Vitamin D and severe therapy resistant asthma in children

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Abstract

Background
There is increasing evidence that low vitamin D levels are implicated in paediatric asthma.

Hypotheses
Children with severe, therapy resistant (STRA) have low serum vitamin D levels which are associated with worse asthma control and airway pathology. In vitro, steroid responsiveness of peripheral blood mononuclear cells (PBMCs) from STRA is improved by vitamin D.

Methods
Serum 25-hydroxyvitamin D (25[OH]D3) was measured in 36 STRA, 26 mild/moderate asthmatics (MA) and 24 non-asthmatic controls, and related to asthma control and exacerbations. 22/36 children with STRA underwent bronchoscopy with assessment of airway inflammation and remodelling. The in vitro steroid responsiveness of PBMC was assessed, in the absence or presence of dexamethasone and vitamin D.

Results
Median 25[OH]D3 levels were significantly lower in STRA (28nmol/L) than MA (42.5nmol/L) and controls (56.5nmol/L). There were positive relationships between 25[OH]D3 levels and %predicted first second forced expired volume (r=0.4, p<0.001) and forced vital capacity (r=0.3, p=0.002). 25[OH]D3 levels were positively associated with asthma control test (ACT) (r=0.6, p<0.001) and inversely associated with exacerbations (r=-0.6, p<0.001) and inhaled steroid dose (r=-0.39, p=0.001) in asthmatics. Airway smooth muscle (ASM) mass was inversely related to 25[OH]D3 levels (r=-0.6, p<0.01). Asthmatic children had diminished levels of bronchoalveolar lavage IL-10 (p<0.001). Their PBMC also demonstrated significantly impaired capacity to secrete IL-10 in culture (p<0.001) and the inclusion of vitamin D, enhanced dexamethasone-induced IL-10 (p<0.05), production. Furthermore vitamin D status correlated with airway IL-10 (r=0.6, p<0.01) and CD4+ FoxP3 T regulatory cells (r=0.6, p<0.01). Children with STRA had significantly higher vitamin D binding protein (VDBP) levels in BAL compared to MA (p<0.05) and controls (p<0.01) and these were positively associated with symptoms (ACT) (r=0.5, p=0.01) and inhaled corticosteroid usage (r=0.6, p=0<0.01).

Summary
Lower serum vitamin D levels in STRA children were associated with increased ASM mass, worse asthma control, lung function and reduced BAL IL-10 and T regulatory cells. In vitro vitamin D enhanced dexamethasone-induced IL-10 production. These data suggest vitamin D supplementation may be useful in STRA and support the need for a clinical trial.
# Table of Contents

Abstract ................................................................................................................................. 2

Table of Contents .................................................................................................................. 3

Publications and abstracts that have resulted to date from the work performed in this thesis ....................................................................................................................... 10

Declaration .............................................................................................................................. 14

Abbreviations ......................................................................................................................... 16

List of Figures .......................................................................................................................... 20

List of Tables .......................................................................................................................... 22

Chapter 1 Introduction ......................................................................................................... 23

1.1 Immunopathology of asthma .......................................................................................... 23

1.1.1 Asthma ..................................................................................................................... 23

1.1.2 T helper cells in asthma ......................................................................................... 26

1.1.3 Th17 cells in asthma ............................................................................................... 28

1.1.4 Regulatory T cells (Tregs) in asthma ....................................................................... 30

1.1.5 Airway remodelling in asthma ................................................................................ 34

1.2 Problematic severe asthma in children ......................................................................... 37

1.2.1 Assessment of steroid responsiveness in children .................................................. 38

1.3 Steroid resistance ........................................................................................................... 42

1.3.1 Mechanism of corticosteroid action ....................................................................... 42

1.3.2 Molecular mechanisms of steroid resistance ......................................................... 43

1.3.3 Steroid resistance in asthma and vitamin D in asthma ......................................... 45

1.4 Vitamin D ....................................................................................................................... 47

1.4.1 Sources of vitamin D ............................................................................................. 47

1.4.2 Vitamin D metabolism ........................................................................................... 48

1.4.3 Definition of vitamin D deficiency and insufficiency ............................................. 50

1.4.4 Prevalence of vitamin D deficiency and insufficiency ........................................... 51

1.4.5 Risk factors for vitamin D deficiency and insufficiency ........................................ 52

1.4.6 Epidemiological evidence of an association between vitamin D and asthma53

1.4.7 Epidemiological evidence of a link between vitamin D and preschool asthma / wheeze ......................................................................................................................... 60

1.4.8 Immunomodulatory effects of vitamin D .............................................................. 63

1.4.9 VDBP ....................................................................................................................... 65

1.4.10 Vitamin D and airway remodelling .................................................................... 67

1.4.11 Vitamin D and lung development ........................................................................ 69

1.4.12 Vitamin D and asthma exacerbations ................................................................ 69

1.5 Summary ......................................................................................................................... 72

1.6 Research Hypotheses, Aims and Objectives ................................................................. 73

1.6.1 Research hypotheses .............................................................................................. 73

1.6.2 Aims ......................................................................................................................... 73

1.6.3 Specific objectives .................................................................................................... 73

Chapter 2 Methods .............................................................................................................. 75

2.1 Study plan and subjects ................................................................................................. 75

2.2 Clinical methods ............................................................................................................ 76

2.2.1 Study consent ........................................................................................................... 76

2.2.2 Height and weight ................................................................................................... 76

2.2.3 Exacerbations and medication usage ...................................................................... 76

2.2.4 Pulmonary function testing .................................................................................... 76
Chapter 3. Relationship between serum vitamin D, disease severity, airway inflammation and remodelling in children with asthma

3.1 Introduction .................................................................92
3.2 Methods ......................................................................93
  3.2.1 Subjects..................................................................93
  3.2.2 Sputum eosinophils and neutrophils .........................93
  3.2.3 Bronchoscopy, BAL and EB .................................93
3.3 Results .......................................................................94
  3.3.1 Subject demographics............................................94
  3.3.2 Serum 25[OH]D₃ levels in asthmatics and controls ....96
  3.3.3 Vitamin D and atopic status...................................99
  3.3.4 Spirometry, BDR and serum vitamin D ..................100
  3.3.5 Asthma control, exacerbations and serum vitamin D levels 103
  3.3.6 Medication dose and serum vitamin D levels ..........106
  3.3.7 Serum vitamin D levels and asthma pathology ........108
  3.3.8 Serum vitamin D levels and disease severity ..........116
3.4 Discussion .....................................................................120
  3.4.1 Principle findings ................................................120
  3.4.2 Strengths and weaknesses ....................................121
  3.4.3 Strength and weaknesses in relation to other studies ....123
  3.4.4 Meaning of the results ........................................125
  3.4.5 Summary..............................................................125

Chapter 4 The effects of vitamin D on IL-10 secretion from PBMC in vitro from asthmatic children and controls

4.1 Introduction ..............................................................127
4.2 Methods ....................................................................130
  4.2.1 Subjects ................................................................130
  4.2.2 Steroid responsiveness ........................................130
  4.2.3 Isolation of cell populations from peripheral blood ...131
  4.2.4 Isolation of total PBMC ........................................131
  4.2.5 Preparation of CD8 depleted PBMC cells ...............131
  4.2.6 CD8 depleted PBMC cell culture..........................133
4.3 Results ......................................................................136
  4.3.1 Serum cytokine levels ..........................................137
  4.3.2 CD8 depleted PBMC from asthmatic children demonstrate impaired IL-10 secretion ...........................................138
  4.3.3 Dexamethasone inhibits IL-13 secretion, but enhances both IL-10 and IL-17A production in cultures of CD8 depleted PBMC .............................................140
  4.3.4 Vitamin D potentiates steroid-induced IL-10, but not IL-17A in CD8 depleted PBMC cultures ........................143
Chapter 6. Relationship between Vitamin D binding protein and asthma

4.3.5 Effect of dexamethasone and Vitamin D on CD8 depleted PBMC cytokine profiles of STRA - related to clinical steroid responsiveness........145
4.3.6 Relationship between serum vitamin D levels and steroid responsiveness ....................................................................................................................152

4.4 Discussion.......................................................................................................................................................................................154
4.4.1 Principle findings .............................................................................................................................................................................154
4.4.2 Strengths and weaknesses ...............................................................................................................................................................157
4.4.3 Strengths and weaknesses in relation to other studies ....................................................................................................................157
4.4.4 Meaning of the results ....................................................................................................................................................................160
4.4.5 Summary .........................................................................................................................................................................................161

Chapter 5. The effects of vitamin D on IL-10 secretion from BAL cells........162
5.1 Introduction .......................................................................................................................................................................................162
5.2 Methods ..........................................................................................................................................................................................163
5.2.1 Subjects.............................................................................................................................................................................................163
5.2.2 Isolation of cells from BAL fluid ......................................................................................................................................................164
5.2.3 BAL cytology ......................................................................................................................................................................................165
5.2.4 BAL cell culture ................................................................................................................................................................................165
5.2.5 Cytokine analysis by CBA .............................................................................................................................................................165
5.2.6 FoxP3 intranuclear analysis by flow cytometry .............................................................................................................................165
5.3 Results ........................................................................................................................................................................................................165
5.3.1 BAL fluid cytokines ......................................................................................................................................................................166
5.3.2 FoxP3 intranuclear staining ............................................................................................................................................................169
5.3.3 The effect of vitamin D and dexamethasone on BAL cell cultures ..................................................................................................171
5.3.4 Serum vitamin D status correlates with BAL IL-10 and CD4+FoxP3 cells. ..................................................................................174
5.3.5 Relationship between airway inflammation and BAL IL-10 in children with STRA. ...........................................................................177
5.4 Discussion................................................................................................................................................................................................178
5.4.1 Principle findings .............................................................................................................................................................................178
5.4.2 Strengths and weaknesses ...............................................................................................................................................................179
5.4.3 Strengths and weaknesses in relation to other studies ....................................................................................................................180
5.4.4 Meaning of the results ....................................................................................................................................................................182
5.4.5 Summary .........................................................................................................................................................................................183

Chapter 6. Relationship between Vitamin D binding protein and asthma severity in children ..................................................................................................................184
6.1 Introduction .......................................................................................................................................................................................184
6.2 Methods ..........................................................................................................................................................................................187
6.2.1 Subjects.............................................................................................................................................................................................187
6.2.2 Measurement of Serum 25-hydroxyvitamin D levels .....................................................................................................................188
6.2.3 Measurement of VDBP levels in the serum and BAL and culture supernatants ............................................................................188
6.2.4 BAL cell cultures .............................................................................................................................................................................189
6.3 Results ........................................................................................................................................................................................................189
6.3.1 Subjects.............................................................................................................................................................................................189
6.3.2 VDBP levels in asthmatics and control ........................................................................................................................................190
6.3.3 VDBP levels and markers of asthma control ..................................................................................................................................192
6.3.4 VDBP levels and serum vitamin D levels ......................................................................................................................................193
6.3.5 Effect of dexamethasone on VDBP production ............................................................................................................................194
6.3.6 Relationship between BAL VDBP levels and airway inflammation and remodelling ........................................................................196
6.4 Discussion................................................................................................................................................................................................198
6.4.1 Principle findings .............................................................................................................................................................................198
6.4.2 Strengths and weaknesses ...............................................................................................................................................................198

5
6.4.3 Possible mechanisms by which excessive VDBP in the airway may limit vitamin D bioactivity with potential immunological consequences .................................. 199
6.4.4 Meaning of the results .................................................................................................................. 200
6.4.5 Summary ........................................................................................................................................ 200

Chapter 7 Discussion ......................................................................................................................... 202
7.1 Main Findings ....................................................................................................................................... 202
7.2 Original hypotheses ............................................................................................................................. 202
7.2.1 Hypothesis 1 ................................................................................................................................... 203
7.2.2 Hypothesis 2 ................................................................................................................................... 204
7.3 Strengths of the study .......................................................................................................................... 205
7.4 Limitations of this study ........................................................................................................................ 206
7.5 Strengths and weaknesses in relation to other studies ....................................................................... 211
7.6 Future work ......................................................................................................................................... 222
7.7 Summary ............................................................................................................................................ 229

References ................................................................................................................................................ 231

Appendix 1. Difficult Asthma Proforma ................................................................................................. 256
Stage 1 ................................................................................................................................................... 256
Stage 2 ................................................................................................................................................... 263
Stage 3 ................................................................................................................................................... 268

Appendix 2. Sputum Processing Form .................................................................................................. 273

Appendix 3 Parent information (asthma) ............................................................................................ 274

Appendix 4 Parent information (control) ............................................................................................. 278

Appendix 5 Child information (control) ............................................................................................... 282

Appendix 6 Child information (asthma) ............................................................................................... 284
Dedicated to the memory of my father
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Dave Richards, and Sarah Dimeloe have been an invaluable guide and great friends. I'm sure I would have been lost wandering in the laboratory without their much appreciated and much needed close guidance.

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Other research colleagues I would like to thank include Hui-leng Tan, Cara Bossley, Ruth O'Reilly, Alies Sjoukes, Andrea McKee, Alex Adams, Caro Minasian, Nico Ullman, and Dr Sarah Brown. My friends who have all inspired and encouraged me at various stages of my career. As always I am grateful to my family, particularly my parents for pushing me and motivating throughout my life always to achieve more. My son Saatwik, who is still perplexed as to why I still have to continue to read and write despite finishing full time education! Finally, to my lovely wife Prachi who supported me when I often despaired with this thesis over the past years.
Publications and abstracts that have resulted to date from the work performed in this thesis

Peer reviewed publications


Kobayashi, Yoshihiko; Bossley, Cara; Gupta, Atul; Akashi, Kenichi; Tsartsali, Lemony; Mercado, Nicolas; Barnes, Peter; Bush, Andrew; Ito, Kazuhiro. Passive smoking impairs histone deacetylase-2 in children with severe asthma. Chest 2013
Abstracts at International Conferences


**Gupta A**, Oates T et al 2011, Vitamin D and airway remodeling in paediatric severe therapy resistant asthma. Winter Meeting of the British-Thoracic-Society, BMJ publishing group, Pages:A18-A18, ISSN:0040-6376


Chambers ES, Urry Z, Nanzer AM 2012, Richards, D. F, **Gupta, A.**, Bush, A.,Saglani, S,Corrigan, C.,Griffiths, C. M.,Hawrylowicz, C. M. Vitamin D increases the frequency of FoxP3+ Tregs in vitro and in vivo: differential role for IL-2 and IL-10, European Congress of Immunology, Wiley-Blackwell, Pages:84-84, ISSN:0019-2805


Declaration

I confirm that I have performed the work described in this thesis. Where colleagues have been involved, their contribution is acknowledged below. I have consulted all cited references. The work has not been submitted elsewhere for a higher degree.

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Contribution of work to this thesis by Atul Gupta (AG) and that performed by other colleagues

Recruitment

All cases identified, notes evaluated, consented and recruited by AG

Difficult asthma assessments

1. All home visits and stage 1 assessments were performed by respiratory nurses Marcella Bracken-King, Pippa Hall, Abby Carlton and Emily Guilmant
2. All stage 2 assessments were performed by AG
3. All stage 3 assessments were performed by AG

Sputum induction and processing

Sputum induction and processing was performed by AG in 10 patients and by Ms Adesimbo Sogbesan in 12 patients.

Bronchoscopies, BAL and EB

Bronchoscopies, BAL and EB were performed by consultant paediatricians for clinical indications. BAL cytology was performed by the histopathology department and biopsies were processed to wax blocks in the Department of Histopathology, Royal Brompton Hospital for clinical indications. Biopsy sections were cut by Mr Timothy Oates, Imperial College London. All markers of airway remodelling and inflammatory cell counts were assessed by Ms A Sjoukes and Mr Timothy Oates

Laboratory analysis
**PBMC culture**

All PBMC cultures were performed by AG.

**BAL culture**

All BAL cultures were performed by AG.

**BAL FACS analysis for CD4+FoxP3 cells**

BAL cells were stained and analysed for CD4+FoxP3 cells by flow cytometry by Mr Dave Richards in 6 subjects, Ms Emma Chambers in 2 subjects and AG in 3 subjects.

**CBA analysis**

AG performed CBA in serum samples under supervision of Mr Dave Richards. BAL CBA was performed primarily by Mr Dave Richards.

**VDBP levels**

BAL VDBP was measured by ELISA by AG and serum VDBP was measured by ELISA by Dr Sarah Dimeloe and Mr Dave Richards.

**Statistical Analysis**

1. Linear regression, logistic regression and power calculation were performed by Mr Winston Banya, Hospital statistician using Stata version 10.1 (Statacorp Texas, USA)

