bronchiectasis.

As part of the LCFC study, thoracic HRCT scans were performed at 12 months of age and changes were very mild. In addition, despite rigorous prior training, interobserver agreement of changes was poor (mean (95%CI) k coefficient 0.34 (0.20 to 0.49)) meaning that the technique is of questionable value in determining longitudinal changes in the lung health of infants (332). In the AREST CF study, just one person interpreted all studies so there was no interobserver agreement assessment. Intraobserver repeatability was fair to
good for diagnosis of bronchiectasis and air trapping (k coefficient 0.64 and 0.55 respectively) and poor for airway wall thickening (k coefficient 0.24)(316).

Whilst this evidence from the AREST CF study appears to suggest that such structural lung change is a good marker of early disease, the apparent resolution of bronchiectasis, methodological concerns regarding inter-and intra-observer repeatability and lack of similar data being seen in all populations casts some doubt over the reliability of thoracic HRCT scans as a source of early lung detection and longitudinal data collection.

7.2 Hypotheses

In this section of my work, I tested my hypothesis that the earlier institution of improved and more aggressive treatments strategies possible in infants diagnosed with CF by NBS leads to a healthy airway during the first year of life.

I also explored vitamin D as an immunomodulatory agent in this cohort testing my hypothesis that vitamin D deficiency will lead to worse clinical outcomes in CF.
7.3 Methods

7.3.1 Patient selection

19 of the infants included in my NBS CF cohort were invited to be part of the LCFC study for which they underwent

- A further bronchoscopy at 12 months of age
- Lung function at 3 and at 12 months of age
- HRCT thorax at 12 months of age.

19 of the 77 infants included in my NBS cohort had data available at 12 months of age and therefore longitudinal evaluation could be made of these infants based on bronchoscopy findings at 3 months.
7.3.2 Longitudinal evaluation

Using available data from the 3 month bronchoscopy, I explored the health of the airways at 12 months to see if 3 month data can be used to inform of the health of the airways at 12 month, and evaluated longitudinal change over the time period where data allowed.
To see if vitamin D levels could be a biomarker and predictive, even if causative, of airway function later on, vitamin D levels from 3 months of age were compared to physiological airway results from 12 months.

### 7.3.3 12 month bronchoscopy

FOBs were carried out at 12 months of age as part of the LCFC study. All children recruited to this study had the procedure carried out at one of 3 of the CF centres and all infants included in my study had this performed at Royal Brompton Hospital. Informed consent was obtained and the procedure carried out as described in section 5.2.

### 7.3.4 HRCT thorax

All thoracic HRCT scans were done under GA at 12 months of age, under the same anaesthetic as the bronchoscopy. CT scan was performed using a standardised protocol devised by the LCFC team with details of not just radiation and scanning but inflation pressures (332) to ensure meticulous scan methodology for standardisation; a team member was present at all scans to ensure good protocol adherence at all centres. All infants included in my study had this performed at Royal Brompton Hospital. CT scan was performed immediately prior to the FOB.

Images were sent to GOSH for review and were assessed by 2 independent radiologists using the Brody scoring system (141, 333). Despite one of the assessors being Dr Brody, and the second assessor being trained in the scoring system by Dr Brody, poor agreement was
seen (332). Each CT thorax was marked on set criteria and the scores for each assessor were kindly made available to me for the 19 patients included in my study.

7.3.5 Lung function

Infants attended GOSH at 3 months and 12 months for lung function testing to be carried out; airway function at raised volume was measured using RVRTC technique and MBW test was performed with inhalation of sulpha-hexafluoride during tidal breathing. Testing was performed at both time points during quiet sleep using oral or rectal sedation and has been described in detail elsewhere (322).

Functional resistance capacity (FRC), FVC and FEV0.5 measurements were obtained from RVRTC and LCI measurements were obtained from MBW testing.

7.3.6 Statistics

As stated previously, non-parametric analysis was used: Mann Whitney for 2 groups, Kruskal-Wallis with Dunn’s correction for multiple groups, Fisher’s exact test for categorical outcomes and Wilcoxon paired test for paired samples. In view of the multiple comparisons undertaken, the null hypothesis was rejected at p<0.01. SPSS v21 (IBM Corporation, New York) and Graphpad prism v6 (Graphpad software, San Diego) were used
7.4 Results

7.4.1 Subjects

19 (25%) of the 77 NBS infant cohort also had a routine bronchoscopy performed at 12 months of age as part of the LCFC study. This subgroup did not differ from the main cohort in terms of age, weight at 3 months or sex.

BALF absolute cell count from the time of the 12 month bronchoscopy was available in just 5/17 and cytokine data in just 6/17 so these parameters were not explored.

Vitamin D was available from blood taken at the 3-month FOB in 12/17 infants and this was compared to later results to establish if vitamin D levels has a role to play in determining future lung health. Median (95% CI of the median) vitamin D level was 79.5 (66 - 87) nmol/L, similar to that of the entire NBS cohort (77 (72 - 84) nmol/L).

7.4.2 Lung function

Mean (95% CI) FEV0.5 z-score was -0.76 (-1.38 - -0.13) at 3 months which improved slightly throughout the first year of life to -0.48 (-1.16 - 0.2) at 12 months of age, although this did not reach statistical significance (p=0.08, Wilcoxon matched paired test).
7.4.3 Infection at 3 months does not predict infection and inflammation at 12 months

The infants who underwent FOB at 3 months were divided into those with and without positive bacterial isolates in their BALF and comparison of the airway infection and inflammation at 12 months of these 2 groups made. 5/17 (29%) infants had culture positive BALF and 12/17 (71%) were culture negative from the bronchoscopy at 3 months. Those with culture negative BALF at 3 months had median neutrophil differential of 9.3% (95% CI 1.7 to 24%) at 12 months which was similar to those with culture positive BALF who had a median neutrophil differential of 8% (95% CI 3.7 - 10.7%) (p = 0.72)(Figure 25).

There was no relationship seen between identification of bacteria at 3 months and identification of bacteria at 12 months. 4/12 (33%) of those with culture negative BALF at 3 months and 2/5 (40%) culture positive infants went on to have positive culture at 12 months of age (Fisher’s exact test p = 0.1) (Figure 26).

Figure 25: BALF neutrophil differential at 12 months according to culture status on 3 month bronchoscopy

There is no difference seen in the BALF neutrophil differential of the BALF at 12 months of age between those were culture negative at 3 months and those who were culture positive. Median neutrophil differential was 9.3% (95% CI 1.7 - 24%) vs. 8% (95% CI 3.7 - 10.7%) respectively (p = 0.72), Mann-Whitney U test.)
7.4.4 Inflammation at 3 months is persistent at 12 months

Of the 19 patients included in this part of the study 10 patients had data available on inflammation within their airways at 3 months of age. 5/10 (50%) of infants had no evidence of inflammation (BALF neutrophil differential ≤4%) and 5/10 (50%) had neutrophilic inflammation (BALF neutrophil differential >4%). I compared these 2 groups to establish if differences could be seen in the data from the 12-month bronchoscopies. 4/5 infants without inflammation had corresponding data available at 12 months and 3/5 infants with inflammation. Of those infants without inflammation at 3 months, 75% (3/4) also had no evidence of inflammation at 12 months and of those with inflammation, all (3/3) had evidence of inflammation at 12 months (Figure 27). There was no statistical difference in these groups (Fishers exact test p = 0.14) but the numbers are very small.
Figure 27: Inflammation at 12 months according to inflammation status on 3 month bronchoscopy
25% of infants without inflammation (neutrophil differential >4%) at 3 months had inflammation at 12 months compared with 100% of those with evidence of airway inflammation at 3 months. There was no statistical difference between these 2 groups, which may be a type 2 error due to the small sample size. Hatched area indicates normal neutrophil differential.

7.4.5 Neutrophilic airway inflammation at 3 months is not associated with functional change at 3 or 12 months

No correlation was seen between BALF neutrophil differential on the 3-month bronchoscopy and any lung function parameter at 3 or 12 months.

7.4.6 Infection at 3 months correlates with functional change at 3 and 12 months

Infants with infection at 3 months had a worse outcome in terms of lung function at 3 months and these changes persisted at 12 months.
At 3 months of age, at a similar time to the infection being identified, infants with infection had a statistically significant worse FRC $z$ score ($p = 0.018$) and FVC $z$ score ($p = 0.029$). No difference was seen in FEV$_{0.5}$ $z$ score (Figure 28).

It might be expected that infants with evidence of bacterial infection would have worse air trapping (demonstrated by higher FVC) than those without infection but this was not the case in this study. A possible explanation is that infants with bacteria had mucus plugging causing them to lose volume hence having lower FVC and FRC than their non-infected peers. Alternatively, early infection may slow lung growth hence lower lung volumes; conversely, small lungs may predispose to more infection? In order to try to address this I looked at a sub-group of those without infection at 3 months ($n = 12$) and compared the lung function at this time according to their microbiological state at 12 months.
Figure 28: Lung function at 3 months of age

Infants with bacteria isolated form their BALF had worse FRC and FVC than infants without infection identified (Mann-Whitney U test). Priori p-value <0.01.
If it is the case that some infants have small lungs and that this group are predestined to get more infection, then a difference in lung volume would already be expected at 3 months. Four of these infants had bacteria identified in their BALF at 12 months; there were no differences in lung volume or lung function between these 4 infants and the 8 infants with no evidence of infection, which does not support this. Decreased volume due to plugging is therefore more likely.

At 12 months, there was a greater difference in lung function seen between those with infection at 3 months and those without, with worse lung function in those with infection, see Figure 29.

FVC z-score \( (p \leq 0.006) \), FRC z-score \( (p < 0.008) \) and LCI score and z-score \( (p < 0.03 \) and \( p < 0.02) \) were statistically significantly worse in those with infection; there was a trend towards worse FRC z-score \( (0 = 0.06) \). By 1 year of age there was no difference in the FEV\(_{0.5}\).

The longitudinal change in lung function in each infant did not differ according to infection isolation at 3 months (Table 12).

**Table 12: Longitudinal change in lung function by infection state on BALF.**

Values are median (95% CI of median) change in lung function between 3 and 12 months. Lung function was worse at 3 months in those with infection but this was not predictive of change at 12 months (Mann-Whitney U test).