2. All other statistical analysis was performed by AG under the guidance of Mr Winston Banya, Hospital statistician using GraphPad Prism 5.02
<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Asthma control test</td>
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<tr>
<td>AHR</td>
<td>Airway Hyperresponsiveness</td>
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<td>ANOVA</td>
<td>Analysis of Variance Test</td>
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<tr>
<td>AP-1</td>
<td>Activator protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin (fluorochrome)</td>
</tr>
<tr>
<td>APC-Cy7</td>
<td>Allophycocyanin-cyano dye 7</td>
</tr>
<tr>
<td>APCs</td>
<td>Antigen Presenting Cells</td>
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<td>ASM</td>
<td>Airway smooth muscle</td>
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<td>ATS</td>
<td>American Thoracic Society</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
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<td>BDR</td>
<td>Bronchodilator reversibility</td>
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<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CBA</td>
<td>Cytometric Bead Array</td>
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<tr>
<td>CoV</td>
<td>Coefficient of variation</td>
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<tr>
<td>CXCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>Cytochrome P450 enzyme 27 B1</td>
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<tr>
<td>DA</td>
<td>Difficult asthma</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotreitol</td>
</tr>
<tr>
<td>EB</td>
<td>Endobronchial biopsy</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FceRI</td>
<td>Fc Epsilon Receptor Type 1 (Immunoglobulin E Receptor)</td>
</tr>
<tr>
<td>FeNO&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Fractional exhaled nitric oxide at flow rate 50ml/second</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Forced Expiratory Volume in 1 second</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt;/FVC</td>
<td>Ratio of Forced Expiratory Volume in 1 second to Forced Vital Capacity</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate (fluorochrome)</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead Box P3</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>FVC</td>
<td>Forced Vital Capacity</td>
</tr>
<tr>
<td>GA</td>
<td>General Anaesthesia</td>
</tr>
<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyl transferase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's Balanced Salt Solution</td>
</tr>
<tr>
<td>hCAP-18</td>
<td>Human Cathelicidin Antimicrobial Peptide</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>ICS</td>
<td>Inhaled Corticosteroids</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon Gamma</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgE</td>
<td>Immunoglobulin E</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL-10-Treg</td>
<td>Interleukin-10-secreting Regulatory T Cell</td>
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<tr>
<td>IM</td>
<td>Intramuclear</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IPEX</td>
<td>Immune Dysregulation Polyendocrinopathy X-linked</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
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<tr>
<td>LABA</td>
<td>Long acting β agonist</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTRA</td>
<td>Leukotriene receptor antagonist</td>
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<tr>
<td>MA</td>
<td>Mild / Moderate Asthma</td>
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<tr>
<td>MAPK</td>
<td>Mitogen–activated protein kinase phosphatase</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RiboNucleic Acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of B cells</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin (fluorochrome)</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on Activation Normal T Cell Expressed and Secreted</td>
</tr>
<tr>
<td>RAST</td>
<td>Radioallergosorbent test</td>
</tr>
<tr>
<td>RBM</td>
<td>Reticular basement membrane</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
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<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
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<tr>
<td>SABA</td>
<td>Short acting β agonist</td>
</tr>
<tr>
<td>SARP</td>
<td>Severe asthma research program</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SM</td>
<td>Smooth muscle</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
<td>SPF</td>
<td>Sun protection factor</td>
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<tr>
<td>SPT</td>
<td>Skin prick tests</td>
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<td>SR</td>
<td>Steroid resistant</td>
</tr>
<tr>
<td>SS</td>
<td>Steroid sensitive</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>STRA</td>
<td>Severe Therapy Resistant Asthma</td>
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<td>TCR</td>
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<td>Type 1 Helper T Cell</td>
</tr>
<tr>
<td>Th17</td>
<td>Interleukin 17-secreting Helper T Cell</td>
</tr>
<tr>
<td>Th2</td>
<td>Type 2 Helper T Cell</td>
</tr>
<tr>
<td>TLC</td>
<td>Total lung capacity</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T Cell</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>1α,25VitD3</td>
<td>1,25-Dihydroxyvitamin D3</td>
</tr>
</tbody>
</table>
25[OH]D3  25-dihydroxyvitamin D
VDBP  Vitamin D Binding Protein
Vs  Volume fraction of airway smooth muscle indexed to reticular basement
Vv  Volume fraction of airway smooth muscle indexed to subepithelium
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Allergic mechanisms in asthma</td>
<td>24</td>
</tr>
<tr>
<td>1.2</td>
<td>T cells involved in the allergic asthma.</td>
<td>25</td>
</tr>
<tr>
<td>1.3</td>
<td>Th17 differentiation and maturation.</td>
<td>29</td>
</tr>
<tr>
<td>1.4</td>
<td>The Royal Brompton Hospital paediatric problematic severe asthma investigation protocol.</td>
<td>41</td>
</tr>
<tr>
<td>1.5</td>
<td>Mechanism of glucocorticoid action by the glucocorticoid receptor and sites of regulation in glucocorticoid insensitivity.</td>
<td>45</td>
</tr>
<tr>
<td>1.6</td>
<td>Metabolism of Vitamin D.</td>
<td>49-50</td>
</tr>
<tr>
<td>1.7</td>
<td>Immunological effects of vitamin D.</td>
<td>63</td>
</tr>
<tr>
<td>1.8</td>
<td>The crystal structure of vitamin D binding protein.</td>
<td>65</td>
</tr>
<tr>
<td>1.9</td>
<td>Characteristic appearances of sputum cytology shown under light microscopy.</td>
<td>81</td>
</tr>
<tr>
<td>2.1</td>
<td>An example of endobronchial biopsy stained with H &amp; E</td>
<td>83</td>
</tr>
<tr>
<td>2.2</td>
<td>Overview of CBA</td>
<td>86</td>
</tr>
<tr>
<td>2.3</td>
<td>An example of gating for FoxP3 intracellular staining</td>
<td>88</td>
</tr>
<tr>
<td>2.4</td>
<td>Serum vitamin D levels in STRA, MA &amp; controls.</td>
<td>95</td>
</tr>
<tr>
<td>2.5</td>
<td>Serum vitamin D levels and season</td>
<td>96</td>
</tr>
<tr>
<td>2.6</td>
<td>Serum vitamin D levels and total IgE levels</td>
<td>97</td>
</tr>
<tr>
<td>2.7</td>
<td>Relationship between vitamin D levels and lung function.</td>
<td>99</td>
</tr>
<tr>
<td>2.8</td>
<td>Relationship between vitamin D levels and BDR.</td>
<td>100</td>
</tr>
<tr>
<td>2.9</td>
<td>Relationship between serum vitamin D level and ACT.</td>
<td>102</td>
</tr>
<tr>
<td>2.10</td>
<td>Relationship between serum vitamin D level and exacerbations.</td>
<td>103</td>
</tr>
<tr>
<td>2.11</td>
<td>Relationship between serum vitamin D levels and steroids.</td>
<td>105</td>
</tr>
<tr>
<td>2.12</td>
<td>No relationship between serum 25[OH]D$_3$ levels and eosinophilic inflammation.</td>
<td>109</td>
</tr>
<tr>
<td>3.1</td>
<td>No relationship between serum 25[OH]D$_3$ levels and neutrophilic inflammation.</td>
<td>110</td>
</tr>
<tr>
<td>3.2</td>
<td>Relationship between serum 25[OH]D$_3$ levels and airway remodelling.</td>
<td>112</td>
</tr>
<tr>
<td>3.3</td>
<td>Endobronchial biopsy sections from a child with STRA.</td>
<td>113</td>
</tr>
<tr>
<td>3.4</td>
<td>Functions of IL-10 relevant to asthma.</td>
<td>122</td>
</tr>
<tr>
<td>3.5</td>
<td>Typical depletion of CD8 T cells isolated by bead-based selection.</td>
<td>126</td>
</tr>
<tr>
<td>3.6</td>
<td>The effect of various concentrations of 1α,25VitD3 on CD8 depleted PBMC cytokine production.</td>
<td>128</td>
</tr>
<tr>
<td>3.7</td>
<td>CD8-depleted PBMC from asthmatics secrete significantly less IL-10 than non-asthmatics, and there is a trend towards increased IL-17A and IL-13 production</td>
<td>132</td>
</tr>
<tr>
<td>3.8</td>
<td>Dexamethasone increases IL-10 secretion, and in STRA inhibits IL-13 secretion, but significantly enhances IL-17A production by PBMCs.</td>
<td>134</td>
</tr>
<tr>
<td>3.9</td>
<td>Vitamin D enhances dexamethasone-induced IL-10 secretion by asthmatic PBMC (A), but does not alter IL-17A (B) or IL-13 secretion (C).</td>
<td>136</td>
</tr>
<tr>
<td>3.10</td>
<td>Effect of dexamethasone and 1α,25VitD3 on PBMC cytokine.</td>
<td>140</td>
</tr>
</tbody>
</table>
profiles of STRA asthmatics delineated by steroid-responsiveness

Figure 4.8 Similar PBMC IL-10 secretion following dexamethasone and 1α,25VitD3 from SR and SS STRA.

Figure 4.9 Relationship between serum vitamin D (25(OH)D3) and steroid responsiveness in children with STRA stratification

Figure 4.10 Serum vitamin D levels and response to a steroid trial in children with STRA.

Figure 5.1 Reduced IL-10 expression in the BAL in STRA.

Figure 5.2 Frequency of regulatory T cells (CD4+FoxP3+ cells) in BAL cells of STRA (n=11) and controls (n=5).

Figure 5.3 The effect of vitamin D and dexamethasone on cytokine secretion profiles of total BAL cell cultures, stimulated with LPS, from non-asthmatic controls (n=4) and STRA (n=9).

Figure 5.4 Relationship between BAL IL-10 levels and serum vitamin D levels.

Figure 5.5 Relationship between BAL CD4+ FoxP3+ cells and serum vitamin D levels.

Figure 6.1 BAL and Serum VDBP in STRA, MA & controls

Figure 6.2 Serum and BAL VDBP.

Figure 6.3 Relationship between BAL and serum VDBP and asthma control, FEV1 and ICS usage

Figure 6.4 Serum vitamin D and VDBP in the serum and BAL.

Figure 6.5 The effect of dexamethasone on bronchoalveolar lavage (BAL) cells of children with STRA (n=7).

Figure 7.1 Potential mechanisms of vitamin D in decreasing the risk for asthma exacerbations.

Figure 7.2 Potential protective effects of vitamin D against asthma morbidity.

Figure 7.3 Overview of vitamin D and its interactions with cells of immune system
# List of Tables

| Table 1.1 | Pivotal human studies describing role of regulatory T (Treg) cell function in asthma and allergies. | 32 |
| Table 1.2 | Serum 25-hydroxyvitamin D (25 [OH]D3) concentrations | 51 |
| Table 1.3 | Risk factors for Vitamin D Deficiency/Insufficiency in children | 52 |
| Table 1.4 | Vitamin D and asthma in children and adults | 56 |
| Table 1.5 | Vitamin D and wheeze in preschool children | 60 |
| Table 1.6 | Response of different target cells to vitamin D | 62 |
| Table 2.1 | Intra-observer sputum eosinophils repeatability | 81 |
| Table 3.1 | Demographic characteristics of subjects | 93 |
| Table 3.2 | Association between serum vitamin D levels and IgE levels | 98 |
| Table 3.3 | Serum vitamin D levels and medication use in all asthmatics | 104 |
| Table 3.4 | Demographic characteristics of STRA children (n=22) in whom asthma pathology was assessed. | 106 |
| Table 3.5 | Association between serum vitamin D levels and airway inflammation in children with STRA. | 108 |
| Table 3.6 | Biopsy cell count results | 111 |
| Table 4.1 | Demographic characteristics of subjects | 129 |
| Table 4.2 | Comparison of serum cytokines in all groups. | 130 |
| Table 4.3 | Relationship between serum vitamin D levels and serum cytokines of those asthmatics with detectable cytokines | 131 |
| Table 4.4 | Demographic of children with complete, partial and non-response to intramuscular steroid (triamcinolone) | 138 |
| Table 5.1 | Clinical Indications of flexible bronchoscopy in non-asthmatic controls | 155 |
| Table 5.2 | Demographic characteristics of subjects | 157 |
| Table 5.3 | Undetectable cytokines in the BAL fluids | 160 |
| Table 5.4 | No association between airway inflammation and BAL IL-10, dexamethasone induced IL-10 secretion in BAL cell culture and % BAL CD4+FoxP3 cells in children with STRA. | 169 |
| Table 6.1 | The functions of VDBP | 177 |
| Table 6.2 | Demographic characteristics of subjects | 181 |
| Table 6.3 | No relationship between BAL VDBP and airway inflammation | 188 |
| Table 6.4 | No relationship between BAL VDBP and airway remodelling | 188 |
Asthma is the most common chronic disease of childhood and its prevalence has increased dramatically over the past few decades.\textsuperscript{2} It is one of the leading causes of morbidity in children worldwide\textsuperscript{3} and has a significant social and financial burden on the individuals and families affected.\textsuperscript{4, 5} It can affect virtually all ages, and varies greatly in severity. Increasingly, the vitamin D pathways, that were thought to be predominantly related to bone health, have been implicated in the pathophysiology of asthma.

The work of this thesis concerns the role of vitamin D in paediatric severe, therapy resistant asthma (STRA). This chapter begins by reviewing the immunopathology of asthma, and then the problem of severe asthma and steroid resistance. There follows a review of current knowledge of the important vitamin D pathways and their role in asthma, and concludes by formulating the hypotheses, aims and objectives of the thesis. A review article based on this chapter has been published in Paediatric Respiratory Reviews.\textsuperscript{6}

1.1 Immunopathology of asthma

1.1.1 Asthma

Asthma is a heterogeneous disease that is characterised by airway hyper-responsiveness, recruitment of inflammatory leukocytes to the lung and tissue remodelling, including excess mucus production and airway smooth muscle hypertrophy and hyperplasia.\textsuperscript{7} It is largely a disease of the conducting airways, which in response to a wide range of exogenous and endogenous stimuli undergo exaggerated constriction, leading to airway hyper-responsiveness, the hallmark feature of the condition. It is classically driven by allergic responses (summarised in Figure 1.1), with enhanced activity of T helper (Th) 2 cells (discussed in the next section), leading to B-cell isotype switching to IgE production, and eosinophilic airway inflammation and airway hyperresponsiveness.\textsuperscript{7, 8} Submucosal eosinophilia has been described in paediatric asthma and preschool wheeze\textsuperscript{9, 10} and de Blic et al\textsuperscript{11} showed marked increase in epithelial eosinophils in symptomatic difficult
Figure 2.1 Allergic mechanisms in asthma. Reproduced from ‘Treatment strategies for allergy and asthma’.12

Abbreviations: Antigen-presenting cells (APCs), T helper 2 (Th2), Interleukin (IL), Regulatory T (T_{Reg}), High-affinity receptor for IgE (Fc\varepsilon RI), Interferon (IFN), T-cell receptor (TCR), Tumour-necrosis factor (TNF)
asthmatic as compared with non-symptomatic patients.

Interactions between multiple factors including early allergen exposure and sensitization, tobacco smoke exposure, infections, diet, genetic predisposition, and pollution result in the development of chronic airway inflammation. A number of different T cell subsets and the cytokines that they secrete are thought to influence the nature and magnitude of the allergic immune response in asthma (summarised in Figure 1.2).

**Figure 1.2 T cells involved in the allergic asthma.** Reproduced from ‘Functions of T cells in asthma: more than just Th2 cells’.
1.1.2 T helper cells in asthma

Chronic airway inflammation in asthma is a multicellular process thought to be driven by CD4+ T lymphocytes. More CD4+ T cells are present in adult asthmatic bronchoalveolar (BAL) and bronchial biopsies as compared to controls.\textsuperscript{15, 16} Naïve CD4+ Th0 cells differentiate into subsets of CD4+Th cells on recognition of the specific signals from antigen presenting cells and dendritic cells. Several subsets of CD4+ T cells are defined on the basis of their cytokine production exist but Th1 cells (involved in cell mediated immunity) and Th2 cells (antibody mediated immunity) are best described. Th1 cells are critical in cellular defence mechanisms to intracellular pathogens and viruses and produce interleukin (IL)-2, interferons (IFN) and tumor necrosis factor (TNF). In contrast, Th2 cells mediate responses to extracellular agents and secrete several mediators including IL-4, IL-5, IL-6, IL-9 and IL-13,\textsuperscript{17} and drive the response to allergen. The Th2 cytokines IL-4 and IL-13 induce B cells to undergo immunoglobulin class switching to produce IgE. During this mechanism, the constant region portion of the antibody heavy chain is changed, but not variable region.

There is now persuasive evidence that Th2 cells are important mediators of asthma and dominate the T cell infiltrate in the airways.\textsuperscript{18} Elevated levels of Th2 cytokine mRNA and protein are expressed in CD4+ T cells from asthmatic BAL.\textsuperscript{19-21} IL-5 mediates eosinophil recruitment to inflammatory sites, survival and eosinophilopoiesis.\textsuperscript{22} IL-5 may also be important in the production of an immune response in asthma since it mediates eosinophil priming, expansion, recruitment and prolonged tissue survival in response to allergens.\textsuperscript{23} IL-4 drives the differentiation of Th2 cells and promotes B cell class switching to IgE production, mast cell proliferation and recruitment of eosinophils.\textsuperscript{24-26} IL-13 promotes B cell IgE production, airway hyperreactivity and mucus production.\textsuperscript{27-29} IL-4 and IL-13 direct the migration of T lymphocytes, monocytes, basophils and eosinophils to sites of inflammation and stimulate B cell class switching (above).\textsuperscript{30} IgE activates mast cells. In animal models of allergic airway disease, antibodies that block the action of Th2 cytokines reduce
pulmonary eosinophilia and airway hyperreactivity (anti-IL-5 blocks eosinophils and anti-IL-13 blocks airway hyperresponsiveness).\textsuperscript{31, 32}

Th2 cells orchestrate the inflammatory response in asthma via the release of cytokines. A large number of studies, both mouse and human, document the expression of Th2-type cytokines, such as IL-4, IL-5, and IL-13, in the allergic lung.\textsuperscript{33} Experimental models of asthma indicate that these Th2 cytokines, are critical in driving key pathologic features of the allergic response, including eosinophilic inflammation, airway mucus production, airway remodelling, and airway hyperresponsiveness.\textsuperscript{33} However, clinical trials of drugs designed to modulate Th2 cytokines have not been universally successful in asthmatic patients. Initial human trials with anti-IL-5 monoclonal antibody were not as successful in adult mild/moderate asthmatics (MA).\textsuperscript{34} However, more recently, double blind placebo controlled trials in adults with severe asthma have shown that anti-IL-5 antibody is effective in reducing exacerbations, specifically in the sub group of patients with sputum eosinophilia.\textsuperscript{35} This is very important as it suggests only a sub-set of patients are likely to respond to such therapy.

The common effects of IL-4 and IL-13 are mediated via the IL-4 Rα receptor.\textsuperscript{30} IL-4 also promotes the differentiation and proliferation of Th2 lymphocytes. IL-13 by activating STAT6 in the airway epithelium\textsuperscript{36} mediates airway hyperresponsiveness and mucus hyperproduction.\textsuperscript{30} The IL-4 analogue pitrakinra binds to IL-4Rα, thus blocking IL-4 and IL-13. This has been shown to reduce the late phase response following allergen challenge compared with placebo in randomised, double-blind, placebo-controlled, phase 2a clinical trials in adults with atopic asthma.\textsuperscript{37} Recently it has been shown that patients with moderate to severe asthma who had specific IL-4 receptor α gene polymorphisms were more likely to have reduced exacerbations, reduced nocturnal awakenings and improved activity following treatment with pitrakinra compared with those without the polymorphisms.\textsuperscript{38} This also reinforces that only a sub-set of patients will respond to such therapy, and possibly tests for such polymorphisms should be performed prior to initiation of this treatment.

27
It has been proposed that severe asthma may in fact represent a spectrum of different pathophysiological subphenotypes. These appear to have a greater or lesser-extent of Th2 activity and may therefore explain inconsistencies in clinical response to both existing and novel pharmacotherapies targeting this axis. In severe asthma, regulation of Th1/Th2 cytokine production has been shown to be different from MA and Th17 related cytokines (discussed in the next section) might be responsible for mediating inflammation. Bossley and colleagues have recently shown that children with STRA have airway eosinophilia without neutrophilia or increased mast cell counts. Importantly, in this cross-sectional study signature Th2 cytokines (IL-4, IL-5 and IL-13) were absent in the majority of children and present only in a small subgroup, suggesting phenotypic heterogeneity. High-dose steroid treatment might have effectively suppressed the signature Th2 cytokines. Similarly, Fitzpatrick and colleagues have shown that severe asthma in children is characterized by a distinct airway molecular phenotype that does not have a clear Th1 or Th2 pattern. They found a mixture of Th1 and Th2 type inflammation in BAL and alveolar macrophages, with BAL IL-13 and IL-6 differentiating severe paediatric asthma from adult controls and RANTES, IL-10, IL-12, chemokine ligand (CXCL) 1 and IFNγ discriminated between STRA and MA in children. In summary, it is evident that severe asthma can no longer be considered simply a Th2 mediated disease but involves a more complex interplay between different T-cell subsets and mediators determined in individual patients by genetic and environmental influences.

1.1.3 Th17 cells in asthma

Recently, additional subsets of Th cells have been described including Th17 cells. The Th17 phenotype is defined by the expression of retinoic acid–related orphan receptor α as a transcription factor and is characterized by secretion of the cytokines including IL-17A, IL-17F, IL-21 and IL-22. Their differentiation is controlled by IL-6, IL-21, IL-23, IL-1β and TGFβ (Figure 1.3). In addition to Th17 cells, other cell types express and produce IL-17A, including hematopoietic cells and structural cells, such as epithelial cells, vascular endothelial cells, fibroblasts, CD8+ T cells, γδ T cells, invariant natural killer T cells, and lymphoid tissue inducer-like cells.
Therefore the precise function of IL-17A may be determined by its cellular source.\textsuperscript{33} IL-17 is involved in granulopoiesis; it is a key cytokine for the recruitment, activation, and migration of neutrophils; and has been shown to regulate neutrophil migration in the lung.\textsuperscript{55} It is postulated that the primary function of Th17 cells is to promote the clearance of certain infections (in particular extracellular bacteria and fungi) at mucosal surfaces.\textsuperscript{56}

**Figure 1.3 Th17 differentiation and maturation.** Reproduced from ‘The Basics of Th17 and Interleukin-6 in Transplantation’ \textsuperscript{57}

More recently, elevated IL-17A has been implicated in asthma.\textsuperscript{58, 59} IL-17A mRNA and protein are upregulated in lung tissue of adults with asthma compared with healthy control subjects.\textsuperscript{58, 60} Using an immunofluorescence assay, Al-Ramli et al have shown the number of IL-17A and IL17F cells are associated with disease severity in endobronchial biopsies (EB) of adult asthmatics.\textsuperscript{61} In addition, the percentage of peripheral Th17 cells and the plasma concentrations of IL-17A and IL-22 increase with disease severity.\textsuperscript{62} Elevated IL-17A levels correlate with increased hyperresponsiveness in patients with asthma.\textsuperscript{63} Overexpression of IL-17F in mouse lung tissue results in enhanced recruitment of neutrophils.\textsuperscript{64} IL-17A is also reported to modulate airway structural cells, leading to tissue remodelling.\textsuperscript{54} IL-17A may also alter airway structural cell function, by promoting smooth muscle motility and contractility.\textsuperscript{65, 66} In mouse models Th17 cells can upregulate Th2 cell-mediated eosinophilic airway inflammation and can enhance airway smooth muscle contraction, proliferation, and airway epithelial permeability to allergen.\textsuperscript{65} IL-17A expression is also reported to correlate with neutrophilic influx in severe adult asthma,\textsuperscript{60} but Bossley and colleagues\textsuperscript{43} and unpublished data (Bossley et al) have
recently shown absence of neutrophilic inflammation in paediatric STRA, thus calling into question the relevance of this mechanism at least in children. In a murine model of ovalbumin-induced allergic inflammation IL-17-producing γδT cells have been shown to mediate the resolution of allergic airway inflammation and airway hyperreactivity while adoptive transfer of Th17 cells had no such effect. The conflicting evidence in mouse models could be due to different mouse strains and allergen sensitization protocols. However, it is now known that IL-17A can be secreted by other cells besides Th17 cells and importantly, IL-17A may play different roles in the airway, depending on its cellular source and context. Specifically, it is not possible to conclude whether in human disease, IL-17A is causative, protective or has mixed roles in different circumstances.

1.1.4 Regulatory T cells (Tregs) in asthma

Allergic inflammation has been hypothesised to represent an abnormal response to harmless airborne particles. These reactions are normally suppressed by a complex series of mechanisms, including regulatory cells and cytokines, which maintain airway tolerance. In asthmatic patients there is a breakdown in these regulatory mechanisms, leading to development of pulmonary inflammation on exposure to innocuous proteins, such as grass, pollens or cat dander. Thus, Tregs are a subset of T cells that are thought to suppress the activity and inflammatory capacity of the innate and adaptive immune system.

CD4+ Tregs ("naturally occurring Tregs") are derived from the thymus and are characterized by expression of the forkhead winged transcription factor FoxP3 (forkhead box P3). In addition to the naturally occurring Treg, CD4+ Tregs may also be induced peripherally. Induced Tregs may be FoxP3 positive (FoxP3+) or FoxP3 negative (FoxP3-) and play an important role in the moderation of autoimmune, infectious, inflammatory and allergic responses to antigens. Tregs control immune responses through several mechanisms including secretion of the anti-inflammatory cytokines IL-10 and TGF-β. They also have a role in the organization and regulation of recruitment of effector T cells to sites of inflammation. The most convincing support for a role of Tregs in preventing
the development of atopic disease stems from studies of the X-linked autoimmunity allergic dysregulation syndrome, also termed immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome.\textsuperscript{77} This is a rare multisystem disorders caused by a genetic abnormality (mutations in the gene coding for the transcription factor Foxp3 which is on the X chromosome). Young boys affected by mutations in this gene present with multisystem problems including enteropathy, endocrinopathy, severe infections, autoimmune disorders and severe allergic manifestations (including food allergies, atopic dermatitis, hyper IgE and eosinophilia) and infants and children with more severe forms of the disease tend to die without bone marrow transplantation or immunosuppression.\textsuperscript{77}

The manipulation of the Treg cell population in the allergic mouse lung by transfer of Tregs (CD4+CD25+FoxP3+) results in abrogation of allergic inflammation and airway hyperresponsiveness.\textsuperscript{78} The defective suppression of Treg is shown in children with moderate asthma. Treg cells isolated from BAL fluid or peripheral blood of asthmatic children were less effective in suppressing proliferation and production of Th2 cytokines and chemokines by CD4+CD25- responder T cells than Treg cells isolated from control subjects.\textsuperscript{79} Treg function may be impaired in asthma\textsuperscript{68, 80, 81} and pivotal human studies describing the role of Tregs in asthma are summarized in Table 1.1
Table 1.1 Pivotal human studies describing role of regulatory T (Treg) cell function in asthma and allergies

<table>
<thead>
<tr>
<th>Author</th>
<th>Type asthma</th>
<th>Control group</th>
<th>Compart-ment tested</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ling et al (^82) 2004</td>
<td>Atopic Adults</td>
<td>Healthy &amp; atopic adults with no symptoms</td>
<td>Peripheral blood</td>
<td>The suppressive activity of Treg cells from the peripheral blood of allergic patients during the hay fever season was reduced</td>
</tr>
<tr>
<td>Hartl et al (^79) 2007</td>
<td>MA Children</td>
<td>Children without lung diseases</td>
<td>BAL</td>
<td>CD4+CD25 high T cells are enriched in BAL compared with peripheral blood. Pulmonary (BAL), but not blood, CD4+CD25high T cells were decreased in number; however, this was restored following inhalation of corticosteroids. The number of CD25high cells correlated with clinical parameters such as lung function (r = 0.85). Lower level of Foxp3 mRNA in peripheral blood from asthmatics as compared with control.</td>
</tr>
<tr>
<td>Akdis et al (^83) 2004</td>
<td>Atopic Adults</td>
<td>Healthy controls</td>
<td>Peripheral blood</td>
<td>Reduced numbers of IL-10+ T cells in the allergic patients</td>
</tr>
<tr>
<td>Lee et al (^84) 2007</td>
<td>Allergic (allergic rhinitis or asthma) Children</td>
<td>Healthy controls</td>
<td>Peripheral blood</td>
<td>Decrease in CD4+CD25+ T cells in the paediatric patients with allergic disease. These findings relate to the use of the CD25 marker to identify Treg. Lower level of Foxp3 mRNA in peripheral blood from asthmatics as compared with control subjects</td>
</tr>
<tr>
<td>Provoo st et al (^85) 2009</td>
<td>MA Adults</td>
<td>Healthy adults</td>
<td>Peripheral blood</td>
<td>Asthmatics have normal numbers of FoxP3+ cells but reduced FoxP3 expression</td>
</tr>
<tr>
<td>Nieminen et al (^86) 2010</td>
<td>Atopic Children</td>
<td>Atopic children</td>
<td>Peripheral blood</td>
<td>Successful allergen immunotherapy is associated with the induction of both FoxP3+ and IL-10 secreting Treg cells</td>
</tr>
<tr>
<td>Scadding et al (^87) 2010</td>
<td>Atopic Adults</td>
<td>Healthy controls</td>
<td>Oral mucosal immune cells</td>
<td></td>
</tr>
</tbody>
</table>

MA: moderate asthma, BAL: bronchoalveolar lavage, IL: interleukin
The consequence of sustained or dysregulated inflammation or ineffective resolution is chronic airway inflammation in asthma.\(^{67}\) The resolution of inflammation in airways is an active coordinated process, which prevents unnecessary tissue damage after clearance of inflammatory stimuli and promotes homeostasis. It depends on the actions of various anti-inflammatory and pro-resolving mediators including IL-10 (below) and clearance of inflammatory cells. The capacity to mount a controlled inflammatory response is central to host defence. However, should inflammation proceed unrestrained, unwanted tissue damage may result.\(^{88-90}\) In summary, airway inflammation in asthma may be due to an excessive pro-inflammatory drive or insufficient anti-inflammatory resolution mechanisms, or both.

IL-10 is a pleiotropic cytokine with broad anti-inflammatory and also immunosuppressant effects on both innate and adaptive immune cells (summarized in Text box 1.1.).\(^{91}\) IL-10 is synthesized and secreted by B cells, mast cells, eosinophils, macrophages and DCs, and all Th cell subsets including CD8+, CD4+FoxP3+ T cells and IL-10-secreting regulatory CD4+ T cells.\(^{73, 92-97}\) Strategies designed to increase the production of regulatory cells might be an effective treatment for asthma.\(^{33}\) Proof of this concept comes from studies of non-allergic beekeepers who are continually exposed to high doses of bee venom antigens. Allergen re-exposure suppressed allergen-specific T-cell proliferation and Th1/Th2 cytokine secretion and was also associated with an *in vivo* switch to an IL-10 secreting phenotype during the beekeeping season. In summary, the allergen re-exposure each year was able to maintain clinical tolerance associated immunologically with restoration of the IL-10 response in culture.\(^{98}\) Allergen-specific immunotherapy is also associated with the development of allergen-specific Treg cells, which are able to control allergic immune responses, and suppressive cytokines, such as IL-10 are thought to play a pivotal role.\(^{86, 67}\) Thus a greater understanding of how immune responses are regulated in the allergic lung may enable manipulation of these regulatory mechanisms to develop novel and improved therapies for asthma.\(^{68}\)
Text box 1.1 Immunomodulatory properties of IL-10

- IL-10 inhibits mast cell activation and cytokine production.\textsuperscript{99-101}
- IL-10 inhibits eosinophil survival and function.\textsuperscript{102}
- IgG4 is an immunoglobulin isotype generally considered to be protective in allergic disease, whereas IgE is an important mediator of the allergic cascade.\textsuperscript{103} IL-10 appears to increase levels of circulating IgG4 relative to IgE.\textsuperscript{104}
- IL-10 inhibits the ability of antigen presenting cells to sustain adaptive immune responses by suppressing macrophage and dendritic cell production of inflammatory cytokines and expression of MHC class II and co-stimulatory molecules.\textsuperscript{105-111}

1.1.5 Airway remodelling in asthma

Airway remodelling was defined by Peter Jeffrey\textsuperscript{112} as ‘the alteration in size, mass or number of tissue structural components that occurs during growth or in response to injury and/or inflammation’.\textsuperscript{112} Remodelling encompasses changes in the epithelium, reticular basement membrane (RBM), extracellular matrix, airway smooth muscle (ASM), blood vessels and mucous glands. This was first described more than 85 years ago by Huber and Koessler in their classic paper on fatal asthma.\textsuperscript{113} Airway remodelling occurs in children with severe asthma\textsuperscript{114-116} and is characterized by increased thickness of the reticular basement membrane (due to an increase in reticulin deposition),\textsuperscript{117} ASM hypertrophy and hyperplasia, extracellular matrix deposition, epithelial shedding, hypertrophy of mucus-secreting glands and increase in size and number of the bronchial vessels within the airways (summarized in Text box 1.2). It is thought that airway remodelling worsens the clinical picture of asthma but some aspects of remodelling may be protective.\textsuperscript{114, 118} For example, a thickened airway wall may be less compliant and better able to resist dynamic compression.

The submucosal eosinophilia is present in paediatric asthma and preschool wheeze\textsuperscript{9, 10} but the presence of submucosal eosinophilia is not itself sufficient to cause
symptoms. In children, Bossley et al. and Payne et al. found no difference in submucosal neutrophils between subjects with difficult asthma and controls. de Blic et al. showed marked increase in epithelial eosinophils and neutrophils in symptomatic difficult asthmatic as compared with non-symptomatic patients. T lymphocytes are central to the pathogenesis of asthma and it may be that these cells also modulate airway remodelling. However, the relationship between inflammation and remodelling is unclear, but they are likely parallel processes, rather than remodelling being driven by inflammation. This hypothesis is supported by the paediatric work of Saglani and colleagues that appeared to show inflammation and remodelling developing in parallel and also no relationship between eosinophils and remodelling was noted by Payne and colleagues. This is also supported by recent evidence from a novel neonatal mouse model of allergic airways disease which has shown that bronchial responsiveness, eosinophilic inflammation and remodelling occur in parallel during inhaled house dust mite sensitization.

The bronchial epithelium acts as a barrier separating the external environment and the lung parenchyma. The bronchial epithelium and underlying RBM have a close spatial and functional inter-relationship and are considered an epithelial-mesenchymal trophic unit. The airway epithelium also plays a central role in the pathogenesis of asthma and the epithelium is emerging as an area of interest as new research shows interactions between the epithelium and the immune system are crucial in driving asthma. There is evidence that dysregulated epithelial repair originates in childhood asthma and is a critical determinant of disease progression. Genetic studies also suggest that the epithelium plays a significant role in the allergic response. Bronchial epithelial cells from asthmatic children function abnormally even in the absence of inflammation with decreased production of TGF-β and the release of anti-inflammatory mediators. There is also some in vitro evidence that an injured airway epithelium stimulates ASM cell hyperplasia. It is therefore possible that children with STRA have inherently functionally different epithelial cells, which thus respond abnormally to infectious, dietary, or environmental stimuli leading to ASM hyperplasia. The identification of key modulators regulating the contribution of ASM function to asthma may provide new
opportunities for therapeutic intervention.

**Text box 1.2 Airway remodelling in children**

<table>
<thead>
<tr>
<th>Changes in reticular basement membrane (RBM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Increased thickness of the RBM present in children with asthma and preschool wheeze.\textsuperscript{115}</td>
</tr>
<tr>
<td>• Increased RBM thickness is not present at a year of age,\textsuperscript{121} but appears as early as 3 years in preschool wheeze.\textsuperscript{10}</td>
</tr>
<tr>
<td>• Increased RBM thickness has been shown in children with mild\textsuperscript{9} and severe\textsuperscript{115} asthma</td>
</tr>
<tr>
<td>• No correlation between RBM thickness and lung function in children.</td>
</tr>
<tr>
<td>• Increased thickness of the RBM is an important pathological feature of asthma but not related to the duration of asthma, or to treatment.\textsuperscript{115}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Changes in epithelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Functional changes in epithelium are considered important in the pathogenesis of asthma.\textsuperscript{123}</td>
</tr>
<tr>
<td>• In children with mild asthma more epithelial shedding than in controls.\textsuperscript{9}</td>
</tr>
<tr>
<td>• Epithelial height is increased in asthmatics.\textsuperscript{126}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Changes in airway smooth muscle (ASM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ASM mass (hypertrophy and hyperplasia) is increased in children with severe asthma.\textsuperscript{116}</td>
</tr>
<tr>
<td>• Positive correlation between ASM mass and bronchodilator reversibility.\textsuperscript{116}</td>
</tr>
<tr>
<td>• Increased ASM mass in children and adult with persistent obstructive pattern.\textsuperscript{127}</td>
</tr>
<tr>
<td>• Inverse relationship between forced expired volume (FEV\textsubscript{1}) and ASM mass in adults.\textsuperscript{127} Thus, ASM mass may be related to function.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Changes in bronchial vessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Increase in size and number of the bronchial vessel size within the airways have been shown in children with mild/moderate asthma.\textsuperscript{9}</td>
</tr>
<tr>
<td>• Dilated and congested blood vessels have been associated with fatal disease in adults.\textsuperscript{128}</td>
</tr>
<tr>
<td>• Study of childhood severe asthma showed the density of the vascular network to be more pronounced in children with fixed airway obstruction.\textsuperscript{129}</td>
</tr>
</tbody>
</table>
1.2 Problematic severe asthma in children

For most children with access to healthcare, asthma can be controlled with low doses of inhaled corticosteroids, sometimes in combination with long-acting β-2 agonist, if regularly and properly administered. There remain, however, a substantial number of children with asthma, who, despite what appears to be appropriate treatment, continue to be symptomatic and at risk of acute exacerbations. Most assessments have proposed that this difficult-to-treat population represents 5-10% of all asthmatics. However, it is difficult to be certain of prevalence as definitions differ between studies. Although this high risk cohort only represents a small portion of all asthmatics, they suffer the greatest morbidity and mortality including exacerbations, admissions to intensive care, need for healthcare utilization and days lost in absence from school or work.

Entry criteria to our paediatric problematic severe asthma clinic at Royal Brompton Hospital (RBH) are one or more of:

1. Persistent (most days, for at least 3 months) chronic symptoms (the requirement because of symptoms for short-acting β-2 agonists at least three times/week) of airways obstruction despite high dose inhaled corticosteroids (Beclomethasone equivalent 800 mcg/day) and trials of add-on medications (these would include long acting β-2 agonist, leukotriene receptor antagonist, oral theophylline in the low, anti-inflammatory dose).

2. Recurrent severe asthma exacerbations despite attempts with medication including trials of allergen avoidance, daily inhaled corticosteroids or intermittent high dose inhaled corticosteroids to abort exacerbations, that have required: either at least one admission to an intensive care unit, or at least two hospital admissions requiring intravenous medication/s, or > 2 courses of oral steroids during the last year, despite the above therapy.

3. Persistent airflow obstruction: post steroid trial, post-bronchodilator Z score < -1.96 or <80% predicted for forced expiratory volume in one second (FEV1) using appropriate reference equations despite the above therapy.

4. The necessity of prescription of alternate day or daily oral steroids to achieve control of asthma.
At RBH, these children with problematic asthma undergo a staged investigation protocol (Figure 1.4) to exclude a wrong diagnosis, asthma with critical co-morbidities and difficult asthma (in which potentially modifiable factors have not been identified and remedied).\textsuperscript{136} The RBH protocol, including a formal assessment in the home (stage 1), results in almost half the referrals being classified as difficult asthmatics, in whom basic management needs to be optimized rather than therapy escalated.\textsuperscript{134, 136} The remaining children with STRA have persistent symptoms, acute severe exacerbations and/or fixed airflow obstruction unresponsive to high-dose steroid therapy and in whom all aspects of basic management have been assessed in detail and found to be satisfactory.\textsuperscript{134} Once the multidisciplinary team is satisfied that the basic management is right but the child is still symptomatic, we use a two stage (stage 2 & 3), invasive investigations protocol (Figure 1.4). This involves performing an assessment of asthma control (using the asthma control test [ACT]), spirometry, exhaled nitric oxide, measured at flow 50ml/s (FeNO\textsubscript{50}) and sputum induction, bronchoscopy, BAL and EB prior to the steroid trial. Triamcinolone is given intramuscularly in theatre while the child is anaesthetised for the bronchoscopy to ensure compliance with the steroid trial. Assessment of asthma control (using the ACT), spirometry, FeNO\textsubscript{50} and sputum induction are then repeated 4 weeks later. The aims of the invasive investigations are to\textsuperscript{134} 1) exclude an additional unsuspected diagnosis; 2) identify discordance between symptoms and lung function or airway pathology; 3) identify evidence of eosinophilic or neutrophilic inflammation; 4) determine whether the child has persistent airflow limitation; and 5) assess the response to a single dose of intramuscular depot triamcinolone to determine whether there is at least partial steroid resistant airway inflammation. The over-arching aim is to try to produce an individualized treatment plan for the child; for example, it would seem illogical to give ever more potent anti-inflammatory medication to a child who is symptomatic but has no evidence of airway inflammation.\textsuperscript{134}

1.2.1 Assessment of steroid responsiveness in children
The adult definition of steroid resistance is <15% increase in FEV\textsubscript{1} after 2 weeks of oral prednisolone (40mg/day) in a patient with an abnormal FEV\textsubscript{1} at baseline and
who can bronchodilate more than 15% with acute use of β2 agonist. This definition is not appropriate for children, who can have normal spirometry despite severe asthma and Bossley and colleagues have recently shown that adult criteria of steroid responsiveness cannot be applied to 50% of children with STRA (manuscript submitted). Unlike adults, there is no universally agreed definition of steroid responsiveness in children or any consensus on the dose, duration and route of administration of treatment, or what end-points of steroid responsiveness to use.

Bush et al recently suggested the following non-evidence-based criteria for steroid responsiveness in children after intra-muscular (IM) triamcinolone and acute administration of inhaled β-2 agonist (Figure 1.4)

1. **Symptom response** - Improvement in ACT score to at least 20 out of 25 or by at least 5 points

2. **Lung function response** - FEV1 rises to normal (>80% predicted) or >15% rise in FEV1% with no residual bronchodilator response (BDR)

3. **Inflammatory response** (if paired induced sputum samples available) - Return of sputum eosinophils to normal levels (eosinophils < 2.5%)

4. **Inflammatory response** (if paired induced sputum samples not available) - Fall in FeNO50 to normal (<24 parts per billion)

**Complete response** is normalization in all three domains

**Partial response** is improvement in one or two domains

**Non-response** is classified as no improvement in any domain.

This proposal is not evidence based and requires validation. Also, the magnitudes of the changes proposed as defining response are arbitrary. The work of this thesis is on children who have been evaluated with this protocol, and are thought to have genuine STRA, rather than difficult asthma. A major strength is the very detailed evaluation of potentially reversible factors to ensure as far as possible that the children had truly severe therapy resistant disease. Adherence was assessed by checking for prescription pick up and respiratory nurses checked the location and
availability of medications in the home (Text box 1.3). Respiratory nurses also checked for allergen and environmental tobacco smoke exposure in the home and asked the children and family to fill in psychosocial questionnaires, and referred those with significant morbidity for professional help. All these assessments had to be completed prior to invasive assessments. If all patients referred with difficult asthma had invasive assessment then children with difficult asthma would have contaminated the cohort. The thorough evaluation, as far as is possible, prevented this contamination of data. This is one of the novel features of this work.