<table>
<thead>
<tr>
<th></th>
<th>Micro +ve</th>
<th>Micro -ve</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FRC (mbw)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>z-score</td>
<td>-0.96</td>
<td>-0.77</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(-1.18; -0.09)</td>
<td>(-1.15; -0.09)</td>
<td></td>
</tr>
<tr>
<td><strong>FEV(_{0.5})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>z-score</td>
<td>0.73</td>
<td>0.42</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(-0.6; 1.23)</td>
<td>(-0.6; 0.91)</td>
<td></td>
</tr>
<tr>
<td><strong>FVC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>z-score</td>
<td>0.26</td>
<td>0.09</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(-1.54; 0.83)</td>
<td>(-0.5; 0.69)</td>
<td></td>
</tr>
<tr>
<td><strong>LCI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.21</td>
<td>-0.79</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>(-1.6; 1.36)</td>
<td>(-1.29; 0.21)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 29: Difference in lung function at 12 months of age between infants culture positive and culture negative at 3 months
Worse functional outcomes at 12 months of age, as measured by FVC, FRC (as measured by plethysmography) and LCI, were seen in infants with bacteria isolated in their BAL at 3 months of age. (Mann-whitney U test; priori p-value p<0.01)
7.4.7 CT scores

CT scores of infants with bacterial infection in their BALF were compared with children without infection and no difference was seen. Due to the discrepancies in observer scores, each score was analysed separately rather than meaning the results.

7.4.8 Vitamin D

Comparison of 3-month vitamin D with 3 and 12-month lung function results showed no relationship.

I also assessed the change over time of airways function and in height and weight parameters over the first year of life as a function of vitamin D at 3 months of age. No relationship was seen with vitamin D and the change in any of these clinical markers between 3 and 12 months of age (Figure 30).
Figure 30: Vitamin D and change in lung function. No relationship was found with vitamin D measured in serum at 3 months and functional airway change or growth over the first year of life. (Spearman correlation; priori p-value < 0.01).
7.5 Discussion

7.5.1 Statement and interpretation of principal findings

I hypothesised that vitamin D deficiency would lead to worse clinical outcomes in CF.

Vitamin D levels did not appear relevant to the evolution of functional change over time; small numbers may account for this, but given previous findings with vitamin D and an absence in association with clinical findings the results support a lack of relevance of vitamin D in early years. However the study does lack deficient infants, so it is not possible to say if really low levels might have been relevant.

From the small numbers available, inflammation persists once it has occurred: all (n = 3) infants with inflammation seen at 3 months also had evidence of inflammation at 12 months whilst those with no inflammation at 3 months (n = 4) generally had no inflammation at 12 months; these figures are in keeping with findings by another group (321).

Infection at the 3-month bronchoscopy did not predict inflammation seen at a year, which may be due to the fact that culture techniques only were used and work using molecular diagnostic testing to identify bacteria might have given a different conclusion.

Another possibility is that because the infection was detected, treatment was given which may have altered the disease course, whereas treatment decisions were not made on inflammatory counts so inflammation outside of detected infection was not treated. If the infection was missed at bronchoscopy then this could be a reason why neutrophilia carries a bad prognosis.
Infection in infancy was associated with worse outcome in lung function at 3 and 12 months. Bacterial infection at 3 months of age was associated with worse functional outcome measures both at the time of infection and 12 months later suggesting that early infection may set the course of CF lung disease. Interestingly, these changes were not seen with inflammation. It is possible that the infection was driving alternate inflammatory pathways not evaluated in this part of the study but this seems unlikely given the wide range and the choice of inflammatory markers evaluated. It is more likely that the study was underpowered to see these changes.

In older children LCI is more sensitive than spirometry but in infants the differences are not clear; some infants with normal LCI can have abnormal spirometry (RVRTC) and vice versa showing that the tests in infants differ to older children and appear to be picking up different disease. Further work is needed to explore this.

When looking at individual longitudinal change no pattern could be seen which is likely to be due to the small numbers but by evaluating functional changes grouped by microbiological state some patterns can be discerned.

7.5.2 Strengths of the study

The main strength of this study is the ability to follow up both inflammatory and functional data of the airways over the first year of life. These data are able to give an indication of the progression of early lung disease. Another strength is being able to evaluate vitamin D levels
at an early age and relate them to progressive change in function over that first year, of life, and to structure age one year.

A huge strength of the LCFC study is the ability to compare these results to those of healthy contemporaneous controls recruited as part of the same study.

7.5.3 Limitations of the study

Only 18 infants were able to be included in this study due to the data being needed at different time points and this was not available for most of the NBS infants. Optimally, more infants would have been included and further work could expand this cohort, and it would be important to do this specifically to address the question of the relationship of future lung function with early infection. Due to funding constraints, the LCFC stopped recruiting to the study and so this limited the numbers available.

All of the infants in the longitudinal study were vitamin D sufficient according to previously defined classifications. Therefore although no relationship was seen with vitamin D and longitudinal change in lung function, it was not possible to examine the effect on lung function over a wide range of vitamin D levels. Vitamin D levels of the infants included in the longitudinal cohort were similar to those in the entire NBS cohort and reflect the fact that levels in younger children are higher than older children. As these infants did have adequate vitamin D levels it does suggest that improving vitamin D levels will not help. I have however been unable to evaluate immunomodulatory effects at very high levels and so am unable to comment if supra-high levels would be of benefit.
Our understanding of “normal” level of neutrophils within the airway of infants is incomplete and levels are thought to be increased compared with adults. A grey area between 4-10% exists and it is therefore difficult to say exactly what constitutes inflammation which makes accurate interpretation of these data difficult. It was reassuring to see that all those with neutrophilia of less than 4% were still less than 4% at 12 months of age. Interestingly both those in the borderline range of 4-10% at 3 months rose to over 10% by 12 months of age. This suggests that those in the borderline range do have more abnormal inflammatory processes occurring and therefore could be classed at having inflammation giving confidence in using this definition of inflammation for analyses in this study. However, the small numbers mandate confirmatory studies.

7.5.4 Findings of the study in relation to other studies

Similarly to another study by Ranganathan et al, CF infant airway function was diminished from a very early age (138) compared to reference values of non-CF infants, but unlike Ranganathan et al’s study where a reduction in FEV0.5 of 20% over 6 months was seen, airway function improved in the LCFC patients recruited into my study. The findings were similar to that of the whole LCFC cohort (332) showing that this sub-cohort were representative of the entire cohort.

In my study the time difference in between the 2 measurements was 9 months rather than 6 months, and my cohort were diagnosed on NBS rather than clinically. These factors may account for this difference and support screening in that lung function improved rather than
decreased over the first year. My results were similar to that of the entire LCFC cohort (322), which also found a reduction in lung function parameters with an improvement in FEV$_{0.5}$ by 12 months of age.

I found a relationship between lung function and infection as was seen by Belessis et al (334); but unlike their study, I did not find an association with inflammatory markers which may be due to the low numbers available in this part of the study.

There is little in the literature about changes in inflammation over the first year of life so comparison to other studies cannot be made. Likewise with vitamin D, data regarding vitamin D and its relationship to infection and inflammation in the first year is not available.

Whilst it is supposed that inflammation leads to functional and to structural change it is possible that these are unrelated as suggested by the authors in one study (167), and there may be reasons other than inflammation that cause lung function to deteriorate.

### 7.6 Conclusions

Although only small numbers were available for the longitudinal study, this data is useful in obtaining information regarding airway inflammation over a longer time period and with which I demonstrated that inflammation persists and early infection is related to later functional deficit of the airways. These findings are very important highlighting the need for early interventions. The findings, similar to the previous chapters, suggest that vitamin D
does not a role to play in immunomodulation in this group and nor does it have a role as a biomarker of airway health.
Chapter 8: The role of vitamin D in airway infection and inflammation

8.1 Introduction

In order to fully explore the relevance of vitamin D in the innate immunity in CF the cohort was expanded to include children with a variety of ages, of disease severities and a greater spread of vitamin D levels.

8.2 Hypotheses

In this section, I address my hypothesis that vitamin D has immunomodulatory effects via antimicrobial peptide pathways; consequently vitamin D deficiency will lead to greater risk of bacterial infection and subsequent inflammation and worse clinical outcomes in CF throughout childhood, in a larger more diverse group of children with CF.
8.3 Methods

8.3.1 Subjects

Patients included in the study met the criteria for one of the 3 following groups. Patients included in each of these groups are discussed in chapter 4.

- CF patients (groups 1a and 1b)
  - n = 113 (a = 31; b = 82)

- Non-CF CSLD patients (group 2a)
  - n = 23

Following initial results, the non-CF CSLD group was expanded by including patients without BALF data, but with vitamin D and lung function results available (subsequently termed group 2b; n = 26).

- Healthy controls (group 3)
  - n = 6

There were no differences in demographic information between the 233 patients eligible for inclusion, and the 143 finally included (section 5.5.1, Table 13).
<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>Non-CF CSLD</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entire eligible group</td>
<td>Included pts only</td>
<td>Entire eligible group</td>
</tr>
<tr>
<td>N</td>
<td>176</td>
<td>113</td>
<td>45</td>
</tr>
<tr>
<td>% of group included</td>
<td>64</td>
<td>51</td>
<td>50</td>
</tr>
<tr>
<td>Age years Median (range)</td>
<td>6.97 (0.21 – 17.6)</td>
<td>7.8 (0.1-17.6)</td>
<td>9.52 (2.78 – 16.26)</td>
</tr>
<tr>
<td>Sex n (% male)</td>
<td>81 (46)</td>
<td>47 (42)</td>
<td>24 (53)</td>
</tr>
<tr>
<td>FEV1 % predicted Median (range)</td>
<td>66.5 (28 – 108)</td>
<td>66 (30 – 101)</td>
<td>74.5 (31 – 95)</td>
</tr>
</tbody>
</table>

Table 13: Patient demographics to compare the included patients with entire potentially included population

8.3.2 Clinical data collection

For the CF cohort, in addition to looking at clinical results from the time of the bronchoscopy, data from annual assessments (AA) were also evaluated. Values were taken from the AA closest to the time of the bronchoscopy (“AA FOB”), the AA the previous year (“AA pre”) and the following year (“AA post”). The data recorded were: spirometric values (FEV1 and FVC), the presence or absence of CF related diabetes, the number of days on intravenous antibiotics (IVAB) and body mass index (BMI) centile; values were collected from documentation on Port CF. These data were reviewed independently to assess respiratory health prior to, at the time of, and after vitamin D measurement; and were reviewed combined to assess respiratory health over a 3 year period to fully explore the relevance of vitamin D to airway health.
The clinical data recorded for each of these time points were recorded as follows:

- The annual assessment (AA) closest to the time of the bronchoscopy (“AA FOB”)
- The AA 12 months prior to the “FOB AA” bronchoscopy (“AA pre”)
- The AA 12 months following the “FOB AA” bronchoscopy (“AA post”).

Microbiological data were collected for a period of 3 years, 12 months before the date of the FOB, 2 years after the date of the FOB. These time periods are defined as follows:

- The 12 months prior to the date of the bronchoscopy (“pre”)
- The 12 months commencing on the day of the bronchoscopy (“year of FOB”)
- The 12 months following this (“post”)

For data assessing inflammation in CF patients under 12 months of age who therefore did not have 12 months data prior to the bronchoscopy, ‘pre’ data were collected from date of diagnosis until the time of the bronchoscopy.

**8.3.3 Statistical analysis**

Statistical analysis was carried out as discussed in generic methods chapter 4. Non-parametric analysis was used: Mann Whitney for 2 groups, Kruskal-Wallis with Dunn’s correction for multiple groups, and logistic regression for binary outcomes. Multiple linear
regressions were performed to determine significant relationships of any to the BALF and serum components measured. The factors tested in all cases were age, gender, total vitamin D level, genotype (phe508del/phe508del versus the rest) and winter (October to March inclusive) versus summer (April to September inclusive). Forward conditional logistic regression with *S. aureus* or *P. aeruginosa* bacterial isolation or not as the binary outcome was performed using age and total vitamin D level as factors. Correlations were performed and reported using Spearman’s ranking correlation coefficient.

Bacterial isolation was first defined as isolation on BALF, and performed again with bacterial isolation being defined as any positive culture of these organisms within the 3-year study period. In view of the multiple comparisons undertaken, the null hypothesis was rejected at *p*<0.01. Unless otherwise stated, results are presented as medians with 95% confidence intervals of the median.

### 8.4 Results

#### 8.4.1 Subjects

142 patients (113 CF, 23 non-CF CSLD and 6 healthy controls) were included in the study (Table 14).
**Table 14: Patient demographics**

Patient demographics of the 3 groups included in the study. There were no differences between the groups except that the proportion of children under the age of 1 year was higher in the CF group (p<0.01). Despite clear numerical differences in FEV$_1$ between the CF group and the healthy controls this did not reach statistical significance, presumably due to the small numbers in the control group.

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>Non-CF CSLD</th>
<th>Healthy</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>113</td>
<td>23</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Age years Median (range)</td>
<td>7.8 (0.1-17.6)</td>
<td>7.8 (2.8-15.5)</td>
<td>12.4 (10.5-15.4)</td>
<td>0.014</td>
</tr>
<tr>
<td>Sex n (%) male</td>
<td>47 (42)</td>
<td>12 (52)</td>
<td>2 (33)</td>
<td>0.57</td>
</tr>
<tr>
<td>F508del *</td>
<td>102 (93%)</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>Pancreatic insufficiency</td>
<td>99 (88%)</td>
<td>n/a</td>
<td>n/a</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin supplements</td>
<td>102 (91%) ‡</td>
<td>Not available</td>
<td>Not available</td>
<td>-</td>
</tr>
<tr>
<td>FEV$_1$ % predicted Median (range)</td>
<td>66 (30 – 101)</td>
<td>69 (25 – 95)</td>
<td>106 (80 – 131)</td>
<td>0.14</td>
</tr>
</tbody>
</table>

* ≥ 1 F508del gene  
† 3 patients missing data  
‡ 1 patient data missing

### 8.4.2 Vitamin D levels

Serum vitamin D levels differed across the three disease groups (p = 0.0024). Despite 91% of CF subjects being prescribed fat-soluble vitamin supplements, 41/113 (36%) were vitamin D deficient (< 50 nmol/L). However, median (95%CI) values in the CF group were similar to those of the healthy controls; 57 (52– 66) and 57 (24 – 74) nmol/L respectively, (Figure 31 and Table 15).

In contrast, the patients with non-CF bronchiectasis, in whom vitamin D is not routinely measured or supplemented (and unlike CF, do not have malabsorption) had lower serum levels (42 (26 - 51) nmol/L p<0.01 vs. CF); 70% of them were deficient (chi squared p value 0.003 vs. CF). There was no relationship with current microbiological state (Figure 31).
Figure 31: Vitamin D levels of the 3 patient groups
The median (95% CI) serum 25(OH)D2 levels were 57 (52 – 66), 42 (26 – 51) and 57 (24 – 74) nmol/L in the CF, non-CF CSLD and the healthy control groups respectively. The non-CF CSLD group had a significantly lower vitamin D level than the CF group (Kruskal–Wallis p<0.01). There was no relationship with current microbiological state. Priori p-value < 0.01.
Table 15: inflammatory markers and antimicrobial peptide levels of the 3 groups

Vitamin D levels, cellular inflammatory markers and antimicrobial peptide levels for the 3 patient groups. Results shown are median (95% CI of the median). Patients with non-CF CSLD had lower levels of vitamin D than the CF patients and the healthy controls. The CF group had higher BALF absolute cell counts than either of the other 2 groups; and the CF and non-CF CSLD groups had higher neutrophil differentials than the healthy controls. There was no difference in serum neutrophils between the 3 different groups.

<table>
<thead>
<tr>
<th></th>
<th>CF</th>
<th>Non-CF CSLD</th>
<th>Healthy</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D$_2$ nmol/L</td>
<td>57 (52-66)</td>
<td>42 (26 – 51)</td>
<td>57 (24 – 74)</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>BALF absolute cell count (x $10^3$)</td>
<td>620 (500 - 850)</td>
<td>364 (115 - 540)</td>
<td>143 (70 – 270)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td>BALF neutrophil differential (%)</td>
<td>31 (19.0 – 41.7)</td>
<td>14.5 (2.0 – 46.3)</td>
<td>1.4 (0.3 - 2.7)</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>BALF neutrophil count (x $10^9$)</td>
<td>166 (45.6 – 306.9)</td>
<td>16.5 (4.2 – 346.5)</td>
<td>2.8 (0.2 – 5.1)</td>
<td>P = 0.0001</td>
</tr>
<tr>
<td>Serum neutrophils (x $10^9$/L)</td>
<td>4.3 (3.7 - 4.9)</td>
<td>4.6 (3.6 – 5.7)</td>
<td>3.9 (2.6 – 4.6)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF LL-37 (ng/ml)</td>
<td>0.5 (0.3 – 0.75)</td>
<td>0.4 (0.3 – 0.8)</td>
<td>0.3 (0.3 – 0.6)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF HBD-2 (pg/ml)</td>
<td>149 (115.8 – 202.6)</td>
<td>51 (&lt;15.6 – 170.5)</td>
<td>120 (&lt;15.6 - 250)</td>
<td>ns</td>
</tr>
</tbody>
</table>

* CF > healthy on Dunn’s multiple comparison test
$ CF >$ non-CF CSLD and healthy
¥ CF and non-CF CSLD > healthy
Δ CF > healthy
8.4.3 Age

The proportion of children under the age of 1 year was higher in the CF group than the 2 other groups (p<0.01). One reason for this is that infants with CF routinely undergo FOB, but this is not the case in non-CF CSLD and the 6 healthy controls were also older. For the patients with CF, vitamin D correlated inversely with age ($r = -0.35, p = 0.001$) (Figure 32) and therefore multiple regression has been done where relevant.

No relationship was seen between vitamin D and age in patients with non-CF CSLD ($r = 0.008, p = 0.96$).

There was no association between FEV$_1$ or FVC with age in either patient group. As it would be expected that lung function would decrease with age in CF children, it may be that bias exists in that patients requiring bronchoscopy at a younger age have more severe disease, or that some younger children may have had poor technique.

For CF patients, as expected, age was also positively correlated with absolute BALF cell count ($r = 0.3, p = 0.002$), neutrophil differential ($r = 0.3, p = 0.002$) and blood neutrophils ($r = 0.4, p = 0.001$). The antimicrobial peptide LL-37 was also strongly correlated with age ($r = 0.38, p < 0.0001$). This relationship was not seen with HBD-2. These relationships were not found in the group with non-CF CSLD.
Figure 32: Vitamin D level by age
For CF patients, as age increases vitamin D level decreases. This was not seen for non-CF CSLD patients. Priori p-value < 0.01.

In order to account for the children with routine bronchoscopies which were done at 3 and at 12 months of age for some infants, post-hoc analyses were performed excluding all children under 2 years of age with no alteration to outcomes. Two years was chosen as some bronchoscopies were performed late and to ensure all routine bronchoscopies were excluded.

8.4.4 Season

For the purposes of ascertaining if a seasonal variation occurred, the year was divided into 2 six-month seasons; “summer” months are defined as April to September and “winter” months as October to March. 74 procedures were carried out in the winter months and 62 in the summer months.
The effect of season on vitamin D level, spirometry, nutrition, infection, cellular and soluble inflammatory markets and antimicrobial peptides were examined and there were no significant differences in any parameters (Mann-Whitney U test). No significant difference was observed between vitamin D level measured in winter months (median (CI) = 55 (44 : 63) nmol/L) and in summer months (63 (53 : 71 )nmol/L)(p = 0.09).

8.4.5 Pancreatic status

99/113 (88%) of the CF patients were pancreatic insufficient. Interestingly, there was no difference in nutritional status between pancreatic sufficient and insufficient patients. Median vitamin D levels were similar for both groups (Figure 33) and no difference was observed in cell counts or FEV1 between the 2 groups.
Figure 33: Vitamin D levels in pancreatic sufficient and insufficient children
Vitamin D levels were similar irrespective of pancreatic status. Median (95% CI) vitamin D was 58 (53 : 66) nmol/L in pancreatic insufficient patients vs. 51 (25 : 79) nmol in pancreatic sufficient patients, \( p = 0.2 \).

8.4.6 Growth

In the CF cohort, height, weight and BMI median z scores were all below zero showing below average nutritional status. Median (95% CI) z-scores for height, weight and BMI were -0.2 (-0.6 : 0.2), -0.3 (-0.6 : -0.1) and -0.5 (-0.7 : -0.1) respectively. Both weight and BMI were lower in the CF cohort than either of the other 2 groups but no difference was observed in height.