Text box 1.3 The nurse–led assessment\textsuperscript{136} (Stage 1 of the protocol)

<table>
<thead>
<tr>
<th>Hospital assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Patient history including triggers, environmental exposures, exacerbations in the past year and family history</td>
</tr>
<tr>
<td>• Skin prick tests (food and aeroallergens, including moulds)</td>
</tr>
<tr>
<td>• Exposure to tobacco smoke using urinary cotinine assay</td>
</tr>
<tr>
<td>• Inhaler technique assessed and corrected</td>
</tr>
<tr>
<td>• Semi-structured psychosocial questionnaire</td>
</tr>
<tr>
<td>• Asthma control test</td>
</tr>
<tr>
<td>• Sweat test (if this had not been previously performed)</td>
</tr>
<tr>
<td>• Spirometry (including bronchodilator reversibility)</td>
</tr>
<tr>
<td>• FeNO\textsubscript{50}</td>
</tr>
<tr>
<td>• Chest X-ray</td>
</tr>
<tr>
<td>• Consent to contact GP and pharmacies regarding medication pick up, and this is performed following the hospital assessment</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Home visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Allergen exposure checked for including presence of allergens patient sensitised to, looking for house dust mite avoidance measures, and advice on exposure reduction</td>
</tr>
<tr>
<td>• Evidence of smoke exposure</td>
</tr>
<tr>
<td>• Adherence assessment; all medications checked including location, ease of access and if they were within their use-by-date</td>
</tr>
<tr>
<td>• Discuss any issues raised by the psychosocial questionnaire</td>
</tr>
<tr>
<td>• Attention to asthma education</td>
</tr>
</tbody>
</table>
Figure 1.4 The Royal Brompton Hospital paediatric problematic severe asthma investigation protocol\textsuperscript{134}

<table>
<thead>
<tr>
<th>Stage 1</th>
<th>Remediable factors are addressed, compliance improved, psychological referrals made (&gt;50% of those assessed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete history</td>
<td></td>
</tr>
<tr>
<td>Psychological questionnaire</td>
<td></td>
</tr>
<tr>
<td>Spirometry (including BDR)</td>
<td></td>
</tr>
<tr>
<td>FeNO\textsubscript{50}</td>
<td></td>
</tr>
<tr>
<td>Sputum induction</td>
<td></td>
</tr>
<tr>
<td>Skin prick tests</td>
<td></td>
</tr>
<tr>
<td>Chest x ray (if not already performed)</td>
<td></td>
</tr>
<tr>
<td>Sweat test (if not already performed)</td>
<td></td>
</tr>
<tr>
<td>Home and school visit</td>
<td></td>
</tr>
<tr>
<td>Evaluation of medication pick up (compliance)</td>
<td></td>
</tr>
</tbody>
</table>

If still symptomatic despite addressing issues in home visit

| Stage 2                                                                                     |                                                                                                               |
| Asthma control test                                                                         |                                                                                                               |
| **Non invasive markers of inflammation**                                                      |                                                                                                               |
| Spirometry (including BDR)                                                                   |                                                                                                               |
| FeNO\textsubscript{50}                                                                         |                                                                                                               |
| Sputum induction                                                                             |                                                                                                               |
| **Invasive testing**                                                                         |                                                                                                               |
| Fibreoptic bronchoscopy                                                                      |                                                                                                               |
| EB/BAL cytology                                                                             |                                                                                                               |
| BAL microscopy                                                                               |                                                                                                               |
| Immune function testing, atopy testing (RAST and total IgE)                                  |                                                                                                               |
| pH study                                                                                    |                                                                                                               |
| **DOSE OF IM TRIAMCINOLONE GIVEN IN THEATRE WHILE CHILD ANAESTETISED**                        |                                                                                                               |

| Stage 3                                                                                     |                                                                                                               |
| Asthma control test                                                                         |                                                                                                               |
| **Non invasive markers of inflammation**                                                      |                                                                                                               |
| Spirometry (including BDR)                                                                   |                                                                                                               |
| FeNO                                                                                       |                                                                                                               |
| Sputum induction                                                                             |                                                                                                               |

**Individualized management plan**
1.3 Steroid resistance

Steroid resistance in asthma represents a major concern, since those patients are most at risk of hospitalization and death from asthma. The molecular mechanisms of glucocorticoid resistance in children are unclear and there is no accepted definition of steroid resistance (see above). Congenital steroid resistance due to mutations in the glucocorticoid receptor (GR) is rare. By definition children with STRA are steroid resistant and acquired steroid resistance is a spectrum and often can be overcome by high steroid doses, albeit at the risk of increased side-effects. The vast majority of studies investigating the potential mechanisms of steroid resistance have been performed in adults and novel therapies aimed at overcoming clinical steroid resistance are pioneered in adult asthmatics. However it is increasingly recognized, both immunologically and in the development of novel therapies, that adult responses cannot be used to accurately predict those of children. This next section describes possible mechanisms of steroid resistance in asthma.

1.3.1 Mechanism of corticosteroid action

Corticosteroids bind with high affinity to GR to form a complex in the cytoplasm, activating the translocation of GRs into the nucleus. The cortisol-GR complex moves swiftly into the nucleus and inhibits inflammation by three molecular mechanisms:

1. Direct genomic effect - binding with specific DNA sequences and influencing nuclear gene expression, thereby decreasing or increasing gene transcription.

2. Indirect genomic effect - via blockade of the activity of nuclear factor-kB (NF-kB), a transcription factor for pro-inflammatory chemokines, cytokines, cell adhesion molecules, and receptors for these molecules.

3. Non genomic effect - activates glucocorticoid signaling through membrane associated receptors and second messengers. There are three main non-genomic mechanisms.
(a) Induction of the production of annexin I, which, by inhibiting the synthesis of cytosolic phospholipase, blocks the release of arachidonic acid from membrane phospholipids and its subsequent conversion to eicosanoids.\textsuperscript{156}

(b) Induction of the mitogen-activated protein kinase (MAPK) phosphatase 1 that dephosphorylates and inactivates members of MAPK cascades, thereby inhibiting the transcription of inflammatory proteins or indirectly inhibiting phospholipase A2-alpha activity.\textsuperscript{156}

(c) Antagonism of the transcriptional activity of NF-kB, thereby inducing repression of cyclooxygenase 2.\textsuperscript{156}

1.3.2 Molecular mechanisms of steroid resistance
The molecular mechanisms of steroid resistance in asthma have been studied mainly in adults and animals and include reduced glucocorticoid binding to GR, reduced GR expression, enhanced activation of inflammatory pathways or lack of corepressor activity. The molecular basis of steroid resistance is summarized in Text box 1.3 and Figure 1.5.

**Text box 1.3 Possible mechanisms of steroid resistance in asthma**

<table>
<thead>
<tr>
<th>Reduced glucocorticoid receptor (GR) function</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Reduced quantity and binding capacity of the GR alpha subunit.\textsuperscript{157, 158}</td>
</tr>
<tr>
<td>• Increased levels of the inhibitory receptor subunit GRβ and of pro-inflammatory transcription factors such as activator protein-1 (AP-1) and nuclear factor of activated T cells (NFAT), both of which undermine GR from DNA-binding, and also inhibit histone acetyl transferase (HDAC) promoter activity in turn causing reduction of glucocorticosteroid effects.\textsuperscript{152, 159, 160}</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Impaired induction of anti-inflammatory genes and/or repression of pro-inflammatory genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Mitogen-activated protein kinase (MAPK) phosphatase-1 dephosphorylates and inactivates MAPK which are pro-inflammatory and are induced by steroids bound to GR. Impaired induction of MAPK phosphatase-1 by dexamethasone results steroid</td>
</tr>
</tbody>
</table>
resistance.\textsuperscript{161}

\textbf{Cytokines}

- PBMC (peripheral blood mononuclear cells) from adult steroid resistant asthmatics fail to secrete IL-10 when treated \textit{in vitro} with dexamethasone, as compared to PBMC from healthy individuals or steroid-sensitive asthmatics.\textsuperscript{162} IL-10 has also been proposed to increase GR alpha.\textsuperscript{163} This suggests that induction of IL-10 is an important part of the anti-inflammatory effect of corticosteroids in asthma.
- PBMC cultures from steroid refractory adult asthmatics synthesise significantly higher levels of IL-17A than steroid sensitive patients and nonasthmatic control cultures.\textsuperscript{164} IL-17 has been associated with steroid resistance in adults.\textsuperscript{54} Recently, IL-17A is also proposed to upregulate GRβ, a dominant negative receptor for steroids and this is proposed to drive steroid resistance.\textsuperscript{165}
- Combination of IL2/IL4 has also been attributed to induce steroid resistance.\textsuperscript{166}

\textit{Increase in free oxygen radicals /oxidative stress}

- Oxidative stress and smoking reduces HDAC2 expression and activity, in turn affecting inflammatory gene induction and repression leading to altered corticosteroid sensitivity. HDAC2 activity has been shown to inversely correlate with steroid sensitivity.\textsuperscript{167}
- Reduced HDAC2 expression in bronchial biopsies and BAL of asthmatics who smoke compared to non-smokers.\textsuperscript{167}

\textbf{Obesity}

- Obese asthmatics do not have the same amount of airway inflammation as non-obese severe asthmatics.\textsuperscript{168}
- Response to ICS reduces with increasing BMI.\textsuperscript{169}
- \textit{In vitro}, dexamethasone induced PBMCs and BAL MAKP-1 expression was reduced in overweight/obese compared with lean asthmatic adults.\textsuperscript{170}

\textbf{Vitamin D} (discussed in detail in next section)

- Vitamin D appears to maintain expression levels of GR in CD4+ T cells and thereby sensitivity to steroids by preventing corticosteroid-induced down-regulation.\textsuperscript{163}
1.3.3 Steroid resistance in asthma and vitamin D in asthma

Vitamin D, once thought mainly to be important for bone metabolism, is now known to interact with a large number of immunological pathways, and has been implicated as being important in many inflammatory respiratory diseases including steroid resistant asthma in adults. However, very little is known about the role of vitamin D in paediatric STRA.\textsuperscript{147}

Corticosteroids \textit{in vitro} induce IL-10 synthesis by peripheral immune cells and CD8+ T cells depleted PBMC stimulated with anti-CD3 antibody, IL-2, IL-4 in the presence of steroids show a dose dependent induction of IL-10 synthesis.\textsuperscript{162} Also, CD8+ T cells depleted PBMC from steroid resistant adult asthmatics fail to show increased IL-10 synthesis following \textit{in vitro} stimulation in the presence of glucocorticoids.\textsuperscript{162}
implying that induction of IL-10 is an critical part of the anti-inflammatory effect of glucocorticoids. Importantly, this defect in corticosteroid-induced IL-10 synthesis was overcome by the addition of vitamin D to the CD4+ T cell culture. Following on, a small pilot study was carried out which demonstrated that oral ingestion of 0.5 mcg vitamin D3 (calcitriol) daily for 7 days by steroid-resistant asthmatic patients restored the CD4+ T cell response to dexamethasone in terms of induction of IL-10 in vitro. Vitamin D also appears to maintain expression levels of GR in CD4+ T cells and thereby sensitivity to steroids.

Sutherland et al have reported an association between lower serum vitamin D concentrations and impaired lung function (reduced FEV₁), increased airway hyper-responsiveness, and decreased in vitro steroid response in a small cohort of adults with mild or MA, whilst higher serum vitamin D concentrations associated with enhanced dexamethasone-induced expression of MAPK phosphatase-1 by PBMC in an apparently IL-10–independent fashion. Another interaction between Vitamin D and glucocorticoid resistance involves the ability to regulate inflammatory gene expression and GR. In vitro, physiologic concentrations of vitamin D added to dexamethasone significantly enhanced MAPK phosphatase-1 expression in PBMCs compared with dexamethasone alone, suggesting that the addition of vitamin D could decrease the dexamethasone dose requirement for steroid response by more than 10-fold. This relationship was stronger in patients who were steroid naïve.

As discussed previously, reduction in Treg numbers has been linked with corticosteroid resistance. Vitamin D has been shown to increase the number of Tregs in peripheral blood and cell culture and in the absence of vitamin D both the number and function of Tregs are reduced. Thus it is possible that the mechanism of the reversibility effect of vitamin D on steroid resistance is via an up-regulation of Treg cells. In a recent adult study of moderate and severe asthmatic individuals who were vitamin D insufficient (defined as less than 75 nmol/l), those who exhibited steroid resistance had a significantly lower numbers of CD4+CD25+FOXP3+ Treg cells in the peripheral blood as compared to the adults with steroid sensitive asthma. Furthermore, in these individuals serum 25(OH)D3
level was strongly associated with FOXP3+ Treg cell number. Thus these data support the hypothesis that vitamin D deficiency in adults hinders the expression of FOXP3 in CD4+ Treg cells, subsequently lowers the steroid-induced IL-10 production by these cells and diminishes the anti-inflammatory effects of glucocorticoids.

It could be postulated that such synergistic effect of vitamin D on corticosteroids in adults observed in vitro might translate to clinical outcomes. In a childhood cohort of mild to moderate persistent asthmatics who received inhaled corticosteroid, lung function (pre-bronchodilator FEV1) improvement, was the greatest in the vitamin D sufficient group (defined as >30 ng/ml) as compared to the vitamin D-deficient group (defined as ≤ 20 ng/ml). Another study, in 616 Costa Rican asthmatic children, showed an inverse association between vitamin D levels and the use of anti-inflammatory medication (either inhaled corticosteroids or leukotriene inhibitors) and Searing et al also found that low levels of vitamin D were significantly associated with inhaled and oral steroid use and total steroid dose in children with asthma. The authors concluded that vitamin D supplementation might potentiate the anti-inflammatory function of corticosteroids in asthmatic patients.

In summary, these studies suggest that insufficient serum vitamin D levels might increase asthma severity, thus requiring increased treatment, or that down-regulation of glucocorticoid pathways due to insufficient vitamin D leads to a need for increased steroid doses. Hence, the next section reviews the current understanding of vitamin D physiology, deficiency and insufficiency.

1.4 Vitamin D

1.4.1 Sources of vitamin D

Vitamin D is derived from skin exposure to ultraviolet B (UVB) rays and the diet. Dietary sources include fish oil, fish, liver, egg yolk and many dietary supplements. As very few foods contain vitamin D, sunlight exposure is the primary determinant of vitamin D status in humans. In a fair skinned person, 20 to 30 minutes of sunlight exposure of the face and forearms at midday is estimated to
generate the equivalent of around 2000 IU of vitamin D. Two or three such sunlight exposures a week are sufficient to achieve healthy vitamin D levels in summer in the UK.\textsuperscript{186} In the absence of adequate sun exposure, at least 800–1000 IU (20–25 µg) vitamin D in the diet per day may be needed to achieve this.\textsuperscript{187}

### 1.4.2 Vitamin D metabolism

Vitamin D synthesis is initiated in the skin by solar UVB radiation (wavelength 290 to 315 nm), activating the precursor 7-dehydrocholesterol, which then circulates in the bloodstream to the liver, where it is converted into its main metabolite, 25-dihydroxyvitamin D (25(OH)D\textsubscript{3}), which has blood levels about 1000 times higher than the active metabolite, 1\(\alpha\),25VitD\textsubscript{3}. A summary of vitamin D metabolism is depicted in Figure 1.6. Until recently, it was thought that the conversion to 1\(\alpha\),25VitD\textsubscript{3} occurred only in the kidneys, but increasing evidence indicates that the cells of most organs express the vitamin D receptor and have the capacity to synthesize 1\(\alpha\),25VitD\textsubscript{3} locally.\textsuperscript{185} This synthesis of 1\(\alpha\),25VitD\textsubscript{3} is dependent on serum 25(OH)D\textsubscript{3} levels, the primary circulating form of vitamin D.\textsuperscript{185}

Systemic 25(OH)D and 1\(\alpha\),25VitD\textsubscript{3} may enter into the cells unbound or complexed with vitamin D binding protein (VDBP) via diffusion or endocytosis.\textsuperscript{188} Once in the cytoplasm, 1\(\alpha\),25VitD\textsubscript{3} binds to its receptor (vitamin D receptor). 1\(\alpha\),25VitD\textsubscript{3} acts through the vitamin D receptor, a nuclear hormone receptor which is expressed in a variety of cell types, including those of the innate and adaptive immune system.\textsuperscript{189} The binding of vitamin D to vitamin D receptor activates the receptor, forms a heterodimer with retinoid-X receptor and the heterodimer subsequently translocates to the nucleus.\textsuperscript{188, 189} In the nucleus the vitamin D receptor – retinoid-X receptor complex binds to a specific DNA sequence on a gene promoter region called vitamin D response elements to transactivate expression of the target genes.\textsuperscript{189} Coactivators of the vitamin D receptor further regulate transcription of vitamin D–dependent innate immune target genes. The genomic effects of vitamin D usually take hours or days to be noticed and are considered the slow effects of vitamin D.\textsuperscript{188}
Figure 1.6 Metabolism of Vitamin D. A) Bone metabolism B) Non-skeletal functions. Reproduced from ‘Vitamin D deficiency’ (Holick NEJM 2007)\textsuperscript{185}

A) Vitamin D – bone metabolism
1.4.3 Definition of vitamin D deficiency and insufficiency

Serum 25(OH)D3 is the best indicator of overall vitamin D status because this measurement reflects total vitamin D from dietary intake and sun exposure, as well as the hepatic conversion of vitamin D from adipose stores. There are no consensus guidelines available on optimal levels of serum 25(OH)D3. Vitamin D deficiency is defined by most experts as a 25(OH)D3 level of less than 50 nmol/L (20 ng per millilitre). The 25(OH)D3 levels are inversely related to parathyroid hormone levels until the former reach 75 to 100 nmol/L (30-40 ng per millilitre), at which point
Intestinal calcium transport increases by 45 to 65% when vitamin D levels increase from an average of 50 to 80 nmol/L (20 to 32 ng per millilitre).\textsuperscript{197} 25\([OH]\)D3 levels between 50-75 nmol/L (20-30 ng per millilitre) are considered indicative of vitamin D insufficiency on the basis of the above data and their association with health outcomes.\textsuperscript{194, 198, 199} 25\([OH]\)D3 levels of 75 nmol/L (30 ng per millilitre) to 100 nmol/L, are indicative of normal vitamin D levels.\textsuperscript{200, 201} Excessive levels or vitamin D intoxication are rare.\textsuperscript{185, 202} The current Institute of Medicine (IOM) guidelines from 2011 recommended a lower limit of 50 nmol/L (20 ng per milliliter) and an upper limit of 125 nmol/L (50 ng/ml).\textsuperscript{203} It should be noted that these values are based entirely on the levels required to maintain bone health,\textsuperscript{185, 190-194} and may be totally different to those needed for immunological and pulmonary health.

**Table 1.2. Serum 25-hydroxyvitamin D (25\([OH]\)D3) concentrations**

<table>
<thead>
<tr>
<th>Vitamin D status</th>
<th>Serum 25-[OH]D3 concentration in nmol/l</th>
<th>Serum 25-[OH]D3 concentration in mcg/l or ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deficient</td>
<td>&lt;50</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Insufficient</td>
<td>50-75</td>
<td>20-30</td>
</tr>
<tr>
<td>Optimal</td>
<td>&gt;75</td>
<td>&gt;30</td>
</tr>
</tbody>
</table>

*To convert to µg/l divide by 2.5.

### 1.4.4 Prevalence of vitamin D deficiency and insufficiency

Vitamin D deficiency is one of the most common medical conditions worldwide with more than 1 billion children and adults at risk.\textsuperscript{204} A recent survey of 7437 adults from across the UK showed 60% had serum 25(OH)D3 concentrations below 75 nmol/L (30 ng per millilitre) in the summer months and 87% were insufficient in the winter.\textsuperscript{205} This survey also demonstrated a gradient of prevalence across the UK, with highest rates of insufficiency in Scotland, northern England, and Northern Ireland.\textsuperscript{205} People living at higher latitudes are known to be at greater risk for vitamin D deficiency.\textsuperscript{206} Depending on the levels used to define vitamin D deficiency, 57% to 93% of the general hospital inpatient population is deficient\textsuperscript{193} and a recent meta-analysis
concluded that use of vitamin D supplements is associated with decrease in total all-cause mortality rates.\textsuperscript{207}

Children and young adults are also potentially at high risk of vitamin D deficiency; 44 (12\%) of 365 infants and toddlers from Boston\textsuperscript{208} and 129 (42\%) of 307 adolescents from Boston\textsuperscript{209} had 25(OH)D3 concentrations below 50 nmol/L (20 ng per millilitre). A recent large, cross-sectional survey\textsuperscript{210} (US National Health and Nutrition Examination Survey from 2001-2004) showed that 61\% of 6275 children aged 1 to 21 years had insufficient levels of vitamin D.\textsuperscript{210} The prevalence of vitamin D deficiency is also reflected in the several hundred children with rickets treated each year in the UK.\textsuperscript{211} However, these children represent a small proportion of all individuals with suboptimal vitamin D levels in the UK population.\textsuperscript{205, 212}

1.4.5 Risk factors for vitamin D deficiency and insufficiency

Vitamin D skin metabolism is influenced by age, skin pigmentation (melanin content of the skin), sun exposure (latitude, season, time outdoors, and clothing), body fat and sunscreen use (for example, use of sun protection factor (SPF) 8 reduces vitamin D production by 92.5\% and SPF 15 by 99\%).\textsuperscript{185, 187} Risk factors for vitamin D deficiency and insufficiency are summarized in Table 1.3.