No relationship was seen between vitamin D and height, weight or BMI z-scores with vitamin D as either a categorical or continuous variable. This was true when considering the year of the FOB (Table 16) and the periods before and afterwards.
Table 16: Nutritional health of the CF cohort by vitamin D status

BMI, height and weight z-scores of CF cohort. Values shown are median (95% CI).

<table>
<thead>
<tr>
<th>Vitamin D</th>
<th>P value</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 50 nmol/L</td>
<td>≥ 50 nmol/L</td>
<td>r = -0.10, p = 0.3</td>
</tr>
<tr>
<td>Height</td>
<td>-0.5 (-1.1: 0.4)</td>
<td>-0.1 (-0.6: 0.3)</td>
</tr>
<tr>
<td>Weight</td>
<td>-0.3 (-0.8: 0.4)</td>
<td>-0.5 (-0.7: -0.1)</td>
</tr>
<tr>
<td>BMI</td>
<td>-0.3 (-0.7: 0.4)</td>
<td>-0.6 (-1.1:-0.3)</td>
</tr>
</tbody>
</table>

8.4.7 Spirometry

Spirometry results were available for 60/113 (53%) CF patients and 16/23 (70%) non-CF CSLD group but not for the others either because they were too young (CF = 50, CSLD = 2) or because spirometry was not performed close to the time of the bronchoscopy (CF = 3, CSLD = 4). Spirometry was performed in just 2 of the healthy controls and therefore analysis of this group was not performed. Median (95% CI) FEV₁ was 66 (62 : 73)% in the CF group and 69 (62 : 85)% in non-CF CSLD. The difference between the groups was not of statistical significance (p = 0.1).

There was no difference in spirometric values between those who were vitamin D sufficient and those vitamin D insufficient in either the CF nor the non-CF CSLD group. In addition, there was no correlation between vitamin D and FEV₁ or FVC in CF or non-CF CSLD groups (Figure 34 and Table 17). The CF and non-CF CSLD groups were then combined and again, no relationship was found (FEV₁ r = -0.06, p = 0.6. FVC r = -0.01, p = 0.9).

The lack of relationship as seen from data from the time of the bronchoscopy persisted if the spirometric values from the AA pre, AA FOB and AA post were analysed, or if all 3 were
meaned. The change in FEV$_1$ over the time period was also evaluated and no relationship with vitamin D seen.
Figure 34: Vitamin D and lung function

Figure a and b show the correlation (Spearman’s correlation coefficient) between serum vitamin D and FEV₁ for CF (a) and CSLD (b) patients. Figures c and d show the FEV₁ for vitamin D deficient vs. vitamin D sufficient patients (Mann-Whitney test). There was no correlation between vitamin D and FEV₁ (a and b) for either group (CF: \( r = 0.03 \), ns; non-CF CSLD: \( r = 0.3 \), ns) and when comparing vitamin D sufficiency and deficiency (c and d), no difference was seen (median (CI) vitamin D in CF: 66 (58-71)% vs. 71.5(61–76)%; ns; non-CF CSLD: 69(36-88)% vs. 70(62–95)%; ns).
Table 17: Clinical parameters by vitamin D status
The table shows Vitamin D levels and clinical parameters for CF and non-CF CSLD patients. Median (95% CI of median) FEV₁, number of days per year spent on IVAB and BMI centile are shown. There was no relationship demonstrated between serum vitamin D level and FEV₁, FVC (figures not shown), the number of days on IVAB or BMI centile.

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D &lt; 50 nmol/L</th>
<th>Vitamin D &gt; 50 nmol/L</th>
<th>Difference between 2 groups (p value)</th>
<th>Correlation with serum vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CF patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ % predicted median (95% CI)</td>
<td>66.0 (58 - 71)</td>
<td>71.5 (61 - 76)</td>
<td>ns</td>
<td>r = 0.03 ns</td>
</tr>
<tr>
<td>FVC % predicted Median (95% CI)</td>
<td>83.5 (69- 93)</td>
<td>82 (72 – 89)</td>
<td>ns</td>
<td>r = -0.08 ns</td>
</tr>
<tr>
<td>Days on IVAB Median (range)</td>
<td>14 (0-56)</td>
<td>12 (0-18)</td>
<td>ns</td>
<td>r = -0.05 ns</td>
</tr>
<tr>
<td>BMI z-score Median (range)</td>
<td>- 0.3 (-3.0 – 2.1)</td>
<td>- 0.6 (-4.0 – 2.6 )</td>
<td>ns</td>
<td>r = -0.19 ns</td>
</tr>
<tr>
<td><strong>Non-CF CSLD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FEV₁ % predicted median (range)</td>
<td>69.0 (36-88)</td>
<td>70.0 (62-95)</td>
<td>ns</td>
<td>r = 0.34 ns</td>
</tr>
</tbody>
</table>

8.4.8 Intravenous antibiotics

These data were collected from the CF AA reports and are therefore only available for the CF cohort. The number of days spent on IVAB in the previous year was collected from AA pre, AA FOB (Table 17) and AA post reports. The data were analysed separately and also meaned. There were no relationships with vitamin D levels.

8.4.9 Infection

Vitamin D levels of each of the 3 groups were related to microbiological status. 64/113 (57%) of the CF patients and 10/23 (43%) of the non-CF CSLD patients had a positive isolate
on their BALF culture. There was no apparent relationship with current microbiological status in either group (Figure 31 and Figure 35). For CF patients, median (95% CI) vitamin D was 63 (52-68) nmol/L in those with sterile BALF, compared with 56 (47 – 63) nmol/L in those who cultured any bacteria or fungi on their BALF. The non-CF CSLD, median vitamin D was 43 (27 – 59) nmol/L for those who were culture negative and 33 (21 – 60) nmol/L in the group with positive isolates on their BALF. By definition, the healthy control group were all culture negative.
Figure 35: Vitamin D level of culture positive and culture negative patients
Vitamin D levels of CF patients who isolated any organisms in their BALF were compared with CF patients with sterile BALF. No difference was seen between the culture negative and culture positive groups (median (CI); 63 (52-68) vs. 56 (47 – 63) nmol/L, ns). This was also true for the non-CF CSLD group (median (CI); 43 (27 – 59) vs. 33 (21 – 60) nmol/L, ns).
As well as evaluating vitamin D status of those who isolated any bacteria on their BALF, comparison was done for those whose samples grew *P. aeruginosa* against those who did not (Figure 36), and those who isolated *S. aureus* against those who did not (Figure 37). In addition to looking at bacterial organisms isolated on BALF, results of all microbiological swabs taken during the study period were examined. 14 (12%) of patients isolated *P. aeruginosa* on their BALF. There was a trend towards a lower median vitamin D level in these patients compared to those without *P. aeruginosa* isolation (median (95% CI) vitamin D level 39 (31 : 57) vs. 62 (53 : 68) but this did not reach the pre-defined significance level of <0.01 (p = 0.014). This trend was not seen in the 1 year or 3 year surveillance follow up periods.

When evaluating the vitamin D level by pseudomonal status in more depth, it was seen that the group with *P. aeruginosa* infection were older than those without although not statistically significantly so. Median age was 10.9 (3.1 : 14.4) years in the *P. aeruginosa* positive group compared with 6.9 (3.4 : 8.7) years in those without *P. aeruginosa* infection (p = 0.16); adjustment for age was done with no alteration to results.

In addition to comparing individuals with *P. aeruginosa* infection with those *P. aeruginosa* negative, I also compared them with individuals who had no organisms at all isolated. These comparisons were done at each time point with similar results. There was no relationship with vitamin D and isolation of *P. aeruginosa* in any of the 3 ways of exploring this; isolation from BALF, from surveillance swabs over a one year follow up period, or the 3 year follow up period.
Figure 36: Vitamin D level of Pseudomonas aeruginosa positive and negative patients

There was no statistically significant difference between the CF patients who isolated *P. aeruginosa* and those who did not in any of the 3 ways used to explore the data. Mean vitamin D at FOB was 39 (31 : 57) vs. 62 (53 : 68) and 53 (38 : 66) nmol/L vs. 59 (53 : 68) nmol/L (p = 0.2, Mann-Whitney) and 55 (45 : 61) nmol/L vs. 63 (53 : 69) nmol/L (p = 0.14) in the 1 and 3 year follow up periods respectively. Priori p-value set at p < 0.01. (Kruskal Wallis with Dunn’s comparison).

Figure 37: Vitamin D level of *staphylococcus aureus* positive and negative patients

Mean vitamin D levels of patients with and without *Staphylococcal aureus* infection at each of the 3 ways of exploring this. Patients with *S. aureus* in their BALF had a median (95% CI) vitamin D of 54 (27 : 58) nmol/L which was similar to the level, 62 (53 : 68) nmol/L, for those without this infection (p = 0.06 Mann-Whitney). For the 1 year follow-up period patients with *S. aureus* did have a lower vitamin D (49 (33 : 57) nmol/L vs. 63 (53 : 68) nmol/L, p = 0.006) but in the 3 year period the difference did not reach statistical significance (mean vitamin D 52 (40 : 58) nmol/L vs. 63 (53 : 73) nmol/L, p = 0.04). However, the apparent relationship disappeared when accounting for age. Priori p-value set at p < 0.01. (Kruskal Wallis with Dunn’s comparison).
For *S. aureus*, there was an initial apparent relationship in the 1-year follow-up period but not in the other 2 analyses; this apparent relationship disappeared when age was assessed as an independent variable.

The *S. aureus* group was also compared to those with no organisms isolated on culture and a similar picture seen to the comparison to those *S. aureus* negative.

### 8.4.10 Cellular and soluble markers of inflammation

BALF total inflammatory cell counts were higher in CF patients than in either of the other 2 groups (p < 0.001) and neutrophil differential was higher in both CF and non-CF CSLD than healthy controls (Figure 38). BALF IL-6 and IL-8 were higher in CF patients although this did not reach our pre-defined statistical cut-off of (p < 0.01). No other differences in cytokine levels were seen.
Figure 38: BALF cellular inflammation seen in the 3 groups
BALF total cell count was higher in the CF group than both the control groups (CF 620 (500: 850), non-CF CSLD 364 (115: 540) and healthy 143 (70 : 270) x10³, p < 0.001).
BALF neutrophil differential was higher in CF than healthy controls (CF 31 (19 : 42), non-CF CSLD 15 (2.0 : 46), healthy 1.4 (0.3 : 2.7)%, p = 0.001). CF patients had a higher BALF neutrophil count than healthy controls (166 (46 : 307) vs. 2.8 (0.2 : 5.1) x 10³, p = 0.0001). (Kruskal Wallis with Dunn’s comparison).

* = p < 0.05
** = p < 0.01
*** = p < 0.001

Priori p-value set at p < 0.01
The cellular and soluble markers of inflammation correlated with infection status; patients with bacteria positive BALF had higher cell counts and pro-inflammatory cytokine levels than patients with no bacteria identified on BALF in CF patients (Table 18). A similar pattern was seen in the non-CF CSLD group.

**Table 18: Cellular and soluble markers of inflammation of the CF cohort by bacterial culture status**

CF patients with bacteria isolated in their BALF had higher BALF total cell count, neutrophil count, neutrophil differential, blood neutrophils, and higher BALF LL-37, IL-10, IL-1β, IL-8 and TNF-α. Values shown are median (95% CI of the median).

<table>
<thead>
<tr>
<th></th>
<th>BALF culture +ve</th>
<th>BALF culture -ve</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALF absolute count (x 10^3/L)</td>
<td>1,035 (730 : 1880)</td>
<td>368 (273 : 525)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BALF neutrophil differential (%)</td>
<td>58 (42 : 71)</td>
<td>14 (8 : 23)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BALF neutrophil count (x 10^3/L)</td>
<td>521 (209 : 1142)</td>
<td>39 (23 : 82)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Serum neutrophils (x 10^9/L)</td>
<td>5.3 (4.3 : 6.3)</td>
<td>3.5 (2.7 : 4.3)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BALF LL-37 ng/ml</td>
<td>0.82 (0.49 : 1.13)</td>
<td>0.35 (0.30 : 0.43)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BALF HBD-2 (pg/ml)</td>
<td>166 (123 : 231)</td>
<td>119 (66 : 204)</td>
<td>0.16</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>3.36 (1.53 : 9.11)</td>
<td>0.5 (0.15 : 0.80)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>GM-CSF (pg/ml)</td>
<td>0.72 (0.26 : 1.90)</td>
<td>0.18 (0.13 : 0.81)</td>
<td>0.02</td>
</tr>
<tr>
<td>INF-γ (pg/ml)</td>
<td>0.14 (0.00 : 1.63)</td>
<td>0.13 (0.13 : 0.13)</td>
<td>0.02</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>1.20 (0.43 : 2.17)</td>
<td>0.20 (0.17 : 0.42)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>43.0 (15.3 : 77.4)</td>
<td>4.22 (1.06 : 8.68)</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>21.4 (9.30 : 31.3)</td>
<td>6.42 (4.21 : 14.2)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>2,347 (1307 : 4163)</td>
<td>442 (234 : 995)</td>
<td>0.0004</td>
</tr>
</tbody>
</table>
Relationships between these cellular and soluble inflammatory markers and vitamin D were explored and no relationship was seen (Table 19).

Table 19: Cellular and soluble markers of inflammation of the CF cohort by vitamin D status

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vitamin D &lt; 50 nmol/L</th>
<th>Vitamin D &gt; 50 nmol/L</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CF patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALF total cell count (X 10^3)</td>
<td>101</td>
<td>652 (500-1,220)</td>
<td>528 (390 - 850)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF neutrophil differential (%)</td>
<td>84</td>
<td>36 (20-56)</td>
<td>25 (15 - 50)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF neutrophil count (X 10^3)</td>
<td>76</td>
<td>207 (40 – 521)</td>
<td>102 (43 – 346)</td>
<td>ns</td>
</tr>
<tr>
<td>Serum neutrophil count (x 10^9/L)</td>
<td>102</td>
<td>4.9 (3.8 - 6.5)</td>
<td>4.2 (3.4 – 4.9)</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Non-CF CSLD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALF total cell count (X 10^3)</td>
<td>16</td>
<td>364 (200 – 500)</td>
<td>460 (60 – 1240)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF neutrophil differential (%)</td>
<td>18</td>
<td>13.4(2.0 – 79)</td>
<td>29.3 (0.0 – 72)</td>
<td>ns</td>
</tr>
<tr>
<td>BALF neutrophil count (X 10^3)</td>
<td>13</td>
<td>8.6(3.3 – 347)</td>
<td>299 (16.4 – 582)</td>
<td>ns</td>
</tr>
<tr>
<td>Serum neutrophil count (x 10^9/L)</td>
<td>18</td>
<td>5.1 (3.6 – 5.7)</td>
<td>2.7 (2.2 – 11.8)</td>
<td>ns</td>
</tr>
</tbody>
</table>

8.4.11 Analyses with alternative vitamin D categories

Post-hoc analyses were carried out using different vitamin D categorical groups to define vitamin D deficiency as described in section 5.6.5. The conclusion was the same, regardless of the cut-off value chosen.

8.4.12 Antimicrobial peptides

BALF levels of LL-37 and HBD-2 were similar in the 3 patient groups (Table 15).
8.4.12.1 Antimicrobial peptides and vitamin D

Counter to the initial hypothesis, no relationship was seen between BALF levels of either of the antimicrobial peptides and serum vitamin D (Figure 39 and Figure 40).

8.4.12.2 Antimicrobial peptides and airway infection and inflammation

BALF LL-37 was higher in patients with infected BALF (Figure 41) and for CF patients levels correlated positively with other markers of inflammation, including cell count \((r = 0.7; \ p < 0.0001)\), neutrophil differential \((r = 0.5; \ p < 0.0001)\) and several of the BALF pro-inflammatory cytokines (Figure 42). This was true when vitamin D was considered as a continuous and a categorical variable. For non-CF patients, a similar but non-statistically significant trend was seen. In neither patient group was there a similar relationship between HBD-2 and vitamin D.

8.4.12.3 Antimicrobial peptides and lung function

No correlation was seen between either LL-37 or HBD-2 and FEV\(_1\) or FVC in CF (Figure 43) or non-CF CSLD patients.
No correlation (Spearman’s correlation coefficient) was seen between serum vitamin D and BALF HBD-2 in either CF ($r = -0.02$, ns) or non-CF CSLD ($r = -0.4$, ns) patients (a,b). In addition, no difference was observed in either group between vitamin D deficient and vitamin D sufficient patients (Mann-Whitney). (Median (95% CI): CF patients; 149 (115 – 235) pg/ml, ns. Non-CF CSLD patients; 139 (15.6 – 1002) pg/ml vs. 15.6 (15.6 – 83)pg/ml ns (c,d). Priori p-value set at $p < 0.01$. 

Figure 39: Vitamin D and HBD-2.
Figure 40: Vitamin D and LL-37. No correlation (Spearman’s correlation coefficient) was seen between serum 25(OH)D2 and BALF LL-37 in either CF ($r = -0.06$, ns) or non-CF CSLD ($r = -0.02$, ns) patients (a,b). In addition, no difference was observed in either group between vitamin D deficient and vitamin D sufficient patients (Mann Whitney). (Median (CI): CF patients; 0.49 (0.37 – 0.86) ng/ml vs. 0.44 (0.37 – 0.94) ng/ml, ns. Non-CF CSLD patients; 0.37 (0.31 – 1.62) ng/ml vs. 0.40 (0.27 – 1.49 ng/ml, ns) (c,d). Priori p-value set at $p < 0.01$. 

a) CF<br>b) Non-CF CSLD<br>c) CF<br>d) Non-CF CSLD
Figure 41: LL37 level in culture positive and culture negative CF patients
CF patients who isolated bacterial or fungal organisms from their BALF had higher levels of LL37 than those with sterile BALF. Median (range) 0.82 (0.3 – 21.2) vs. 0.35 (0.1 – 6.7 ng/ml. (Mann-whitney test, priori p-value p < 0.01).
Figure 42: LL37 and inflammatory markers
A positive correlation was seen with LL-37 and BALF absolute cell count, BALF neutrophil count and differential and inflammatory cytokines. (Spearman’s correlation coefficient).
Figure 43: LL37 and HBD-2 and FEV1 in CF patients
No relationship was observed between HBD-2 or LL37 and FEV1 in CF patients. There was no correlation (Spearman’s correlation coefficient) with LL37 and FEV1 (r = -0.14, p = 0.3) nor LL37 and FVC (r = -0.15, p = 0.3). This lack of relationship was also seen for HBD-2 and FEV1 (r = 0.01, p = 0.9) and HBD-2 and FVC (r = -0.07, p = 0.6).
8.5 Discussion

8.5.1 Statement and interpretation of principle findings

8.5.1.1 Overall findings

Contrary to my hypothesis, I have demonstrated that there are no detectable adverse outcomes in a range of clinically relevant pulmonary parameters in association with Vitamin D deficiency.

8.5.1.2 Vitamin D level

Over half of our CF population did not achieve optimal vitamin D levels (≥ 75 nmol/L), despite most (91%) being prescribed supplements. This may reflect the decreased absorption seen in this group of patients, meaning inadequate levels achieved with routine supplementation and that higher supplemental doses are required. 12% of the CF cohort were pancreatic sufficient but it is known that pancreatic sufficient patients still have some impairment to pancreatic function and this group also had suboptimal vitamin D levels. In fact vitamin D levels were similar in the pancreatic sufficient and insufficient groups. Another reason for the low levels despite prescribed supplementation may be a reflection of decreased adherence. Studies have found that vitamins are some of the least adhered to medications prescribed to patients with CF (335). This group of patients are also frequently prescribed antibiotics with photosensitivity as a side effect meaning that sun-protection is essential to prevent burning, but also decreases vitamin D exposure. Finally, patients with CF have been shown to have lower levels of vitamin D binding protein (270), which are not
related to levels of vitamin D. Vitamin D binding protein is required for Vitamin D transportation and low levels may lead to less tissue vitamin D.

When comparing CF patients with the 2 control groups, it was interesting, and unexpected, to observe that CF children and healthy controls have a significantly higher vitamin D level than non-CF CSLD (p < 0.01). 34% of CF patients and 33% of healthy controls were vitamin D insufficient (< 50 nmol/L) compared with 67% of non-CF CSLD, an admittedly small group, but an effect not accounted for by ethnicity or age. This group of patients have no known gastrointestinal problems or reasons for malabsorption. Further work is needed to understand this finding, which is outwith the scope of the thesis.