**Table 1.3 Risk factors for Vitamin D Deficiency/Insufficiency in children\textsuperscript{147}**

<table>
<thead>
<tr>
<th>Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lack of sunlight exposure (very little vitamin D is produced in people living in areas at beyond a latitude of 35° from October to March)</td>
</tr>
<tr>
<td>2. Non-white ethnicity (pigmented skin)</td>
</tr>
<tr>
<td>3. Concealing clothing (for example use of veil, headscarf)</td>
</tr>
<tr>
<td>4. Use of sun screen</td>
</tr>
<tr>
<td>5. Obesity</td>
</tr>
<tr>
<td>6. Institutionalized individuals who spend relatively large amount of time indoors.</td>
</tr>
<tr>
<td>7. Liver disease, malabsorption, short bowel, drugs such as phenobarbitone, cholestyramine, orlistat</td>
</tr>
</tbody>
</table>

52
The next section reviews the epidemiological evidence of the association between vitamin D and asthma.

1.4.6 Epidemiological evidence of an association between vitamin D and asthma

Vitamin D deficiency and asthma are both common, and share risk factors such as urban residence, black ethnicity and obesity. This has generated interest in exploring a link between vitamin D deficiency and asthma.

Black et al reported a positive correlation between serum vitamin D and FEV₁ and forced vital capacity (FVC) in a cross sectional study of 14,000 adults. An association between lower serum vitamin D concentrations and impaired spirometry is also reported in children and adults with mild to moderate asthma. A high prevalence of vitamin D deficiency (54%, 46 out of 85) and insufficiency (86%, 73 out of 85) was noted in a recent cross sectional case control study of inner-city African-American children with asthma. Significantly lower vitamin D levels were also noted in asthmatic children compared to healthy controls. A study of 616 Costa Rican asthmatic children between the ages of 6 and 14 years showed an inverse association between serum vitamin D and total IgE levels, and peripheral blood eosinophil counts. A high frequency of vitamin D insufficiency (35%) in 1024 North American children with mild-to-MA has also been reported. In addition, vitamin D insufficiency was associated with a lower FEV₁ compared with children with sufficient levels. Vitamin D levels are known to change with demographic and lifestyle variables and this study did not report repeated vitamin D measurements over time, an issue that limits our ability to understand the extent to which fluctuations in vitamin D levels over time influence asthma.

Recently, Searing et al have shown the prevalence of vitamin D insufficiency was 47% (47 / 100) in children with asthma who were primarily from latitudes above 35°N, and 17% (17/100) of asthmatic children were vitamin D deficient. These percentages were higher than those in the equatorial population from Costa Rica, in which 175 (28%) of asthmatic children were vitamin D insufficient. The differences
between these studies support the association of an increased risk of vitamin D deficiency in populations living at higher, northern latitudes. Searing et al\textsuperscript{173} also found that vitamin D levels were positively correlated with FEV\textsubscript{1} percent predicted and the FEV\textsubscript{1}/FVC ratio.\textsuperscript{173} Total IgE and the number of positive aeroallergen skin prick test responses showed a significant inverse correlation with vitamin D levels. More recently a positive association between vitamin D levels and asthma control (assessed by childhood ACT)\textsuperscript{224} and negative association with exercise induced bronchoconstriction has also been shown in Italian asthmatic children.\textsuperscript{225}

Although these studies suggested a role for vitamin D in asthma control, some authors have also suggested that vitamin D may promote, rather than ameliorate the asthmatic phenotype.\textsuperscript{226} Although these studies suggested a role for vitamin D in improving asthma control, Wjst et al have suggested that vitamin D may promote, rather than ameliorate the asthmatic phenotype.\textsuperscript{227} In many studies, it is uncertain whether vitamin D is critical in airway inflammation or is just an indirect biomarker for asthma morbidity. A large, population-based, cross-sectional survey found that corticosteroid therapy of any kind (not just for asthma) is associated with low levels of vitamin D, possibly because exogenously administered corticosteroids accelerate degradation of vitamin D.\textsuperscript{228} The potential relationship between vitamin D deficiency and asthma is further confounded by the strong association with physical activity, which is linked to an individual’s level of exposure to sunlight\textsuperscript{229} and disease severity, which means that it is unclear whether vitamin D is simply an indirect marker of physical activity or disease severity and, therefore, is an innocent spectator in disease pathogenesis. Also, whether there is a direct effect of airway inflammation in asthma on circulating vitamin D levels is not known. Given that there is local upregulation of the conversion of 25(OH)D to 1,25(OH)\textsubscript{2}D during infection or inflammation one might predict that immediate circulating levels / store of 25(OH)D would be depleted. It is possible that in the setting of chronic airway inflammation systemic vitamin D levels may be reduced as a direct result of the inflammatory response, which further confounds the positive association between vitamin D deficiency and disease severity.\textsuperscript{230-232} In summary, associations between asthma and vitamin D could represent causality, reverse causation, or be a consequence of
inflammation or treatment.

Regular vitamin D supplementation (2000 IU/day) in the first year of life increased the risk of developing atopy, allergic rhinitis, and asthma when assessed at age 31 years. However, this study was limited by the absence of data on maternal intake of vitamin D and assessment of childhood asthma or atopy. Mothers reported the frequency and dose of vitamin D supplementation and daily dose was calculated based on this information. Furthermore, this finding may be related to the very high dose of vitamin D supplementation. Another study from the United Kingdom has shown that pregnant women with higher levels of vitamin D have offspring with an increased risk of eczema at 9 months and asthma at 9 years of age; however, this study also has important limitations (only 30% [178 out of 596] follow-up at age 9 years and relatively small numbers [n=596]). In another study of 922 newborns, low cord blood vitamin D levels were associated with an increased risk of respiratory infections at three months of age and wheeze by five years of age, but again were not associated with doctor diagnosed asthma. This study was limited by unavailability of vitamin D status during pregnancy and childhood and information regarding respiratory infections and wheeze were captured from interviews with mother rather than medical evaluation. In summary, the evidence to suggest that higher vitamin D levels reduce the incidence of asthma is conflicting and observational studies of vitamin D and asthma are summarized in Table 1.4. These variable results may be secondary to differences in the absolute amount of vitamin D exposure, the baseline vitamin D status (vitamin D deficiency versus insufficiency) and the timing of exposure (naive immune cells versus mature cell lines). Furthermore, the effect of vitamin D status on asthma and allergic diseases may only exist among a subgroup of subjects with particular genotypes. This is supported by a recent study investigating the effect of low cord blood vitamin D and gene interactions on the development of food sensitization using a prospective birth cohort design. They noted vitamin D deficiency alone, was not associated with food sensitization, however, when individual genetic susceptibility was also considered, vitamin D deficiency increased the risk of sensitization among children with certain
high-risk genotypes. This underscores the need to consider individual genetic susceptibility in assessing effects of vitamin D deficiency. Polymorphisms exist in the genes encoding the vitamin D receptor, VDBP and enzymes involved in vitamin D metabolism. Some genetic studies have reported associations between vitamin D status and asthma. Genome scans for asthma have identified possible linkages on 17 different chromosomes, including chromosome 12, region q13-23. Because the vitamin D receptor gene spans 63kb of the long arm of chromosome 12, some geneticists have postulated an association between vitamin D receptor polymorphisms and genetic susceptibility for asthma and allergy, and more specifically an area towards the centromeric end of chromosome 12q, which includes the vitamin D receptor gene. Other potential candidate asthma genes map to this region, including STAT-6 (a signaling molecule in Th2 cells), IRAK3 (involved in IL-1 signaling) and c-kit (mast cell growth factor receptor). However, studies of a number of single nucleotide polymorphisms (SNPs) in these genes have not identified a link with asthma.
Table 1.4 Vitamin D and asthma in children and adults. *Updated from Vitamin D and asthma in children*  

<table>
<thead>
<tr>
<th>Effect of vitamin D</th>
<th>Study Limitations</th>
<th>Population studied</th>
<th>Children / adults</th>
<th>Study type</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log10 ↑ in serum vitamin D level associated with ↓ hospitalizations, ↓ anti-</td>
<td>Cross sectional study (inability to exclude reverse causation)</td>
<td>Costa Rica Asthma</td>
<td>616 children, aged 6-14</td>
<td>Cross sectional</td>
<td>Brehm et al 183</td>
</tr>
<tr>
<td>inflammatory medication, and ↓ markers of allergy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D insufficiency is common in children with mild-to-moderate asthma and</td>
<td>Lack of repeated measures of vitamin D</td>
<td>North American Severe Asthma</td>
<td>1024 Children</td>
<td>Sub study of Childhood Asthma</td>
<td>Brehm et al 222</td>
</tr>
<tr>
<td>is associated with higher odds of severe exacerbation over a 4-year period</td>
<td></td>
<td></td>
<td></td>
<td>Management Program (CAMP) study</td>
<td></td>
</tr>
<tr>
<td>Vitamin D insufficiency is associated with reduced asthma control</td>
<td>Inability to exclude reverse causation, small sample size</td>
<td>Italian Asthma</td>
<td>75 children (5-11yrs)</td>
<td>Cross sectional</td>
<td>Chinellato et al 240</td>
</tr>
<tr>
<td>Lower serum Vitamin D levels associated with reduced lung function and increased</td>
<td>Inability to exclude reverse causation, small sample size</td>
<td>Italian Asthma</td>
<td>45 children (5-11yrs)</td>
<td>Cross sectional</td>
<td>Chinellato et al 225</td>
</tr>
<tr>
<td>airway reactivity to exercise</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D level was positively correlated with lung function and inversely</td>
<td>Inability to exclude reverse causation, lack of adjustments to confounders.</td>
<td>Denver Colorado Asthma</td>
<td>100 children</td>
<td>Cross sectional</td>
<td>Searing et al 241</td>
</tr>
<tr>
<td>correlated with total IgE, atopy and steroid use</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D level at 6 years was associated with asthma in boys at 14 years of age. Children (particularly males) with inadequate vitamin D are at increased risk of developing atopy, and asthma</td>
<td>Significant loss to follow up</td>
<td>Australia</td>
<td>989 - 6 yrs old, 1380 14 yrs old (689 children were seen longitudinally at both 6 and 14 years)</td>
<td>Longitudinal and Cross-sectional</td>
<td>Hollams et al 242</td>
</tr>
<tr>
<td>Vitamin D deficiency associated with asthma</td>
<td>Small sample size</td>
<td>Washington, DC</td>
<td>106 children (6-20 yrs)</td>
<td>Case control study Cross sectional design</td>
<td>Freishtat et al 215</td>
</tr>
<tr>
<td>Regular vitamin D supplementation (2000 IU/day) in the first year of life increased the risk of developing atopy, allergic rhinitis, and asthma by age 31 years</td>
<td>Lack of serum vitamin D levels in infancy, no study visits between 4-31 year of age and 29% were lost to follow up</td>
<td>Finland</td>
<td>7648 adults</td>
<td>Cross sectional study at 31 years of age</td>
<td>Hypponen et al 243</td>
</tr>
<tr>
<td>Vitamin D deficiency associated with decreased lung function in newly diagnosed asthmatics</td>
<td>Inability to exclude reverse causation</td>
<td>Chinese</td>
<td>Adults</td>
<td>Cross sectional</td>
<td>Li et al 244</td>
</tr>
<tr>
<td>No association</td>
<td>Low vitamin D level in all subjects,</td>
<td>United Kingdom</td>
<td>160 Adults</td>
<td>Case control study Cross sectional design</td>
<td>Devereux et al 245</td>
</tr>
<tr>
<td>Vitamin D level was positively correlated with lung function and glucocorticoid response</td>
<td>Small sample size, Inability to exclude reverse causation</td>
<td>Denver, Colorado</td>
<td>54 adults</td>
<td>Cross sectional</td>
<td>Sutherland et al 246</td>
</tr>
<tr>
<td>Severe and uncontrolled asthma in adults is associated with vitamin D insufficiency and deficiency</td>
<td>Inability to exclude reverse causation</td>
<td>Germany</td>
<td>280 Adults</td>
<td>Cross sectional design</td>
<td>Korn et al 247</td>
</tr>
<tr>
<td>Association between vitamin D receptor polymorphisms and asthma</td>
<td>Small sample size</td>
<td>Tunisia</td>
<td>155 children 225 control</td>
<td>Case control study</td>
<td>Maalmi et al 248</td>
</tr>
<tr>
<td>No association</td>
<td>Inability to exclude reverse causation.</td>
<td>Worcester, USA</td>
<td>263 children with asthma 284 controls</td>
<td>Retrospective, Case control study</td>
<td>Menon et al(^{249})</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------</td>
<td>----------------</td>
<td>-----------------------------------</td>
<td>-------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>No association between maternal late pregnancy vitamin D status and either asthma or wheeze at age 6 years.</td>
<td>Only single serum measurement, incomplete follow up</td>
<td>Southampton, United Kingdom</td>
<td>451 children</td>
<td>Prospective</td>
<td>Pike et al(^{250})</td>
</tr>
<tr>
<td>Vitamin D insufficiency is associated with severe asthma exacerbations, independently of racial ancestry, atopy and time spent outdoors.</td>
<td>No information on adherence to prescribed steroids</td>
<td>Puerto Rico</td>
<td>560 children</td>
<td>Cross sectional study</td>
<td>Brehm et al(^{251})</td>
</tr>
<tr>
<td>Low serum vitamin D levels were associated with asthma atopy and asthma, predominantly in boys, at age 6 and 14.</td>
<td>Inability to exclude reverse causation.</td>
<td>Western Australia</td>
<td>689 children were assessed at both ages</td>
<td>Longitudinal cohort study</td>
<td>Hollams et al(^{252})</td>
</tr>
<tr>
<td>Serum vitamin D levels were inversely associated with asthma</td>
<td>Inability to exclude reverse causation.</td>
<td>Iran</td>
<td>50 asthmatic children and 50 controls</td>
<td>Cross-sectional study</td>
<td>Alyasin et al(^{253})</td>
</tr>
<tr>
<td>Cord Vitamin D levels are inversely associated with risk of wheezing but no association with asthma at 5 years.</td>
<td>Short duration of follow up</td>
<td>New Zealand</td>
<td>1105 children, 823 assessed at 5 years</td>
<td>Population based birth cohort studies</td>
<td>Camargo et al(^{235})</td>
</tr>
<tr>
<td>Vitamin D levels were inversely associated with wheeze and asthma</td>
<td>Inability to exclude reverse causation.</td>
<td>US</td>
<td>6857 children &amp; adults</td>
<td>Cross sectional study (NHANES 2005-6)</td>
<td>Keet et al(^{254})</td>
</tr>
<tr>
<td>Vitamin D concentration at age 4 years was inversely associated with asthma at 4-8, whereas vitamin D measurement at age 8 was positively associated with asthma at age 8.</td>
<td>Small sample size, diagnosis of severe asthma was based on parental questionnaire</td>
<td>Netherlands</td>
<td>372 at 4yr and different 8 year sub group of 328</td>
<td>Cohort study – cross sectional as well as longitudinal PIAMA birth cohort of 4146 pregnant women.</td>
<td>Oeffelen et al(^{255})</td>
</tr>
</tbody>
</table>
Importantly, the observational nature of these studies precludes an assessment of cause and effect. Epidemiological cross-sectional studies suggest an association but do not prove causality. Longitudinal and interventional studies in children and adults are needed to elucidate any relationship more clearly.

1.4.7 Epidemiological evidence of a link between vitamin D and preschool asthma / wheeze

Prenatal vitamin D status is thought to affect the development of the fetal lung and immune system.\textsuperscript{256, 257} Thus it is possible that vitamin D deficiency/insufficiency in early life predisposes individuals to asthma. A birth cohort (n=2000) in Scotland showed children of healthy mothers who had lower vitamin D intake had an increased risk of recurrent wheeze at age 5 years. However, there was no association with spirometry or FeNO\textsubscript{50}.\textsuperscript{258} In another epidemiological study of 1194 mother-child pairs, children born to healthy women living in an inner city who had vitamin D deficiency during pregnancy were at increased risk for recurrent wheeze at 3 years of age.\textsuperscript{259} A limiting factor to all these studies was the use of food frequency questionnaires and not serum levels of vitamin D. Observational studies of vitamin D and preschool wheezing are summarized in Table 1.5.

In summary, \textit{in utero} and early life vitamin D deficiency/insufficiency is associated with an increased risk of wheezing. However, association does not prove causation, and further evaluation needs to be undertaken to clarify the exact role of vitamin D in the pathogenesis of wheeze and asthma.
Table 1.5 Vitamin D and wheeze in preschool children

Updated from Vitamin D and asthma in children⁶

<table>
<thead>
<tr>
<th>Effect of vitamin D</th>
<th>Limitations</th>
<th>Age group</th>
<th>Study type</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low serum vitamin D is associated with increased risk of viral co-infection in wheezing children</td>
<td>Cross sectional study, uncertain diagnosis</td>
<td>284 Preschool children (1-2.8 years)</td>
<td>Substudy of the VINKU project, Finland</td>
<td>Jartti et al 260</td>
</tr>
<tr>
<td>Cord blood levels of vitamin D are inversely associated with risk of respiratory infections and childhood wheezing but no association with asthma at 5 years.</td>
<td>Short duration of follow up</td>
<td>Birth cohort of 1105 children, 823 assessed at 5 years of age</td>
<td>New Zealand birth cohort Population based cohort studies</td>
<td>Camargo et al 235</td>
</tr>
<tr>
<td>Mothers in highest quartile of vitamin D intake had lower risk for recurrent wheeze in child at age 3 years</td>
<td>Short duration of follow up</td>
<td>Birth cohort study of 2128 children, 1194 children assessed at 3</td>
<td>Mother-child pre-birth cohort</td>
<td>Camargo et al 235</td>
</tr>
<tr>
<td>Mothers in highest quintile of vitamin D intake had lower risk for child at age 5 years to have ever wheezed, wheezing in the previous year, and persistent wheezing. No association of vitamin D levels with asthma, spirometry, or atopic sensitization</td>
<td>No maternal serum vitamin D levels during pregnancy</td>
<td>2000, mother &amp; child pair 1335, aged 2 years 1212, aged 5 years</td>
<td>Mother-child pre-birth cohort</td>
<td>Devereux et al 258</td>
</tr>
<tr>
<td>Maternal 25-hydroxyvitamin D concentrations &gt;30 ng/mL associated with an increased risk of eczema at 9 months and increased risk of asthma at 9 years</td>
<td>70% lost to follow up, no study visits between 9 month and 9 years</td>
<td>596 mother &amp; child pair 440 children aged 9 months 178 children aged 9 yrs</td>
<td>Mother-child pre-birth cohort</td>
<td>Gale et al 234</td>
</tr>
</tbody>
</table>
| Maternal vitamin D intake from foods during pregnancy is negatively associated with risk of asthma and allergic rhinitis | No maternal serum vitamin D levels | Birth cohort study of 3565 children, 1669 children assessed at 5 yrs | Finnish birth cohort study | Erkkola et al. 
261 |
| --- | --- | --- | --- | --- |
| Atopic manifestations were more prevalent in the group with higher intake of vitamin D | Small n numbers No vitamin D levels | 123 children, aged 6 years | Postal questionnaires | Back et al. 
262 |
| Maternal intake of vitamin D associated with reduced risk of wheeze | No maternal serum vitamin D levels during pregnancy | 1002 mother and child pairs, 763 assessed at 16-24 m | Birth cohort study | Miyake et al. 
263 |
| Cord serum vitamin D levels were inversely associated with the risk of transient early wheezing by the age of 5 years but no association with asthma | Parental questionnaires could result in misclassification No vitamin D levels during childhood | 239 newborns | EDEN birth cohort | Baiz et al. 
264 |
| No difference between supplemented and control groups in risk of wheeze atopy, eczema and lung function or FeNO<sub>50</sub> between supplemented groups and controls. | Possibility of insufficient dose (even after supplementation, only a small percentage of offspring (13% daily group, 3% bolus group) had cord vitamin D levels in the sufficient range) Supplementation only started at 27 weeks of gestation (? late). Risk of reporter’s bias (not blinded) | 180 pregnant women at 27 weeks gestation | Randomised controlled trial to either no vitamin D, 800 IU ergocalciferol daily until delivery or single oral bolus of 200,000 IU cholecalciferol, | Goldring et al. 
265 |
1.4.8 Immunomodulatory effects of vitamin D

Vitamin D may be important for maintenance of pulmonary health through its influence on innate and adaptive immune responses and the promotion of regulatory T cells (summarized in Figure 1.7). Recent immunological and epidemiological studies have shown that vitamin D has numerous cytokine-modulating effects through multiple different cells of the immune system and airway (summarized in Table 1.6).