*Seasonal effect*

As vitamin D is largely obtained by a pathway involving sunlight, it would be expected that vitamin D levels would be higher in the summer months when there is more direct exposure of skin to sunlight, but this was not seen, for reasons that are not clear. A possible explanation is that since most CF patients are taking oral vitamin supplements, this may obscure the seasonal variation related to endogenous synthesis. However, in healthy individuals over 80% of vitamin D is from sunlight requiring direct skin exposure, without sunscreen. This of course, necessitates time spent outdoors with bare skin exposed in order to allow sunlight rays to penetrate the skin. There do not appear to be any studies addressing the sunlight exposure of CF children versus sunlight exposure of non-CF children.
Age effect

I also examined the effect of age on vitamin D level and found a weak, but statistically significant, inverse relationship between in the CF group. There are a number of reasons why younger children may have higher levels of vitamin D than older children. Several foods, including formula milks, are fortified with vitamin D and children in the younger age range may be more likely to have such foods as part of their diet. Also, younger children may be more compliant with their medications, as these are administered by parents (336). It is also possible that older children may have a higher treatment burden preventing them from spending as much time outside as the younger children.

Unlike the CF cohort, vitamin D was not related to age in the non-CF CSLD group. This population are not routinely prescribed supplements, which might lend support to adherence being the main influence of the age relationship in CF. Alternatively, their treatment burden may be less, in particular treatment with antibiotics requiring sun protection (particularly ciprofloxacin due to less Pseudomonal infection in this group).

The apparent relationship of more Pseudomonal infection in vitamin D deficiency with infection did not persist through different time points as would be expected if they were real. These apparent trends and relationships could be entirely accounted for by age.

8.5.1.3 Antimicrobial peptides

There is no relation between Vitamin D deficiency and BALF LL-37 and HBD-2 levels, nor any relationship between these peptides and outcomes despite biological plausibility, given the genes contain VDREs.
The antimicrobial peptides HBD-2 and LL-37 have previously been shown to be induced by vitamin D, but there was no relationship between these peptides, measured in BALF, and serum vitamin D.

The majority of the work finding induction of cathelicidin by vitamin D has been conducted in-vitro rather than in-vivo, and clearly not all results translate. In-vivo other environmental factors can alter relationships. Induction of cathelicidin could occur but increased proteolysis could mean that the protein gets broken down immediately and therefore increases in protein levels are not seen. In addition, most in-vitro experiments measure the CAMP gene expression, not LL-37 protein levels. An increase in gene expression does not necessarily translate to increase in protein level; the production of the LL-37 protein requires proteinase 3 to cleave it from its propeptide and the presence of this enzyme, present in neutrophils, is therefore also required for an increase in LL-37. In addition, most authors describe a 2-fold increase in CAMP, which may not equate to significantly increased protein production in-vivo (245). However, Yim et al (1) did demonstrate an increase in protein levels and a 10-fold increase in gene expression so this does not seem to be the only explanation for the lack of effect seen in my study. In the work by Yim et al, the concentration of vitamin D far exceeded the concentration that would be seen in-vivo providing an explanation as to why effects may not translate.

Other explanations for a lack of effect seen in-vivo is the possibility that there are other influences on gene expression present in the airways that over-ride the potential increased expression by vitamin D in a competitive manner.
There is a lack of data on induction of LL-37 by vitamin D *in-vivo*; a study evaluating the level of LL-37 before and after administration of high dose vitamin D found an increase in gene expression but similarly to my study, no increase in protein levels (225). Grossman et al also found no effect on LL-37 levels with a large dose of vitamin D in a double –blind randomised control trial (292).

LL-37 was found to be related to age in the CF group. LL-37 is released by neutrophils and macrophages as well as epithelial cells and older children have more inflammatory cells present. Therefore, the increase in LL-37 may be a consequence of an increased number of cells rather than a direct age effect. This study did not allow these two possibilities to be distinguished.

Whilst no relationship was found with LL-37 and vitamin D, I did find that LL-37 levels correlated with other markers of inflammation. BALF total cell count, neutrophil count and neutrophil differential, serum neutrophils and BALF cytokines all demonstrated a positive correlation with LL-37. No relationship was seen with LL-37 and lung function (FEV₁ and FVC) as LL-37 is stored in neutrophils, this relationship might have been anticipated. Therefore, rather than being a marker of benefit to lung health, perhaps LL-37 should be viewed as another biological marker of airway inflammation. The positive findings seen here, again, add further weight to the negative results with LL-37 and vitamin D. They demonstrate that assays were performed correctly, samples stored and processed correctly and the negative results seen were not due to a defect in the methodology, but due to a true absence of relationship because vitamin D is irrelevant, or not relevant at these levels.
8.5.2 Strengths of the study

One strength of this study is that the mechanistic differences were confirmed by clinical findings. We found no mechanistic difference between vitamin D sufficient and deficient groups, and no associations between vitamin D and clinical parameters.

In order to ensure a signal was not missed, and as the optimal vitamin D level for respiratory health is unknown, different cut-off values were used to define cohorts, but with no change in outcome. Some groups have used a higher cut-off of 75nmol/L to distinguish deficient from insufficient patients (44, 227, 253, 275, 278). The insufficient group have sometimes been sub-divided but again, different cut-off values have been used; some groups have used <25nmol/L and 25-<75nmol/L (278) and others of <50nmol/L and 50-<75nmol/L (227, 253, 275). This discrepancy in values used shows the lack of consensus as to an appropriate vitamin D level that should be accepted. The performance of post-hoc analyses with several cut-off values increases the likelihood that negative findings were not due to differences in definitions of vitamin D deficiency.

To maximise the reliability of the conclusions, large numbers (113) of CF patients with a wide range of disease severity were studied. However, the numbers in the non-CF CSLD and healthy control group were smaller due to the relatively less frequent performance of FOB in these patients.

Finally, many different parameters were explored, including clinical and biological aspects. A thorough exploration of immunological and clinical benefits was therefore performed. Although many other potential pathways could have been examined, the total absence of a
signal of low vitamin D levels with any clinically significant downstream measurement led my to conclude that such a search was unlikely to be fruitful.

8.5.3 Weaknesses of the study

Whilst a large number of CF patients were recruited to the study, due to the opportunistic nature of the study, a smaller number of control patients were recruited.

A limitation worthy of discussion is that few CF patients beyond the first year of life undergo FOB at a time of clinical stability. Patients are recruited opportunistically, when having clinically indicated bronchoscopy, biasing towards the exacerbating state. It is possible therefore that these data cannot be directly extrapolated to the stable CF lung. However, as CF is a condition with frequent exacerbations, which impact directly chronic respiratory health, these findings are of likely relevance.

Additionally, the BALF data are cross-sectional, reflecting a single time point; there are no data on the longitudinal changes of many of the markers measured in this study. Microbiological culture status at the time of bronchoscopy may be affected by the use of antibiotics and sampling errors; future studies should use molecular techniques if the relationship of Vitamin D on the whole microbiome is to be studied, although it has to be said that the relevance of molecular studies to clinical management has yet to be determined.

Microbiological results may also be affected by sampling errors as the BAL samples were routinely obtained from the right middle lobe and lingula, whilst some studies have
reported that whereas inflammation and inflammatory changes are homogenous throughout the lung, there is considerable geographical variability amongst airway regions with regards to bacterial culture (110).

BALF proteases were not measured and with hindsight, neutrophil elastase would be a useful addition to my study and would have allowed fuller consideration of whether vitamin D is only relevant at certain stages of the disease process. VDBP levels are not available so the amount of free vitamin D available for action cannot be calculated. VDBP can be measured in serum using commercially available ELISA kits but due to the lack of relationship seen with vitamin D and the innate immune markers with clinically relevant outcomes, in this study a decision was taken not to investigate this vitamin D related marker.

It may be that whilst serum levels of vitamin D are not relevant, BALF levels are; and another weakness to this study is that BALF vitamin D levels were not measured. Previous investigators within the department had unsuccessfully attempted to perform this assay and so no further attempts were of made as part of this study. It is therefore not possible to comment on whether local levels would have had an impact but if they are important, it is not via LL-37 and HBD-2.
8.5.4 Findings in relation to other studies

8.5.4.1 Vitamin D levels

Similarly to our study, many other investigators have reported low levels of vitamin D in CF and the comparability of my results to these is a further validation of the results I obtained. A review of 29 studies of CF patients over the past 20 years documented that just one study found the median vitamin D to be in the optimal range (≥ 75 nmol/L) (267). The vitamin D levels of our patients were higher than that of many other studies, with 18 of these 29 studies reporting a lower median vitamin D than that seen in our population (57 nmol/L). However, the 6 studies included in this review with younger patients (median age <12 years) all had similar or higher median vitamin D levels (55 – 87.75 nmol/L). A recent study (290) looking at 175 CF children found that 39% were vitamin D sufficient (≥ 75 nmol/L), compared to 25% in our study, and 18% were deficient (<50nmol/L) compared to 34% in our study. These differences were statistically significant (Fishers exact test <0.01); the study was conducted in a sunnier climate, which may at least partially explain the higher levels seen.

Several groups have found that satisfactory levels can be achieved with adequate supplementation. A trial done in Liverpool, UK, found that a weekly dose of 50,000 international units of vitamin D3 increased the median serum vitamin D from 17.6 to 78.6 nmol/L (337) and an observational study of 26 CF children found that vitamin D level of over 75 nmol/L were seen in half of the pancreatic insufficient children (338). Interestingly, this was only achieved in 40% of the pancreatic sufficient children which may reflect the fact that pancreatic sufficient CF children are not always routinely supplemented with the fat
soluble vitamins. This was also seen in a study of 148 Boston children with 60% of pancreatic insufficient patients achieving vitamin D levels over 75 nmol/L but only 50% of pancreatic sufficient children (339). In my study, median vitamin D levels were similar in pancreatic sufficient and insufficient groups; 25% of pancreatic sufficient and 21% of pancreatic insufficient patients achieved vitamin D level over 75 nmol/L patients reflecting the need for supplementation in both groups. Vitamin D deficiency is commonly seen in the healthy population and so the pancreatic sufficient do not differ from this population in this regard, although may have subclinical insufficiency predisposing them to deficiency. It is therefore believable that if healthy population are deficient then so too might the pancreatic sufficient cohort.