Table 1.6 Response of different target cells to vitamin D

<table>
<thead>
<tr>
<th>Target Cells of vitamin D</th>
<th>Effector function of vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td>All T cells</td>
<td>Inhibit T cell proliferation&lt;sup&gt;266&lt;/sup&gt;</td>
</tr>
<tr>
<td>Th1</td>
<td>Inhibit Th1 cytokine release&lt;sup&gt;267-269&lt;/sup&gt;</td>
</tr>
<tr>
<td>Th2</td>
<td>Conflicting evidence for enhancement&lt;sup&gt;266&lt;/sup&gt; and inhibition&lt;sup&gt;270&lt;/sup&gt; of Th2 responses, which may pertain to dose.&lt;sup&gt;278&lt;/sup&gt;</td>
</tr>
<tr>
<td>Th17</td>
<td>Inhibit Th17 cytokine release&lt;sup&gt;271&lt;/sup&gt;,&lt;sup&gt;272&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tregs</td>
<td>Induces IL-10 synthesis&lt;sup&gt;163, 171, 178&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Increase in the frequency of FoxP3 cells&lt;sup&gt;177, 273&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Enhance TGF β synthesis&lt;sup&gt;274&lt;/sup&gt;</td>
</tr>
<tr>
<td>B cells</td>
<td>Nonlinear association between serum vitamin D levels and IgE&lt;sup&gt;202&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Enhanced production of the immunomodulatory cytokine IL-10&lt;sup&gt;275&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Renders monocyte derived dendritic cells more immature and tolerogenic&lt;sup&gt;276&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Induces IL-10 synthesis&lt;sup&gt;276&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bronchial smooth muscle cells</td>
<td>Inhibit cytokine synthesis and release&lt;sup&gt;277&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Decrease lung inflammation&lt;sup&gt;268, 278&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Inhibit bronchial smooth muscle cell proliferation and remodelling (eg, matrix metalloproteinase-9 (MMP-9))&lt;sup&gt;279-281&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Inhibit the differentiation, maturation, and homing of mast cells to allergic airways&lt;sup&gt;282&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Induce IL-10 synthesis&lt;sup&gt;283&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 1.7 Immunological effects of vitamin D. Reproduced from ‘The impact of vitamin D on Regulatory T cells’ (Chambers and Hawrylowicz, 2011)\(^\text{179}\)

ILT3 - Immunoglobulin-like transcript 3, PD-L1- programmed cell death ligand 1, CTLA 4- cytotoxic T-lymphocyte antigen 4, TLR—Toll-like receptor

Vitamin D appears to influence T cell function. Essentially all studies using mouse or human cells agree on the capacity of vitamin D to inhibit T cell proliferation and production of Th1 associated cytokines.\(^{284-287}\) However the effect of vitamin D on Th2 cytokines is less clear and there are contradictory reports.\(^{269, 288-290}\) Inconsistent results may be due to the differences in target cell types, and timing and dose of vitamin D administration.\(^{220, 289, 291}\) For example a study on whole human PBMC responses to allergen with high concentration of vitamin D (10\(^{-6}\)M) found that Th1 cytokines were suppressed
while Th2 cytokine secretion (IL-5 and IL-13) was enhanced. However, studies using 10^{-7}M to 10^{-9}M (lower) concentrations of vitamin D showed suppression of both Th1 and Th2 cytokines. A counterpart to this probably exists in vivo in humans. Hypponen et al recently showed that a significant, but nonlinear relationship exists between serum vitamin D and IgE, where a very high and very low serum vitamin D associated with higher levels of IgE. More recently vitamin D has been shown to inhibit IL-17A production in animal models and moderate to severe adult asthmatics which may be important in steroid resistant airway disease.

Vitamin D also appears to promote the function of Tregs. Vitamin D enhances the production of the anti-inflammatory cytokine IL-10 by human T cells in vitro and in vivo both directly and synergistically with corticosteroids. Vitamin D can also increase IL-10 gene expression. Enhancement of IL-10 synthesis in B cells by vitamin D has also been reported. Studies by Adorini and colleagues demonstrate the capacity of vitamin D to induce IL-10 production by dendritic cells. Taher et al demonstrated a beneficial effect of vitamin D administration to mice post allergic sensitization, but prior to allergen challenge, when delivered together with allergen immunotherapy. Moreover, using antibodies (anti-IL-10 receptor and anti-TGFβ-neutralizing monoclonal antibodies) to block TGFβ and IL-10 reversed these beneficial effects.

1.4.9 VDBP

The vitamin D axis not only includes vitamin D but also incorporates VDBP and the vitamin D receptor. VDBP is a glycosylated α-globulin, with 458 amino acids folded into a disulfide-bonded, triple-domain structure (Figure 1.8). Most of the vitamin D in the circulation is bound to VDBP with high affinity. Vitamin D is taken up into cells by diffusion of unbound vitamin D across cell membranes, and by endocytosis of that bound to VDBP. Circulating vitamin D is also bound to albumin. Since the affinity of albumin for vitamin D is lower than that of VDBP the vast majority is bound to VDBP.
The diagram shows the structure of VDBP. Domain I, which binds vitamin D, is shown in purple, II in blue and III in brown. Actin binds to elements within zones II and III. The arrow like elements within each structure represent alpha helices, and the disulphide bonds linking elements within the structure can just be seen in orange.

Figure 1.8 The crystal structure of vitamin D binding protein.

‘Reproduced from The vitamin D axis in the lung: a key role for vitamin D-binding protein’.

299
VDBP is a multifunctional protein. The major functions of VDBP are the binding, solubilization and serum transport of vitamin D. The relationship of VDBP and vitamin D concentrations is not yet clear. Low vitamin D concentrations were reported in the VDBP knockout mouse model but VDBP does not influence vitamin D’s biologically active pool (vitamin D available to enter cells and tissues).  

VDBP also possesses direct functions independent of vitamin D binding that may be of particular relevance in airway inflammation. VDBP is expressed in the plasma membrane of neutrophils, contributes to macrophage activation, augments monocyte and neutrophil chemotaxis to C5-derived peptides and acts as a scavenger protein to clear extracellular G-actin released from necrotic cells. (The functions of VDBP are discussed further in chapter 6 and summarised in table 6.1) Recently VDBP has also been identified in BAL. Wood and colleagues recently noted that circulating VDBP levels inversely correlate with FEV₁ in an adult cohort with COPD, and that sputum VDBP contributes to macrophage activation. They used an innovative in vitro assay to assess alveolar macrophage phagocytic capacity (efferocytosis) by engulfment of fluorescently-labelled apoptotic neutrophils. They showed that exogenous VDBP in culture enhanced macrophage activity in a dose-dependent manner, and that sputum from adults with COPD increased macrophage phagocytic activity to an extent that positively correlated with the amount of VDBP present. The anti-inflammatory role of VDBP, together with the relationship with serum vitamin D, may therefore be important in children with asthma but there have been no reports comparing VDBP levels of healthy children and those with asthma. Despite the wealth of serum vitamin D studies, there has been relatively little work concerning the role of VDBP in children with asthma.

1.4.10 Vitamin D and airway remodelling

As discussed earlier in this chapter, increased ASM mass is a feature of airway remodelling and in vitro studies have shown that vitamin D may influence airway remodelling by inhibiting ASM proliferation in adult asthma and COPD and alter expression of genes (such as MMP9 and
ADAM metallopeptidase domain 33 [ADAM33]) involved in airway remodelling. *In vitro* Damara et al\textsuperscript{255} have shown that in primary human ASM culture, twice as many asthmatic ASM cells entered into S phase of the cell cycle as compared to non-asthmatic cells.\textsuperscript{255} They also showed that stimulation with platelet-derived growth factor increased DNA synthesis in asthmatic ASM cells as compared to normal ASM cells and importantly, treatment with vitamin D decreased the platelet-derived growth factor induced DNA synthesis and reduced the number of ASM cells in the S-phase in both asthmatic and non-asthmatic cells.\textsuperscript{255} In another *in vitro* study of human primary ASM cells isolated from adult asthmatic and non-asthmatic subjects using flow cytometry, immunocytochemical staining and colorimetric proliferation Song et al\textsuperscript{311, 313} demonstrated that asthmatic ASM cells had greater proliferation rate as compared to the controls, as determined by proliferating cell nuclear antigen staining. In the presence of vitamin D, asthmatic ASM cells *in vitro* inhibit ASM proliferation rate to normal.\textsuperscript{288, 290} Additionally, vitamin D reduced the mRNA and protein expression of MMP9 and ADAM33 genes (both involved in airway remodelling) to a level comparable to that of non-asthmatic ASM cells.\textsuperscript{311, 313} Bosse et al\textsuperscript{279} have shown *in vitro* that vitamin D increases glucocorticoid bioavailability in bronchial smooth muscle cells suggesting a further beneficial role for vitamin D in the treatment of asthma.

A recent study has shown that in a mouse model vitamin D receptor deletion upregulated matrix metalloproteinases, which are critically involved in the airway remodelling process, in BAL and whole lung homogenates.\textsuperscript{314} Further, *in vitro* vitamin D has been shown to inhibit the profibrotic effects of TGFβ on lung fibroblasts and epithelial cells in cell culture.\textsuperscript{315} Recently, Lai et al\textsuperscript{316} have also shown that in a mouse model, vitamin D treatment reduced pulmonary inflammation and attenuated established structural changes of the airways. They showed that vitamin D treatment could efficiently reduce ASM mass, subepithelial collagen deposition, epithelial thickening and prevent goblet cell hyperplasia.\textsuperscript{316} In another recent study, Agrawal and colleagues\textsuperscript{317} examined the effects of dietary supplementation with vitamin D on a murine model of allergic airways inflammation. Evidence of severe airway remodelling (airway
thickening, smooth muscle cell thickening, collagen deposition, epithelial hyperplasia with abundant cytoplasmic as well as airway lumen filled with mucin) was noted in the vitamin D deficient group compared to vitamin D-sufficient and Vitamin D supplemental groups. In summary, in vitro and animal work suggest vitamin D may have an impact on airway remodelling in asthma.

1.4.11 Vitamin D and lung development

If vitamin D is important in disease onset then it is most likely to exert its effect in utero or early in post-natal life. Vitamin D has been shown to stimulate alveolar type II cells and surfactant production and may regulate alveolarization. Gaultier et al have shown reduced lung compliance in pups born to vitamin D deficient rats. These data are consistent with a recent study that showed decreased lung function (as assessed by plethysmography and the forced oscillation technique) and altered lung structure without changes in somatic growth in mice born to mothers with vitamin D deficiency. Given the effect of vitamin D on lung growth in vivo and the role that structural deficits are likely to play in the onset of chronic lung disease and the prognosis of an individual who is genetically susceptible to the development of obstructive lung disease, it may be that vitamin D deficiency may predispose to pulmonary morbidity by altering early lung development.

1.4.12 Vitamin D and asthma exacerbations

Viral respiratory infections are the commonest cause of asthma exacerbations in children and adults. Increasing evidence has suggested that viruses may cause more than just simple respiratory infections. In humans, rhinovirus infection induces airway inflammation, increases asthma exacerbation severity, and results in more severe infections in asthmatics when compared to normal subjects. There is emerging evidence that low serum vitamin D levels are associated with more upper respiratory infections, especially among patients with asthma, and with increasing asthma severity. A prospective cohort study that measured serial concentrations of vitamin D in 198 healthy adults observed that individuals
with a 25(OH)D3 concentrations > 95 nmol/L (38 ng/ml) had a twofold reduced risk of acute viral respiratory tract infections.\textsuperscript{330}

Emerging evidence indicates that vitamin D mediated effects on innate immunity, particularly through enhanced expression of the human cathelicidin (hCAP-18) antimicrobial peptide, is important in host defence against respiratory tract pathogens.\textsuperscript{331-334} In particular, hCAP-18 enhances microbial killing and acts as a chemoattractant for neutrophils and monocytes. Other antimicrobial products regulated by vitamin D are defensin-\(\beta\)-2 and -4. \textit{In vitro}, the induction of hCAP-18 in respiratory epithelial cells by vitamin D enhances antimicrobial activity against respiratory pathogens.\textsuperscript{334, 335} Vitamin D can potentially modify the signaling pathways that bind respiratory viruses. Vitamin D decreases the expression of ICAM-1, the major cellular receptor for human rhinovirus in human PBMC.\textsuperscript{336} Both cathelicidins and defensins have a broad spectrum of antimicrobial activity and kill bacteria by disruption of microbial membranes and also act as chemoattractants for other inflammatory cells and contribute to wound repair. A study of Mycobacterium tuberculosis infection in human monocytes found that activation of toll-like receptors (responsible for recognizing microbial ligands) led to upregulation of the vitamin D receptor and cytochrome P450 enzyme 27 B1 (CYP27B1), the enzyme responsible for converting 25[OH]D3 to active form of vitamin D.\textsuperscript{332} The endogenous production of active form of vitamin D by CYP27B1 and subsequent action through the vitamin D receptor led to the induction of cathelicidin, thus demonstrating a mechanism of the antimicrobial activity of vitamin D.\textsuperscript{332} In addition to enhancing antimicrobial peptide expression, vitamin D also induces autophagy in human macrophages.\textsuperscript{337} Autophagy is the ingestion of sequestered material inside phagosomes, which is important in the defense against infections. Natural killer cells also play a critical role in the innate immune system and are able to kill infected cells. Vitamin D induced cathelicidin was required for cytotoxic activity of natural killer cells against tumor cells in a transplantable mouse melanoma model.\textsuperscript{338} As discussed earlier in this chapter, vitamin D can also enhance IL-10 production in adults, thus providing a potential mechanism by which vitamin D can decrease inflammation while increasing antimicrobial action.
The association of vitamin D with asthma exacerbations triggered by acute viral respiratory infections is supported by intervention trials demonstrating decreased respiratory tract infections in children receiving vitamin D supplementation. Majak et al in a double blind, placebo controlled study assessed the effect of vitamin D supplementation in the time period from September to July on asthma symptom score, lung function and the number of exacerbations in 48 vitamin D sufficient Polish children (aged 5-18 yrs) with newly diagnosed asthma. Despite the lack of any significant differences between the study groups as far as the absolute changes of vitamin D levels were concerned, vitamin D supplementation decreased the number of asthma exacerbations triggered by acute respiratory tract infections. Findings from this small clinical trial must be interpreted with caution due to non-standardized assessment of asthma exacerbations and lack of significant improvement in symptom score. In another multi-centre, randomized, double blind placebo-controlled clinical trial in 430 Japanese school children, vitamin D supplementation appeared to have a stronger effect in the sub-cohort with underlying asthma. Vitamin D supplements (1200 IU daily for 4 months) led to a relative risk reduction in asthma exacerbations of 93% compared with children given placebo. These observations are supported by a study by Brehm et al in 1,024 children with mild to MA in which vitamin D insufficiency was associated with higher odds of any hospitalization or emergency department visit (odds ratio 1.5) and a Costa Rican study which showed that asthmatic children with higher vitamin D levels had fewer hospitalizations in the previous year (odds ratio =0.05, 95% confidence interval =0.004-0.71, P=0.03). A recent systematic review of clinical studies investigating the association between vitamin D deficiency and susceptibility to acute respiratory infection in humans was published. A total of 39 studies (4 cross-sectional studies, 8 case-control studies, 13 cohort studies and 14 clinical trials) were evaluated. Observational studies predominantly reported statistically significant associations between low vitamin D status and increased risk of both upper and lower respiratory tract infections, However, results from randomised controlled trials were conflicting, reflecting heterogeneity in dosing regimens and baseline vitamin D status in study
populations. The potential for vitamin D to increase pulmonary defence against respiratory infections may reduce the triggering of asthma exacerbations caused by respiratory tract infections.

1.5 Summary
Vitamin D deficiency and insufficiency are increasingly being recognized in the general population, and have been largely attributed to lifestyle changes (reduced exposure to sunshine due to working indoors or the use of protective clothing and sunscreen; and changes in diet) over the last few decades. There is growing appreciation of the likely importance of vitamin D as a pleiotropic mediator that contributes to pulmonary health by inhibiting inflammation, in part through maintaining regulatory T cells, direct induction of innate antimicrobial mechanisms and by altering lung development in early life.

Vitamin D deficiency and asthma are common conditions that share risk factors such as urban residence, black ethnicity and obesity. Epidemiologic data suggest that low serum vitamin D in children with asthma is associated with more symptoms, exacerbations, reduced lung function, increased medication usage and severe disease. In vitro studies have demonstrated that vitamin D enhances steroid responsiveness in adult asthmatics.

An increasing amount of data has now shed light on the role of vitamin D in asthma. However, there are several areas about which little is known. First, the prevalence of vitamin D deficiency and insufficiency in children with STRA and the relationship between vitamin D levels and markers of asthma severity, including clinical and therapeutic disease variables has not been explored. Secondly, we know little about the relationship between vitamin D levels and airway inflammation and remodelling in children and adult with STRA, nor about the relationship between steroid resistance and vitamin D in these children. The aims of this thesis are to try and address some of these gaps in our understanding.
1.6 Research Hypotheses, Aims and Objectives

1.6.1 Research hypotheses

i. Children with STRA have lower serum vitamin D levels than MA and non-asthmatic controls, and these are associated with worse clinical parameters of asthma control including lung function and symptoms, and worse pathological markers of asthma severity and airway remodelling.

ii. In vitro, steroid responsiveness of PBMCs from children with STRA can be improved by addition of Vitamin D

1.6.2 Aims

i. To compare serum vitamin D levels in children with STRA, MA and age-matched non-asthmatic controls, and to investigate the relationship between serum vitamin D levels and markers of asthma severity.

ii. To investigate the relationship between vitamin D levels and airway inflammation and remodelling in BAL and EB from children with STRA.

iii. To assess the differences in glucocorticoid response between STRA, MA and control PBMC and BAL cells in cell culture and to ascertain whether vitamin D modulates the effects of glucocorticoids; and to determine whether addition of vitamin D restores steroid sensitivity in children with STRA.

1.6.3 Specific objectives

i. To recruit children with STRA, MA and non-asthmatic controls attending the Royal Brompton Hospital, and assess their asthma control (measured by the ACT), exacerbations, lung function, medication usage, and serum vitamin D levels.

ii. To obtain BAL and EB from children with STRA undergoing clinically indicated bronchoscopy as part of investigations to assess steroid responsiveness and to use BAL and EB to study cellular content and markers of airway inflammation (BAL cell counts, cytokine measurements, mucosal eosinophils and neutrophils) and remodelling
(RBM thickness and ASM mass) and correlate with concurrent serum measurements of vitamin D status.

iii. To use BAL whole cell and PBMC cultures to evaluate the effects of vitamin D and dexamethasone alone and in combination on IL-10 production and to correlate the responses with evidence of steroid responsiveness in STRA.

The following chapter describes the clinical protocols and laboratory methodological techniques that are common to several sections of this thesis. Laboratory techniques unique to each study are described in the relevant results chapter. Details of the some clinical methods used, including subject selection and recruitment are also described in the individual results chapters.
Chapter 2 Methods

The main aims of this thesis were to investigate the relationship between serum vitamin D levels and markers of asthma severity, airway inflammation and remodelling in children with STRA, as well as to assess whether in vitro addition of vitamin D to PBMC in cell cultures restores steroid sensitivity in these children. This chapter reports those methods which are common to all of the results chapters in this thesis (clinical assessment, non-invasive measurement of inflammation, bronchoscopy). Detailed immunological methods which were used to generate the results are reported in the subsequent chapters with the corresponding results, for ease of reference.

2.1 Study plan and subjects

Three subject groups, aged 5-16 years were studied:

**Non-asthmatic controls** comprised either children with no respiratory disease whose parents had consented for a small (<1 ml/kg, maximum 20 ml) blood sample to be taken during an elective cardiac surgical procedure or children undergoing a clinically indicated bronchoscopy for upper airway symptoms. The actual indication for flexible bronchoscopy in each child is presented in Table 5.1, chapter 5

**MA** - mild/moderate asthmatics well controlled on low dose (<800mcg/day beclomethasone equivalent) inhaled corticosteroids and were free of exacerbation for 3 months.

**STRA** - were defined as previously described in chapter 1 section 1.2 and flow diagram summarizing stage 1-3 in Figure 1.4. Briefly, persistent (≥ 3 months) symptoms (requiring rescue bronchodilator ≥ 3 days per week) despite treatment with high dose inhaled corticosteroids (> 800 microgram/day of beclomethasone equivalent) and trials of add on drugs (long acting β2 agonists, leukotriene receptor antagonists and oral theophylline in a low, anti-inflammatory dose) and / or recurrent severe asthma exacerbations and / or persistent airflow obstruction (post oral steroid, post-inhaled
bronchodilator Z score < -1.96 for FEV$_1$ despite above therapy); all children had poor control despite high-dose therapy, and had been through a detailed assessment to exclude wrong diagnosis, important co-morbidities, and to optimize adherence and other aspects of basic management, as far as possible.$^{134}$

Subjects were excluded from the study if they were taking vitamin D supplements.

2.2 Clinical methods
Since not all children participated in every study, the demographic details of the groups studied are described in their appropriate results chapters.

2.2.1 Study consent
The study was approved by the Royal Brompton and Harefield Research Ethics Committee (09/H07008/48). Informed consent was obtained from parents and age-appropriate assent from children.

2.2.2 Height and weight
The child's height without shoes was measured in centimeters to one decimal place using a calibrated wall-mounted Harpenden stadiometer (Chasmor, London, UK). Weight was measured in kilograms to one decimal place with calibrated electronic scales (Marsden, Taiwan) with the child wearing minimal clothing.

2.2.3 Exacerbations and medication usage
Regular medications and doses were recorded for the asthmatics. Acute exacerbations were defined as episodes necessitating physician or parent initiated high dose oral steroids for at least 3 days, and the number of exacerbations in the previous six months was recorded.

2.2.4 Pulmonary function testing
Spirometry was performed using Vitalograph Compact (Vitalograph Buckingham, UK). The spirometer was calibrated on the day of the study with
a one litre syringe. If it was the subject's first attempt at spirometry (healthy controls) a period of training was given. The child was familiarised with the equipment which was then demonstrated. The child was observed closely to ensure the manoeuvre was performed optimally. Manoeuvres were rejected if the subject was thought to use sub-optimal effort, if a full inspiratory breath was not taken, if there was coughing during the first second, if a plateau was not reached, or if the blow was terminated prior to full expiration. If the child had difficulty performing the manoeuvre then interactive computerised incentive spirometry was used (Vitalograph Pneumotrac, Spirotrac IV software). As this machine was situated in another room and was often in use for busy outpatient clinics it was not possible to use it routinely. The highest sum of FVC and FEV\textsubscript{1} of the 3 manoeuvres was recorded to define the best flow volume loop, as per ATS/ERS guidelines. All spirometry results were compared to appropriate recent reference populations. The procedure was repeated in order to achieve 3 satisfactory measurements. Spirometry was required to meet ATS criteria for acceptability and reproducibility.

2.2.5 BDR

Spirometry was performed at baseline and 15 minutes after the administration of 1mg (10 puffs) of salbutamol via a large volume spacer in subgroup of children with STRA undergoing stage 2 & 3 assessment (Figure 1.4). BDR was defined as change in percentage predicted FEV\textsubscript{1} from baseline:

\[
100 \times \frac{\text{FEV}_{1\text{post bronchodilator}} - \text{FEV}_{1\text{pre bronchodilator}}}{\text{FEV}_{1\text{pre bronchodilator}}}
\]

A positive BDR was defined as FEV\textsubscript{1} improvement of >12% of baseline. This formula is used in our institution on children on a daily clinical basis, as do other lung function laboratories over the country. A relatively high dose of salbutamol was used to ensure as far as possible the top of the dose response curve had been reached, and also to protect against hypertonic saline induced bronchoconstriction during subsequent sputum induction.
2.2.6 Symptom Questionnaire

All parents, and when age-appropriate, the children with asthma also completed the childhood ACT questionnaire. The ACT is a 5 point questionnaire, marked out of a total of 25. Low score is reflective of suboptimal asthma control and a score of 20 and above was taken to represent adequate control. It is a simple tool to assess control and is used to document asthma control over a 4 week period. This scoring system has been validated and correlates well with specialists’ evaluation of asthma control.

2.2.7 FeNO50

FeNO50 was measured prior to lung function using a chemiluminescence analyser (NIOX Aerocrine AB, Solna, Sweden). A constant temperature of 22°C was maintained in the room in which the analyser was stored to ensure optimal operating conditions. The ambient nitric oxide (NO) was recorded prior to each reading and was always <300ppb in accordance with the manufacturer’s recommendations. FeNO50 was always measured before forced expiratory manoeuvres such as spirometry as they may affect FeNO50 levels.

The procedure was explained and demonstrated to the child and all measurements were made with the child sitting with the disposable mouthpiece held in their hand in front of them. A nose clip was not used. Children had several practise runs to optimise their technique before proceeding to actual measurement. For the measurement, NO-free air (generated by the NIOX module internally from ambient air) was inhaled to near total lung capacity over a period of 2 to 3 seconds through the mouth-piece of the instrument. Then the child was asked to exhale slowly, being encouraged to maintain a constant flow by a visual display on a screen (blowing a little girl in a balloon between 2 cliffs). There was automatic control of exhaled flow rate to a constant 50 ml/s, making it easier for the child to comply. The exhaled NO value is the mean NO level during a 3 second NO plateau. Three acceptable readings (defined as agreement to within 10%) were performed with at least 30-second intervals between manoeuvres and
FeNO\textsubscript{50} was calculated as the mean of three correctly performed exhalations. Normal FeNO\textsubscript{50} is less than 24 parts per billion.\textsuperscript{146}

2.3 Laboratory Methods

2.3.1 Serum 25-hydroxyvitamin D
Serum 25-hydroxyvitamin D level was measured in all subjects using a 2 dimensional high performance liquid chromatography system - tandem mass spectrometry (2D LC-MS-MS), by the biochemistry department at the RBH. Serum 25-hydroxyvitamin D levels are considered the best circulating biomarker of vitamin D metabolic status because this form has a longer half-life (2-3 weeks) than 1\alpha,25VitD3 (6-12 hours) and reflect contributions from all sources of vitamin D (i.e. diet and sun exposure).\textsuperscript{351} Serum 25-hydroxyvitamin D levels were measured in duplicate and the lowest limit of detection was 3 nmol/L and upper limit of detection was 100nmol/L.

Vitamin D deficiency was defined as serum 25-hydroxyvitamin D <50nmol/L (20ng per milliliter)\textsuperscript{185,352} and serum levels between 50-75 nmol/L (20-30 ng per millilitre) were defined as vitamin D insufficiency.\textsuperscript{185,352} Based on changes in parathyroid hormone levels and intestinal calcium transport values of less than 75nmol/L have been suggested as insufficient.\textsuperscript{185} The date the 25-hydroxyvitamin D level was measured was recorded to assess seasonal variation between subjects. Subjects were grouped as follows; winter (December to February), spring (March to May), summer (June to August) or autumn (September to November).

2.3.2 Serum total and allergen specific IgE
Serum IgE was analyzed by Beckman Access 2 immunoassay analyser and allergen-specific IgE levels (normal <0.35 IU/ml) to cat, dog, tree pollen, \textit{Dermatophagoides pteronyssinus}, \textit{Aspergillus fumigatus}, grass, egg, milk and peanut were analyzed by Phadia Immunocap 250 analyser in the biochemistry department at the RBH. Recently it has been postulated that it is
useful to quantify atopy rather than just reporting it as present or absent. This was done by summing the results of specific IgE for food and aeroallergen.³⁵³

### 2.3.3 Sputum induction

For subjects with a post-bronchodilator FEV₁ > 65% predicted, sputum induction was performed using 3.5% saline inhalation for four 5 min periods as part of clinical investigations. For subjects with a post-bronchodilator FEV₁ < 65% predicted, sputum induction was performed with 0.9% saline. I performed sputum induction and processing in 10 patients and in the remaining 12 patients was performed by Ms Adesimbo Sogbesan, sputum technician at RBH.

Children were asked to blow their nose and rinse their mouth to reduce squamous cell contamination before sputum induction was started. The children were instructed how to cough and clear their throat in order to obtain the best sample. The recorded baseline FEV₁ was the highest manoeuvre from 3 satisfactory spirometric curves. Children were seated comfortably and asked to inhale the saline solution for periods of increasing duration from an ultrasonic nebuliser (De Vilbliss 2000, Somerset). FEV₁ was measured after each inhalation period and the higher of 2 readings was selected. After each 5 minute nebulisation period children were encouraged to cough and expectorate sputum into a falcon tube. If the FEV₁ had not fallen below 10-15% of the baseline value, further aliquots of 3.5% saline were nebulised. Symptoms such as cough or wheeze were recorded at each stage. If an inadequate sample of sputum was produced the process was continued for up to a maximum of 15 minutes.

Children whose FEV₁ had decreased by more than 15% were given salbutamol (1000µg) through a large volume spacer and spirometry was repeated 10 minutes later to ensure return to baseline values before discharge.

*Sputum processing*
Sputum samples were stored on ice until ready for processing. All samples were processed in less than 4 hours. The sample was poured into a petri dish and sputum was selected by identifying mucoid plugs and gelatinous portions. A selected rather than whole sample processing method was used to reduce salivary contamination because this improves intra-observer repeatability of differential cell counts.119 The selected sputum was returned to a clean pre-weighed tube and weighed using a microbalance. A stock dithiothreitol (DTT) (0.1%) was then added to the sputum in the ratio of 4 ml DTT to 1g sputum. The mixture was gently aspirated with a 3ml disposable wide bore plastic pipette and then gently agitated on a rolling mixer (DenleySpiramix 5, Denley Instruments, Colchester, UK) for 15 minutes. The sample was filtered using a 48µm pore nylon mesh filter. Phosphate buffered saline (PBS) (in the ratio of 4 ml PBS to 1g original sputum weight) was used to wash out the tube and added to the filtrate (through the filter). The filtrate was centrifuged at 4°C for 10 minutes at 400g (1500rpm). The remaining pellet was re-suspended in 1ml PBS and mixed by gentle vortexing. 20µl of sputum cell suspension was added to 20µl trypan blue (1 in 2 dilution factor). 10µl of the mixture was placed on a haemocytometer and examined using a light microscope (Leitz, Wetzlar, Germany) (improved Neubauer, BDH, Leicestershire, UK). All leukocytes (viable stained yellow and dead stained blue) and squamous cells were counted in the bottom left middle and top right quadrants of the haemocytometer grid. The total leukocyte count per ml was calculated using the following formula:

Total count = no of viable & dead leukocytes X trypan blue dilution factor X10^4

After calculation of the total leukocyte count the sputum cell suspension was adjusted to 200,000 cells/ml by diluting with PBS. The mixture was centrifuged (Shandon Cytospin Preparation System, Thermo Shandon, Cheshire, UK) at 450rpm for 3 min. If the total leukocyte count was less than 2 x 10^5 /ml aliquots of 200µl or 400µl of sputum cell suspension were loaded. 1-4 slides were left to air dry for 30 minutes and then fixed with methanol. The slides were then stained with a modified Wright Giemsa stain Reastain®
Quick-Diff staining kit (Reagena Ltd. Toivala, Finland). Characteristic images of cells are shown in Figure 2.1
Figure 2.1 Characteristic appearances of sputum cytology shown under light microscopy (magnification x400) (a) normal count showing macrophages (b) eosinophilic (c) neutrophilic.

Photograph taken by Dr Cara Bossley.

**Cell counts**

The 400 cells were counted and expressed as % total inflammatory cells and total cell counts. Eosinophilia was defined as ≥2.5% eosinophils; neutrophilia as ≥54% neutrophils.³⁵⁴

The intra-observer sputum eosinophil variability, percentage of eosinophils counted in the same slides on 3 separate occasions from 3 different subjects (blinded to previous results) is shown in Table 2.1

**Table 2.1. Intra-observer sputum eosinophils repeatability.** This show acceptable coefficient of variation (CoV) of less than 10%

<table>
<thead>
<tr>
<th>Slide</th>
<th>Count 1</th>
<th>Count 2</th>
<th>Count 3</th>
<th>Average</th>
<th>CoV</th>
</tr>
</thead>
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<tr>
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<td>4</td>
<td>3.7</td>
<td>4.3</td>
<td>4</td>
<td>7.5</td>
</tr>
<tr>
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<td>9.8</td>
<td>10.4</td>
<td>10.4</td>
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</tr>
<tr>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2.2</td>
<td>2.1</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Mean CoV 6.2

CoV-Coefficient of variation
2.4 Fibreoptic bronchoscopy

This was performed under general anaesthetic (GA) administered by a consultant anaesthetist, and the procedure was undertaken by a paediatric respiratory consultant as part of clinical investigations. The anaesthetic technique was at the discretion of the individual anaesthetist, and usually a laryngeal mask was preferred. Intravenous access was obtained and a blood sample was taken in all subjects. Standard monitoring included a continuous electrocardiogram, oxygen saturation, end tidal carbon dioxide and systemic arterial blood pressure.

An Olympus BF-XP40 BF-MP60 (4.0 mm videobronchoscope) or BF-P20D (4.9 mm) bronchoscopes (KeyMed, Southend-on-Sea, Essex, UK) were used as appropriate to the size of the child. BAL was performed using 3 aliquots of 1ml/kg 0.9% sterile saline (to a maximum of 40mls per aliquot) instilled into the right middle lobe and the returns pooled. BAL fluid was sent to the Royal Brompton Hospital pathology laboratory for cytology, bacterial culture and viral immunofluorescence. The larger single use 2mm disposable cupped forceps (FB-231D, Olympus, KeyMed, UK) were used to take up to 4 EB under direct vision from the subsegmental bronchi of the right lower lobe.

2.4.1 BAL cytology

Quantification of inflammatory cells in BAL was performed by the histopathology department at the RBH. BAL was centrifuged at 2000 rpm (400g), 4°C for 10 minutes. The cell pellets were re-suspended in 1ml PBS and mixed for 15 seconds using vortex. Cell viability was then assessed using the trypan blue exclusion method. 50µl of cell suspension was added to 50 µl of trypan blue. 20 µl of this suspension was then put onto an improved Neubauer Haemocytometer. Number of live cells (white), dead cells (blue) and squamous cells were noted. A total cell count could then be calculated by multiplying the average number of live and dead cells by the dilution used. A suspension of approximately 100,000 cells per ml was made up using PBS. Eosinophilia was defined as eosinophil percentage > 1.19. Neutrophilia was defined as neutrophil percentage >3.5.
2.4.2 Processing of endobronchial biopsy

Biopsies were fixed in formal saline, and processed to paraffin within 24 hours by the histopathology department at the RBH. The 5µm sections were stained with haematoxylin and eosin (H&E) to determine biopsy quality. Sections were examined under a light microscope (Leica Microsystems, Milton Keynes, UK). To be categorized as “evaluable”, a biopsy had to fulfill the following criteria: (i) presence of identifiable epithelium, RBM with associated submucosa; (Figure 2.2) (ii) good orientation; (iii) minimal crush, edema or blood within the biopsy. Biopsies were evaluated by Mr T Oates. Assessments of tissue remodelling were performed on H&E stained sections by Mr T Oates and Ms A Sjoukes using computer aided image analysis and equations from design-based stereology, as described previously.

Morphometry of airway remodelling

Evaluable H&E stained sections were used to quantify airway remodelling, including RBM thickness, epithelial shedding and smooth muscle mass.

Figure 2.2 An example of endobronchial biopsy stained with H & E; showing epithelium, reticular basement membrane and sub-mucosa.

Photograph taken by Dr Cara Bossley.
RBM thickness

RBM was measured in sections stained with H&E using computer aided image analysis at x 400 magnification by Ms. A Sjoukes. Forty point-to-point repeated measurements were taken of RBM thickness at right angles to the basement membrane, at regular intervals of 20 micrometer (µm) in randomly selected sections. Results are the mean of the 40 measurements per patient in µm.

Epithelial shedding

The length of incomplete epithelium was measured as a percentage (%) of the total epithelial length (assessed by the length of basement membrane) at x 200 magnification by Ms A Sjoukes. The epithelium was considered incomplete when the basement membrane was completely denuded or when it was only covered by a single layer of basal cells with no intact ciliated cells or goblet cells. A minimum length of 1mm epithelium was assessed.

Smooth muscle volume fraction

Smooth muscle quantification was performed by Ms Sjoukes using equations from design-based stereology. The volume fraction of smooth muscle (sm) was measured using a weibel grid at x 200 magnification. Stereological data were calculated as follows:

\[ Vv \text{ (sm/submucosa)} = \frac{\sum \text{points on sm}}{\sum \text{points on sm} + \text{points on submucosa}} \]

Proliferating Cell Nuclear Antigen (PCNA) – for smooth muscle proliferation

PCNA is a sensitive marker of smooth muscle proliferation. It has been demonstrated to detect ASM hyperplasia, and an associated change in muscle mass. PCNA staining was performed in paraffin sections by T Oates and A Sjoukes as previously described. Positively stained smooth muscle nuclei were counted in every biopsy at x 400 magnification and divided by the total number of smooth muscle nuclei and expressed as a percentage (%).
Quantification of tissue inflammation
Mucosal inflammation was quantified in sections stained with congo red (eosinophils), and immunohistochemistry was used to assess neutrophils (neutrophil elastase)\(^{363}\) and mast cells (mast cell tryptase).\(^{364}\) All cells with positive nuclear staining were counted in the submucosa in every biopsy at x 400 magnification. Mast cells were also counted within the smooth muscle in every biopsy by A Sjoukes. The data is presented in \(\text{mm}^2\) per area of submucosa or smooth muscle (for mast cells alone).

2.5 Cytokine Analysis by Cytometric Bead Array (CBA)
Cytokines were analysed using the CBA kit (CBA, Human Inflammation kit, BD Biosciences, Pharmingen, San Diego). This is a commercial, flow cytometer based immunoassay that allow for simultaneous multiple analyte measurements to be made from small serum, BAL and tissue culture supernatant samples. It uses a matched antibody pair that permits analysis of multiple cytokines simultaneously within a sample (Figure 2.3). The lower limit of detection was 1.5pg/ml for all cytokines. CBA was performed as per the manufacturer’s instructions and by Mr David Richards in BAL. I also performed CBA in serum samples under the close supervision of David Richards. For beads to be identified and analysed separately each analyte bead is labelled with a unique combination of the fluorochromes allophycocyanin (APC) and allophycocyanin-cyano dye 7 (APC-Cy7). Multiple analyte measurement is possible because the beads are tandem conjugated with 2 fluorescent dyes. By varying the labelling concentration of these dyes, it is possible to distinguish between the analyte beads based on the their fluorescence when measured in the APC and APC-Cy7 channels. The sample/standard are incubated with antibody coated beads, which capture secreted cytokines. The detection antibodies fluoresce within the phycoerythrin (PE) emission spectrum at a frequency determined by the quantity of detection antibody bound to captured cytokine. A 16-point standard curve containing all relevant cytokines was prepared in CBA sample buffer (BD Biosciences) from the top standard, which was 50000 pg/ml for all cytokines.
Test steps
Samples (50µl) were added to a 96-well plate and incubated with 50µl of capture beads 10% Bovine Serum Albumin (BSA, Fischer Scientific), 0.5% TWEEN-20 (Sigma) and 2mM EDTA (Sigma)) with gentle shaking (500rpm) for the first 15 minutes and then incubated for 3 hours at room temperature in the dark. After 3 hours the plate was then centrifuged at 500g for 5 minutes at 4°C and then beads were resuspended with 200µl of CBA buffer. The plate was again centrifuged for 5 minutes at 500g / 4°C. The beads were then incubated with 50µl antibody-detection reagent with gentle shaking (500rpm) for the first 15 minutes and then incubated for a further 2 hours at room temperature in the dark. Finally, beads were washed twice and resuspended in 150µl FACSflow for analysis using a BD Fortessa flow cytometer with high-throughput sampler plate-reader platform. Data were read off as mean fluorescence intensity (MFI) values, which were analysed using FlowJo (version 9.2, TreeStar Inc) and GraphPad Prism (version 5 for Mac OS X, GraphPad Software Inc.).