Seasonal variation in vitamin D levels has been reported (266, 340) but not universally (339); it may be that ingested supplements make sunlight and therefore season less important.

Other investigators have found increased levels in BALF in patients with severe asthma, a correlation with inflammatory markers and a deactivation of vitamin D in-vitro with the addition of VDBP (341). The results may imply that increased VDBP impairs vitamin D activity by reducing bio-availability, or it may exert its chemotactic effects on neutrophils and macrophages increasing the inflammatory state of the airways thereby and contributing the severity of disease in asthma (342); or it could be a marker of inflammation rather than a causative agent as VDBP protein expression is known to be increased by inflammatory cytokines (343).
There was no relationship between spirometry and vitamin D unlike the findings of a large study in Scandinavia (286). This study found a positive correlation between vitamin D and FEV₁ ($r^2 = 0.308$, $p = 0.025$) and an inverse relationship with vitamin D and IgG ($r^2 = 0.376$, $p<0.001$). However, the correlation coefficient was small and the study involved around 800 patients. By exploring the spirometry of patients, not just at the time of the vitamin D measurement, but over a 3 year period we are confident that our conclusion of no relationship being present is correct for our population. In addition, other clinical parameters such as BMI and days on IVAB, showed no relationship to vitamin D. We cannot account for the reasons in the difference between the Scandinavian cohort and our population. However, in our study, we looked at possible mechanistic as well as clinical effects and found no difference in either.

In a pilot double-blind RCT of 30 adults with CF, subjects experiencing a pulmonary exacerbation received vitamin D or placebo (344) and there was a trend towards improved clinical outcomes with patients supplemented with vitamin D compared with placebo. Our patient group did not have levels comparable to this and there is a possibility that clinical benefit could have been observed at much higher serum levels. As most patients never achieve such a high level, whether in clinical practice or research studies, it is not known if this finding would be reproducible across other settings and it is possible that immunomodulation for CF patients requires much higher levels than the level required for bone health. The fact that only a trend towards positive effects was seen even at such high levels makes it less certain that any true relationship exists, and more likely that, as in our
study, there is not a significant benefit of higher vitamin D levels in respiratory health in CF. It may be that vitamin D levels required for bone health and immune health are different.

Similarly to other studies (290, 339, 345) we did not find any association between vitamin D and clinical parameters or inflammatory markers. One of these studies (290) examined the vitamin D levels of 130 children and, comparable to the findings of my study, they found no association with vitamin D and lung function (FEV₁, FVC and FEV₁/FVC). They did find that more pulmonary exacerbations were seen in adolescents with vitamin D deficiency (< 75 nmol/L) than those not deficient (≥ 75 nmol/L). However, this finding was not seen in other age groups and was not associated with a difference in lung function so the significance of this finding remains unclear. Another possibility is that low level of vitamin D may be a marker of poor treatment adherence and it is the poor adherence that leads to exacerbations, not the low vitamin D.

Hall et al. compared sputum inflammatory markers with vitamin D in adult patients with non-CF bronchiectasis (227) and found that levels of the pro-inflammatory cytokines IL-8, IL-1β and TNF-α were higher in vitamin D deficient patients compared to those vitamin D sufficient (≥75 nmol/L) whilst we found no such relationship. Although this study examined sputum whereas we examined BAL, one would expect similar findings in both and so would not expect this to account for the differences in findings. However, more proteolysis is expected in CF than in non-CF bronchiectasis, which may account for the lack of effects seen. We used different vitamin D cut-off values to those used in this study but on post-hoc analysis using the cut-off values used in this study we found no difference in vitamin D deficient and sufficient groups. Our study examined children with CF and this study
examined adults with non-CF bronchiectasis; therefore the study populations were different. The different results seen in these 2 studies are likely due to the different population groups studied. If this relationship is indeed true, the relationship between vitamin D and inflammatory markers is therefore either not applicable in children or not applicable to CF patients. One explanation for a lack of relationship being seen in children as compared to adults is that this group may have relatively healthy airways. It may be that more severe inflammation is needed for vitamin D to demonstrate an effect; that at lesser levels of inflammation other factors have more of an impact and so no additional effect is observed with vitamin D. Alternatively, it may be that the underlying CF pathology and the presence of abnormal CFTR with its consequences have an impact with other disease specific factors within the airway negating any benefit that would otherwise be seen.

8.5.4.3 Bacteriology

Unlike my cohort of CF and non-CF CSLD paediatric patients, vitamin D deficiency was associated with increased bacterial infection in adults with non-CF bronchiectasis (227). A possible explanation for the difference in findings could be due to CF airways being more complex with multiple defects contributing to disease processes, or adult patients having altered immune function. The findings in my non-CF cohort may be due to lack of power but the absence of a trend in any parameter supports a true lack of relationship.

One factor to consider in comparing my results with published data is that publication bias means that positive findings are more likely to appear in the literature; it is therefore not known whether other groups have found similar results to mine but the negative studies
have just not been published. This is a common factor within medical literature and not unique to this study.

A study conducted in infants and pre-school children with CF (345) found that low vitamin D was associated with increased risk of lower respiratory infection with *S. aureus*. A separate study found that patients with *P. aeruginosa* had a lower median serum vitamin D than those without (339). This study was conducted in Boston, USA and examined 148 children (339). The median vitamin D of patients in this study was higher than in our study with a median vitamin D of 81 nmol/L, compared with 57 nmol/L in our study. Just 43% of subjects had a level less than 75 nmol/L; 75% of the subjects in my study had levels below this. This study found that patients who had ever isolated *P. aeruginosa* had a lower median serum vitamin D than those without *P. aeruginosa* (69.3 nmol/L vs. 82.3 nmol/L, p = 0.02). However, significant overlap was seen between the two groups, the p-value was greater than the value considered significant in my study and the effect was not seen for *S. aureus*. More meaningfully, in neither of these were any downstream inflammation consequences of this apparent increased bacterial infection demonstrated, and therefore it may be questioned as to whether these findings were real. The authors of one of these studies (339) comment themselves that whilst the difference was statistically significant it may have been of doubtful *clinical* significance as median values in the groups were so close. They also commented that even if an association did exist, it does not imply causation and suggest it could be a reflection of medication adherence.

Although we did find lower vitamin D with *S. aureus* and *P. aeruginosa* at some time points within the study, in logistical regression models these relationships disappeared once age
had been accounted for, and we found no relationship between vitamin D level and the presence of any bacterial or fungal organisms. The lack of this relationship being seen in all settings and its relationship to age make us confident that at least in our cohort, there is no true relationship between vitamin D and bacterial isolation.

Unfortunately, due to a change in methodology of viral detection during the study period, we were not able to accurately explore this.

8.5.4.4 Antimicrobial peptides

The authors of another study (124) also found that LL-37 level increased with BALF total cell count and BALF neutrophil count and similarly to our study they found no association with LL-37 and HBD-2.

I did not find a relationship between LL-37 and FEV\textsubscript{1} and this was similar to the finding in the study previously described (124) although they did find a negative association with MEF\textsubscript{25}\% (r = -0.33, p=0.016) implying increasing small airways disease with increasing levels of LL-37. These data were not collected in this study as it is a measure with far greater variability than FEV\textsubscript{1}. Future work should use LCI to examine the effect on small airways.

These results suggest that rather than increased levels of LL-37 meaning more antimicrobial activity and subsequent better lung health, LL-37 is increased in diseased states. It is likely that LL-37 is not a causative factor of poorer lung health, but a marker of disease. As LL-37 has immunomodulatory actions as well as antibacterial, it could add to the increased inflammatory state within the airway thereby having adverse effects. However, longitudinal or intervention studies are required to differentiate causation from association.
The presence of a vitamin D response element in the promoters of camp and defB2 (the genes encoding LL-37 and HBD-2) suggests that vitamin D is relevant to LL-37/HBD-2 and in-vitro data has shown increased expression of LL-37 and to a lesser extent HBD-2 with vitamin D in CF cells (213). LL-37 and HBD-2 gene expression can be measured using real-time quantitative PCR from extracted DNA. Some of the study subjects had BALF cells suspended in RNALater® and stored at -80 degrees available and may have been possible to perform this technique from these cells; measurement of gene expression rather than just protein levels would have been an interesting addition to the study to examine if gene expression correlates with vitamin D level. It is possible that vitamin D promotes gene expression of the antimicrobial peptides but these molecules are degraded quickly and subsequently a relationship with the protein cannot be seen. However, even is such a relationship does exist, this does not translate to a clinical effect and is thereby somewhat irrelevant.

One small RCT involving 30 CF patients treated with either intramuscular vitamin D or placebo at the time of a pulmonary exacerbation found no difference in plasma LL-37 levels between the groups, although they did report a treatment-associated reduction in TNF-α (292). Similarly to this study we found no effect of vitamin D on LL-37 level, contrasting with in-vitro data of vitamin D induced expression of LL-37 in CF cells. This finding is also in agreement with previous work which found no relationship between serum LL-37 (225) and serum vitamin D. It is possible that the expression of LL-37 and HBD-2 is induced by vitamin D, but that these peptides are degraded in the proteolytic environment of the CF airway and therefore no relationship is found with peptide levels. Against this, LL-37 correlated positively with other markers of inflammation including neutrophil numbers. Depending on
survival time on the airway surface one might also expect to see a relationship between vitamin D and infection, which was clearly lacking in our group. Another study found an association with BAL LL-37 and BAL vitamin D in allergic patients but this was only seen after allergen challenge (346) and only with BAL vitamin D, not serum vitamin D. LL-37 levels significantly increased after allergen exposure, which may be due to the influx of inflammatory cells that occurs as a result of the allergic response. Although LL-37 is predominantly stored in neutrophilic granules, other inflammatory cells including mast cells express this peptide. It was interesting that this association was only seen with BAL vitamin D and suggests that local action and local levels are more important than circulating levels.

It is worth noting, that whilst in-vitro studies have shown LL-37 to be induced by vitamin D, experimental conditions have been based on exposures of a much higher concentration of vitamin D than that seen in in human physiological conditions. Also, such studies use the active form of vitamin D (1,25(OH)D2, and are therefore not relying on the enzymatic conversion of the inactive form (25(OH)D2). One study used a concentration of 1,25(OH)D2 of 10-100 nM compared with the 50-100 pM seen in-vivo, a 1000-fold increase (221). It is therefore difficult to make a direct comparison.

It is possible that the expression of LL-37 and HBD-2 is induced by vitamin D, but that these peptides are degraded in the proteolytic environment of the CF airway and therefore no relationship is subsequently apparent with peptide levels. Against this, LL-37 correlated positively with other markers of inflammation including neutrophil numbers, although they could have initially been much higher.
We chose two innate defence peptides based on *in-vitro* evidence that their promoters contain VDRE and increased expression in response to vitamin D (213). It is possible that vitamin D affects other defence proteins that have not been explored here, although in the absence of detectable down-stream consequences, any such mechanisms may be of questionable clinical relevance. If there is a biological effect of vitamin D on any of these pathways, the benefit is not translated to a clinical one.

### 8.5.5 Conclusions

In conclusion, I hypothesised that levels of antimicrobial peptides in BALF would be related to vitamin D status in children with CF, which would provide an explanation for the reported improvements in lung function via improved airway defence to bacterial infections. I aimed to explore the relationships between serum vitamin D levels and a) levels of inflammatory mediators, antimicrobial peptides and bacterial infection in the airway and b) clinical parameters including lung function, requirement for intravenous antibiotics and BMI. The data demonstrate that there is no relationship between serum levels of vitamin D and BALF levels of either HBD-2 or LL37 and no effect on airway inflammation and no detectable clinical or physiological effects of vitamin D deficiency.
Part D: Conclusions
Chapter 9: Final discussion, conclusions and future work

9.1 Principal findings

I hypothesised that the earlier institution of improved and more aggressive treatments strategies possible because of NBS would lead to improved airway health and that vitamin D has immunomodulatory effects via antimicrobial peptide pathways with vitamin D deficiency leading to worse clinical outcomes. I set out to describe the spectrum of airway disease in a large cohort of young infants diagnosed on NBS and was able to recruit 77 infants and well described the infection and inflammation seen in their airways cross-sectionally, and described longitudinal changes with relation to structural and functional change in 19 of them. I also set out to describe vitamin D levels in children with CF and compare them to 2 control groups and was able to recruit 113 children with CF, 23 patients with non-CF CSLD and 6 healthy controls. I collected BALF samples and blood samples in order to measure vitamin D, inflammatory markers and antimicrobial peptides.

There was no association between serum vitamin D and airway levels of LL-37 and HBD-2, or any marker of airway inflammation and infection, in children with CF, nor any correlation with clinical outcomes. This discrepancy between my hypothesis and my results could be because (a) Vitamin D is irrelevant; (b) it is only relevant at certain stages of the disease, for example early on when fewer proteases are seen in the BALF and in the initial response to infection; (c) only tiny amounts of Vitamin D are needed for optimal immune health, and all patients were on the top flat part of the dose-response curve; (d) much higher levels of
Vitamin D are needed to show an effect, all patients being at the bottom flat part of the curve; (e) Local levels of Vitamin D are relevant, and unrelated to systemic levels.

The optimal level of vitamin D for immunological functioning is not known and it could be that all recruited children were either all above, or all below this, which is why no difference was seen. As the children spanned a wide range of vitamin D it would be difficult to find children with supra-high or supra-low levels to use as a comparison as the vitamin D ranges in this study are comparable with the literature. Biologically, given the known toxic effects of high dose vitamin D, it seems unlikely that much higher doses than conventionally attained would be necessary for immunological health, so (d) above, although theoretically an explanation, seems least likely to be true.

Numerous polymorphisms exist in the VDR, which binds to vitamin D allowing it to enter the cell to exhibit its effects. Some polymorphisms may be more effective than others at carrying out these conversions and it may be this activation that is relevant rather than the availability of free, inactive, vitamin D.

As vitamin D is actually LOWER in non-CF bronchiectasis, a condition in which better outcomes are expected; this supports (a) and the idea that Vitamin D is irrelevant to airway health in chronic suppurative lung disease, at least in the range studied. The lack of correlations with vitamin D and clinical outcomes (FEV₁, FVC, BMI, days on antibiotics; infection state) further support the likely lack of any clinical significance of Vitamin D levels in CF.
I addressed the possibility of vitamin D only being relevant at certain stages of disease by splitting into early and later disease and found no effects of Vitamin D. The results presented in this thesis include children of all ages. Post-hoc analyses confirmed there was no change in the conclusions when children under the age of 2 years were excluded from the group in order to look at the relationship of vitamin D in those with more established disease. Perhaps more importantly, post-hoc analyses of CF patients <2-years-old were performed. Innate host defence mechanisms are thought to be of particular importance in the early years, before the body has had time to develop acquired antibody immunity but even in this age group, vitamin D level did not correlate with markers of inflammation. Results of these 2 post-hoc analyses demonstrated no alteration to findings.

Two innate defence peptides were chosen based on in-vitro evidence that their promoters contain VDRE and there was increased expression in response to vitamin D (213). However, it is possible that there are effects in other defence proteins which have not been explored here, although in the absence of detectable down-stream consequences, any such mechanisms may be of questionable clinical relevance.

### 9.2 Strengths of the study

A strength of this piece of work is the large number of children and different disease severities studied with the same conclusion being drawn from each sub-study. Some of the data that I was able to collect was longitudinal adding further to the strength of the results.
Inflammatory cells, both cellular and soluble correlated with clinical features. For example, those with infection in their BALF had higher pro-inflammatory cytokines, more airway neutrophilia, a greater number of total inflammatory cells in their BALF and higher serum neutrophils than those BALF culture negative. Also, total cell count in BALF correlates with numerous BALF cytokines. The fact that this expected result was demonstrated (an important positive control) gives confidence to the result obtained demonstrating an absence of relationship between inflammation and vitamin D, and also demonstrates that the study was adequately powered to show relationships.

9.3 Weaknesses of the study

The opportunistic nature of the study limited selection over patient recruitment, particularly in the controls. Limitations due to financial and sample availability meant it was not possible to repeat assays if no reading was obtained meaning that full results were not available for every recruited child.

Although I had some longitudinal data a number of the studies were unavoidably cross-sectional. I also did not recruit any adults to the study, it is conceivable that in end-stage disease the pattern may be different, but I have no data to comment on this.

Another limitation is the variability seen with the HBD-2 assay and in future work I would try to perform all assays at the same time point rather than on different runs to try to limit the
inter-run variability. However, given the rest of the laboratory data and the fact that my conclusions rest on clinical endpoints, I do not believe that the problems with this assay affect my core conclusions.

9.4 Future work

9.4.1 Vitamin D

Vitamin D measured in serum does not correlate with clinical outcomes, but local levels may not relate to systemic (341). If levels of 1,25(OH)D in BALF were found to be relevant then perhaps a future direction would be to explore vitamin D analogues as therapeutic agents. 1,25(OH)D analogue use rather than vitamin D itself may avoid potential limitations in dose due to toxicity and also have the benefit that it would not be reliant on conversion enzymes and negate the effects of the polymorphisms seen in the VDBP. In order to achieve this it would be necessary to reliably be able to measure vitamin D and VDBP in BALF and future work could develop these assays.

It would also be interesting to ascertain if polymorphisms in the VDBP gene are CF disease modifiers because to my knowledge this has never been looked for.

It would be interesting to observe if an increase in LL-37 is seen after inflammatory response due to infection as well as to allergen exposure as was previously demonstrated; both would result in influx of inflammatory cells and so one would expect similar findings. By
experimenting with CF and non-CF cells, it would be possible to detect if a difference was due to underlying pathology such as increased proteolysis or the pro-inflammatory state seen in the CF airway. Clearly such an experiment would not be possible in humans and so such experiments would need to be conducted in animals or cell studies although both methods pose difficulties in translating findings to human subjects. Even if a difference was seen it is difficult to see how this translates to clinical relevance given the absence of findings in this work. If a direct \textit{in-vitro} effect is seen, it may be worthwhile looking at possibility of administering very high doses of vitamin D or vitamin D analogues.

9.4.2 Early inflammation

More work on infants, early lung function and inflammation with longitudinal monitoring is needed to further understand the airway and understand how early new treatments need to be given. Delay in such treatments may be advantageous due to unknown side effects on the developing organs, but if significant inflammation is consistently seen that sets the course for life then it may be that waiting is not possible. Further evaluation will enable decisions to be made when such treatments become available by providing information as to whether some infants are predestined to a more complicated course, or if early exposure to bacteria sets the course of more severe disease. Early and longitudinal measurement of inflammatory markers alongside lung function is required but poses difficulties due to the invasive nature of obtaining airway samples. This study has shown BALF LL-37 to correlate with other clinical parameters and I would like to go on and compare the levels in BALF to serum to address the question; how can we find a non-invasive biomarker for those who will
do badly in order to enable early aggressive and prompt treatments to alter their
disease progression?

9.5 Conclusions

If vitamin D was exerting a biologically important effect on the innate immune system, regardless of the mechanism of action, we would expect to see downstream consequences with decreased cellular markers of inflammation and decreased pro-inflammatory cytokines. This was not the case in this study and no relationship was demonstrated between vitamin D and the BALF innate markers. If vitamin D is involved in the induction of such defence peptides in-vivo, the impact of this on protein levels may be limited in the degradative environment of the inflamed airway. The fact that we also had an absence of relationship with vitamin D and inflammation/infection in our non-CF population makes it less likely to be a disease specific effect and more likely that vitamin D bears no significant benefit on respiratory health in children with chronic suppurative lung diseases.

In conclusion, having set out to test the hypotheses that vitamin D deficiency leads to worse clinical outcome I found that, contrary to my hypotheses no such signal was seen. Although for reasons of bone health optimising vitamin D levels in CF is important, there is no relevance of serum Vitamin D levels to immunological or inflammatory airway health, at least in children.
10 References


17. Durr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. BiochimBiophysActa. 2006;1758(9):1408-25.


32. CF Trust. Standards for the Clinical Care of Children and Adults with cystic fibrosis in the UK 2011.
33. UK Newborn Screening Programme Centre. A laboratory guide to newborn screening in the UK for cystic fibrosis (June 2009) 2009.


116. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, et al. Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and


231. Sutherland ER, Goleva E, Jackson LP, Stevens AD, Leung DY. Vitamin D levels, lung function, and steroid response in adult asthma. Am J Respir Crit Care Med. 2010;181(7):699-704.