2.6 FoxP3 intranuclear analysis by flow cytometry
FoxP3 staining was performed with a PE conjugated FoxP3 antibody and the FoxP3 staining buffer set, (Ebiosciences, San Diego, USA) according to manufacturer’s instructions. BAL cells were first stained for surface expression of CD4 and CD25 or the matched isotype control antibody. BAL
cell pellets were resuspended in 1ml FACS Flow containing 1% Mouse serum. A 50µl of cell suspension was taken to count cells on a haemocytometer and cell concentration was adjusted to 1x10^6/ml and specific antibodies were added at 20µg/ml (2microlitre per 10^6 cells) according to manufactures instructions and were incubated in the dark on ice for 30 minutes. Samples were then washed twice at 200g for 5 minutes at 4°C in 250 microlitres of FACSflow. The cells were resuspended in 250 microlitres FACSflow and 10,000 live cells were analysed for fluorescence on a FACSCalibur, following compensation, using the CellQuest software. Dead cells were excluded by forward and side scatter gating. Quadrant markers were based upon background staining of matched isotype control antibodies. For the FoxP3 staining, BAL cells were washed twice in ice-cold FACS flow (200g at 4°C for 5 minutes). The cell pellet was resuspended by pulse vortex, 1 ml of FoxP3 fixative (freshly diluted, 1:4) was added and incubated in the dark at room temperature for 45 mins. Cells were washed twice in 1 ml of FoxP3 permeabilisation buffer (diluted 1:10 times) and centrifuged at 200g for 5 mins. Fluid was decanted and 5µl mouse serum was added to each sample and then incubated at room temperature for 10 minutes. Five µl of PE rat IgG2a control antibody was then added to the appropriate tube and 5µl PE conjugated anti-human FoxP3 antibody to another tube. After mixing the contents of each tube by vortex, they were incubated in the dark at room temperature for 45 minutes. Cells were then washed with 1 ml of FoxP3 permeabilisation buffer (diluted 1:10 times), and centrifuged at 200g for 5 mins. Liquid was decanted and cell pellets were washed again with FACSflow and centrifuged at 200g for 5 mins. Liquid was again decanted and 500µl of FACSflow was added to the cells. Resuspended cells were analyzed by flow cytometry using FACScan (Becton Dickinson, Abingdon, GB). Forward scatter was increased for the FoxP3 analysis to 2.5 to compensate for cell shrinkage (as a result of the staining process). Quadrant markers were set according to the matched isotype control antibody staining (Figure 2.4). The gating and instrument settings were controlled in every experiment and maintained throughout the whole study in all samples analyzed. Calculations were performed with Cell Quest software (Becton Dickinson).
Figure 2.4. An example of gating for FoxP3 intracellular staining

Flow cytometry density plots to demonstrate the gating strategy employed to analyse the frequency of FoxP3 in BAL cells. BAL lymphocytes were identified according to forward (FSC) and side scatter (SSC). Cells were next gated on the basis of positive expression of CD3 and CD4. Quadrant markers were set according to the isotype matched isotope control (rat IgG2a).

2.7 Statistical Analyses

For the clinical relationship (discussed in chapter 3) again there are insufficient published data with which to inform a power calculation, however a retrospective power calculation was performed (the limitations of this approach are acknowledged). The mean vitamin D levels for the controls, MA and STRA were 59.9, 46.5 and 29.8 respectively and the within group mean square was 304.66 giving a pooled standard deviation of approximately 17.5. Based on these means and SD, the power to achieve the effect size reported in this study is greater than 90%. The clinical study was therefore adequately powered for the data relating to serum vitamin D and clinical status. The laboratory studies were novel studies therefore the previous work was not relevant to power calculations. Also, laboratory studies were opportunistic and limited by the sample volumes.
Categorical data were analyzed using the Chi squared or Fishers exact tests. Data were assessed for normality using the D’Agostino and Pearson test. For nominal data, between group differences for normally distributed data were analyzed using the student’s t test. The Mann-Whitney U test was used for skewed (non-normally distributed) variables. Differences between 3 groups were assessed using one way analysis of variance (normal data) or Kruskal-Wallis test (non-normal distribution) followed by a Bonferroni correction to compare differences between groups. Correlations were assessed using the Pearson correlation (normal data) or the Spearman's rank correlation (skewed data). To assess the association between serum vitamin D levels and severity of disease, linear regression was used for continuous variables and logistic regression was used for categorical variables. When a non-normal variable remained skewed even after log transformation, this variable was dichotomized and used in the logistic regression. For both types of regression analyses, 2 models (an unadjusted and a multivariable model were constructed). To assess the effect of two factors simultaneously repeated measures two way analysis of variance (ANOVA) test was used. Statistical significance was reported at \( p<0.05 \).

Linear regression, logistic regression and power calculations were performed by Mr. Winston Banya, Hospital statistician using Stata version 10.1 (Statacorp Texas, USA). AG performed the rest of the statistical analysis using GraphPad Prism 5.02 under the guidance of Mr. Winston Banya.

The next chapter compares serum vitamin D levels in children with STRA, MA and non-asthmatic controls and describes the relationship between serum vitamin D levels and clinical markers of asthma severity, airway inflammation and remodelling.
Chapter 3. Relationship between serum vitamin D, disease severity, airway inflammation and remodelling in children with asthma

3.1 Introduction

In the previous chapter, methods common to all chapters of this thesis were described. The overarching aim of this thesis is to investigate the relationship between vitamin D and childhood STRA. This chapter compares serum vitamin D levels in children with STRA, MA and non-asthmatic controls and describes the relationship between serum vitamin D levels and clinical markers of asthma severity, airway inflammation and remodelling. Some of these finding have been reported in the American Journal of Respiratory and Critical Care Medicine.\textsuperscript{366}

Little is known about vitamin D levels and their impact on disease control and airway pathology in children with STRA. As discussed in chapter 1 section 1.1.5 an increase in ASM mass is a key feature of airway remodelling in asthma.\textsuperscript{116, 367-369} Importantly, increased ASM hypertrophy and hyperplasia has been demonstrated in EB from children with severe asthma and is significantly related to bronchodilator responsiveness.\textsuperscript{116} However, to date, there is little evidence that any asthma therapies affect airway remodelling. \textit{In vitro} studies have shown that vitamin D may influence ASM remodelling by exerting an inhibitory effect on ASM growth and contractility.\textsuperscript{280, 281, 311} The relationship between airway pathology in asthmatics and serum vitamin D levels has not been reported.

3.1.1 Hypothesis

The hypothesis tested in this chapter was that the children with STRA have lower serum vitamin D levels than MA and non-asthmatic controls, and these are associated with worse clinical parameters of asthma control including lung function and symptoms, and worse pathological markers of asthma severity including airway remodelling.
3.1.2 Aims

i. To compare serum vitamin D levels in children with STRA, MA and age-matched non-asthmatic controls, and to investigate the relationship between serum vitamin D levels and markers of asthma severity.

ii. To investigate the relationship between serum vitamin D levels and airway inflammation and remodelling in BAL and EB from children with STRA.

3.2 Methods

3.2.1 Subjects
Children aged 5-16 years with STRA (n=36), MA (n=26) and non-asthmatic controls (n=24) were recruited prospectively (Table 3.1). A sub-group of STRA (n=22/36) underwent stage 2 and 3 investigations according to the severe asthma protocol at RBH (as described in chapter 1 Figure 1.4). This includes performance of fibreoptic bronchoscopy under general anaesthesia, with BAL and EB. The other 14/36 children with STRA had previously undergone flexible bronchoscopy and stage 2 and 3 investigations. MA had doctor diagnosed asthma being managed at the RBH treated at steps 2-3 of the British Thoracic Society guidelines. Non-asthmatic controls comprised either children with no respiratory disease whose parents had consented for blood tests during an elective surgical procedure (n=18) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (n=6).

3.2.2 Sputum eosinophils and neutrophils
Sputum induction, processing and cell counts were performed in children with STRA (n=22) as described in chapter 2 section 2.3.3. Sputum induction was not undertaken in MA and controls, as it was not clinically indicated.

3.2.3 Bronchoscopy, BAL and EB
All bronchoscopies, BAL & EB were performed under general anaesthesia in children with STRA (n=22) as part of their clinical investigations as described in chapter 2. The histopathology department at the RBH performed
quantification of inflammatory cells in the BAL for clinical purpose. BAL fluid was centrifuged at 300 g at 4°C for 10 minutes. The supernatant was removed and stored at – 80°C and the cell pellets were resuspended in RPMI-1640 medium (Sigma) with 10% fetal calf serum. Slide preparations for differential percentage counting of cells were made in a Shandon cytocentrifuge (Cytospin II; Shandon Ltd, Runcorn, Cheshire, UK) using aliquots of the lavage cell suspension, adjusted to 0.5 x 10⁶ cells/ml. Preparations were stained with May-Grunewald Giemsa. Differential counts were made from a minimum of 400 cells. EB were fixed in formal saline and processed to paraffin blocks within 24 hours by the histopathology department at the RBH as previously described.³⁵⁵ 5µm thick sections were cut at 25-50µm intervals. Sections were stained with H&E and were assessed for morphology and quality. Adequate sections were used to assess airway remodelling (RB³⁶¹, epithelial shedding⁹ and smooth muscle mass¹¹⁶). Mucosal inflammation was quantified using specific stains; congo red for eosinophils, neutrophil elastase for neutrophils³⁶³ and mast cell tryptase for mast cells.³⁶⁴ All biopsies were cut and stained by Mr. Timothy Oates, and analyzed by Ms. Alies Sjoukes, Imperial College London.

3.3 Results
3.3.1 Subject demographics
The demographic data of the children in whom serum vitamin D levels were measured are presented in Table 3.1. There were no significant differences between children with MA, STRA, and non-asthmatic controls in age, ethnicity, gender distribution or BMI. As expected, children with STRA were on higher doses of ICS, had poor asthma control, worse lung function and had more frequent exacerbations.
Table 3.1. Demographic characteristics of subjects

No significant differences between children with MA, STRA, and non-asthmatic controls in age, ethnicity, gender distribution or BMI. As expected, STRA had worse lung function and more symptoms and exacerbations, and were more bronchodilator responsive, despite being on more treatment. The normal controls were less likely to be atopic and had a lower IgE than the asthmatics.

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=36)</th>
<th>MA (n=26)</th>
<th>Controls (n=24)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11.5 (9.5, 14)</td>
<td>12.5 (11,13)</td>
<td>10.5 (9-13)</td>
<td>0.24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male (%)</td>
<td>21 (58%)</td>
<td>11 (42%)</td>
<td>15 (62%)</td>
<td>0.17&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>BMI (Kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>19.5 (16, 24)</td>
<td>19 (16, 25)</td>
<td>18 (15.7, 23)</td>
<td>0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White (%)</td>
<td>23 (64%)</td>
<td>22 (84%)</td>
<td>21 (88%)</td>
<td>0.056&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Non-white (%)</td>
<td>13 (36%)</td>
<td>4 (16%)</td>
<td>3 (12%)</td>
<td></td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; (%predicted)</td>
<td>76 (63, 85)</td>
<td>88 (84, 95)</td>
<td>94 (90, 97)</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVC (%predicted)</td>
<td>88 (78, 97)</td>
<td>103 (96,110)</td>
<td>96 (94,108)</td>
<td>&lt;0.002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; / FVC ratio</td>
<td>73 (68, 84)</td>
<td>84 (78, 92)</td>
<td>92 (89, 96)</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Atopic&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>32 (88)</td>
<td>20 (77)</td>
<td>3 (12)</td>
<td>&lt;0.001&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>530 (196, 645)</td>
<td>717 (254, 1377)</td>
<td>12.5 (8, 51)</td>
<td>&lt;0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BDR&lt;sup&gt;+&lt;/sup&gt; (%)</td>
<td>15 (7, 25)</td>
<td>4 (3.5, 6)</td>
<td>-</td>
<td>0.065&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACT^</td>
<td>11 (8.5, 14)</td>
<td>18 (17, 21)</td>
<td>-</td>
<td>&lt;0.001&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>ICS dose% (microgram/day)</td>
<td>1600 (1000-2000)</td>
<td>600 (500-800)</td>
<td>-</td>
<td>&lt;0.001&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
<tr>
<td>Median exacerbations **</td>
<td>3 (2,4)</td>
<td>1 (0,2)</td>
<td>-</td>
<td>&lt;0.001&lt;sup&gt;x&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Definition of abbreviations: BMI = body mass index; FEV<sub>1</sub> = forced expiratory volume in 1 second; FVC = forced vital capacity; BDR= Bronchodilator response; ACT= asthma control test;

<sup>o</sup> Values are given in Median (interquartile range) for continuous variables or as number (%) for binary variables.
<sup>+</sup>One or more positive allergen-specific IgE responses
<sup>**Median exacerbations in last six months requiring oral steroids</sup>
3.3.2 Serum 25[OH]D₃ levels in asthmatics and controls

Serum 25[OH]D₃ levels are considered the best circulating biomarker of vitamin D metabolic status because this form has a longer half-life (2-3 weeks) than 1α,25VitD₃ (6-12 hours) and reflect contributions from all sources of vitamin D (i.e. diet and sun exposure).³⁵¹ As described in chapter 2 section 2.3.1 serum 25[OH]D₃ level was measured in all subjects using a 2 dimensional high performance liquid chromatography system - tandem mass spectrometry, at the RBH. Serum 25[OH]D₃ levels (median [IQR]nmol/L) were significantly lower in children with STRA (28[22-38])nmol/l than those with MA (42.5[29-63])nmol/L and non-asthmatic controls (56.5[45-67])nmol/L (p<0.001 for both) (Figure 3.1A). The prevalence of vitamin D deficiency (25[OH]D₃ level < 50nmol/L) was 94%, 54% & 33% in STRA, MA and controls respectively (p<0.001) (Figure 3.1B) and 97% of children with STRA, 92% of MA and 83% of controls had insufficient serum 25[OH]D₃ levels (25[OH]D₃ level <75nmol/L).

Vitamin D metabolism is affected by season (factors affecting sun exposure). The date the 25[OH]D₃ level was measured was recorded to assess seasonal variation between subjects. Subjects were grouped as follows; winter (December to February), spring (March to May), summer (June to August) or autumn (September to November). There was no significant impact of season of sample collection on serum vitamin D level in the three groups (Figure 3.2).
Figure 3.1 Serum vitamin D levels in STRA, MA & controls.

Figure 3.1A Serum 25-hydroxyvitamin D levels were lower in STRA and MA as compared to non-asthmatic controls (Kruskal-Wallis test $p<0.0001$). Bar represents median values. Mann Whitney U test, followed by a Bonferroni correction was used to compare differences between groups. Figure 3.1B shows a higher prevalence of vitamin D deficiency (serum 25-hydroxyvitamin D levels less than 50 nmol/L) in STRA compared to MA and controls ($p<0.001$, calculated by Chi-square test)
Figure 3.2. Serum vitamin D levels and season
(a) No significant difference in season and sample collection in the three groups when combined \(p=0.13\), measured by Chi-square test. (b) No significant difference in season and serum 25(OH)D\(_3\) levels \(p=0.07\), measured by Kruskal-Wallis test.
3.3.3 Vitamin D and atopic status

A history of atopic disorders was taken at recruitment. Food allergies were determined by clinical history, formal food challenges were not performed. Blood for total IgE and specific IgE was taken at the same time as vitamin D levels. Serum 25[OH]D\textsubscript{3} was inversely related to serum total IgE (\(r=-0.3, \ p=0.01\)) (Figure 3.3), specific IgE to cat (\(r=-0.27, \ p=0.01\)), dog (\(r=-0.29, \ p=0.01\)), tree pollen (\(r=-0.28, \ p=0.02\)), *Dermatophagoides pteronyssinus* (\(r=-0.3, \ p=0.01\)), and *Aspergillus fumigatus* (\(r=-0.36, \ p=0.009\)) (Table 3.2).

Recently it has been postulated that it is useful to quantify atopy rather than just reporting it as present or absent. This is done by summing the results of specific IgE for several aeroallergens.\textsuperscript{353} In a *post hoc* analysis, there was a significant inverse relationship between serum 25[OH]D\textsubscript{3} levels and sum of specific IgE to aeroallergen (cat, dog, tree pollen, *Dermatophagoides pteronyssinus*, *Aspergillus fumigatus* and grass) (\(r=-0.28, \ p=0.009\)) but not to sum of specific IgE to food allergens (egg, milk and peanut). Blood test for serum eosinophil count was taken at the same time as serum vitamin D levels. There was no relationship between serum 25[OH]D\textsubscript{3} levels and serum eosinophil and vitamin D levels.

![Figure 3.3 Serum vitamin D levels and total IgE levels.](image)

**Figure 3.3 Serum vitamin D levels and total IgE levels.**

Significant negative association between serum vitamin D levels and total IgE levels (IU/ml) (\(r=-0.3, \ p=0.01\)) Correlation was determined by the Spearman rank correlation coefficient.
Table 3.2. Association between serum vitamin D levels and IgE levels

Serum 25(OH)D₃ was inversely related to serum total IgE ($r=0.3$), specific IgE to cat ($r=-0.27$), dog ($r=-0.29$), tree pollen ($r=-0.28$), *Dermatophagoides pteronyssinus* ($r=-0.3$), and *Aspergillus fumigatus* ($r=-0.36$). *Correlation (r) was determined with the Spearman rank correlation coefficient.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Correlation (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE (IU/ml)</td>
<td>86</td>
<td>-0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Specific IgE to cat</td>
<td>79</td>
<td>-0.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Specific IgE to dog</td>
<td>75</td>
<td>-0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>Specific IgE to tree pollen</td>
<td>67</td>
<td>-0.28</td>
<td>0.02</td>
</tr>
<tr>
<td>Specific IgE to <em>Dermatophagoides pteronyssinus</em></td>
<td>73</td>
<td>-0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Specific IgE to <em>Aspergillus fumigatus</em></td>
<td>51</td>
<td>-0.36</td>
<td>0.009</td>
</tr>
<tr>
<td>Specific IgE to grass</td>
<td>57</td>
<td>-0.14</td>
<td>0.3</td>
</tr>
<tr>
<td>Specific IgE to egg</td>
<td>37</td>
<td>-0.04</td>
<td>0.7</td>
</tr>
<tr>
<td>Specific IgE to milk</td>
<td>35</td>
<td>-0.09</td>
<td>0.58</td>
</tr>
<tr>
<td>Specific IgE to peanut</td>
<td>34</td>
<td>-0.07</td>
<td>0.7</td>
</tr>
<tr>
<td>Serum Eosinophil count %</td>
<td>84</td>
<td>-0.08</td>
<td>0.4</td>
</tr>
</tbody>
</table>

3.3.4 Spirometry, BDR and serum vitamin D

There was a positive correlation between serum 25(OH)D₃ levels and % predicted FEV₁ ($r=0.43$, $p<0.001$) (Figure 3.4A) and FVC ($r=0.32$, $p=0.002$) (Figure 3.4B) when the subjects from all the three groups were combined. However, correlation analyses within each patient group were not significant (controls- $r=0.3$, $p=0.08$; MA- $r=0.3$, $p=0.18$; STRA - $r=0.1$, $p=0.5$), probably limited by the low numbers of total participants and the lack of vitamin D sufficient STRA. Serum 25(OH)D₃ was significantly and inversely associated with % BDR ($r=-0.4$, $p=0.003$) and positive BDR (Figure 3.5A & 3.5B)
Figure 3.4 Relationship between vitamin D levels and lung function.
Positive association between serum vitamin D levels and %predicted FEV$_1$ (r=0.43, p<0.001) (A) and FVC (r=0.32, p<0.002) (B). Lower serum vitamin D levels were associated with higher bronchodilator response (BDR), (r=-0.40, p=0.003)
Correlation was determined by the Spearman rank correlation coefficient.
Figure 3.5 Relationship between vitamin D levels and BDR.

Lower serum vitamin D levels were associated with higher bronchodilator response (BDR), \((r=-0.40, p=0.003)\) (A) and positive BDR (FEV\(_1\) improvement of at least 12\%) \((p<0.001)\) (B). BDR (%) = percentage increase of FEV\(_1\) after inhalation of 1,000 \(\mu\)g of salbutamol. Correlation was determined by the Spearman rank correlation coefficient. Mann Whitney U test, was used to compare differences between groups.
3.3.5 Asthma control, exacerbations and serum vitamin D levels

A positive relationship was found between 25(OH)D$_3$ level and ACT ($r=0.6$, $p<0.001$) (Figure 3.6), whereby higher serum 25(OH)D$_3$ was associated with better asthma control as assessed by ACT in all asthmatics. Lower serum 25(OH)D$_3$ levels were associated with increased acute asthma exacerbations in the previous six months ($p<0.001$) (Figure 3.7).

The severity of asthma (defined by asthma symptoms [assessed by ACT] and frequent exacerbations) was so closely associated with serum vitamin D levels it was difficult to separate the two. For example, dividing the STRA group into those who were well controlled and those who had poor control as shown by an asthma control test of less than 20, left only one patient with well-controlled STRA so no meaningful comparisons are possible.
Figure 3.6 Relationship between serum vitamin D level and ACT

Children with higher serum vitamin D levels had less asthma related symptoms ($r=0.6, \ p<0.001$) Correlation was determined by the Spearman rank correlation coefficient.
Figure 3.7 Relationship between serum vitamin D level and exacerbations.

Children with lower serum vitamin D levels had more acute exacerbations in the last 6 months ($r=-0.6$, $p<0.001$) Correlation was determined by the Spearman rank correlation coefficient. Kruskal-Wallis test and Mann Whitney U test, followed by a Bonferroni correction was used to compare differences between groups.
3.3.6 Medication dose and serum vitamin D levels

Of the different therapies received by children with MA and STRA, the use of daily maintenance oral steroids ($p<0.001$), oral theophyllines ($p=0.02$), and leukotriene receptor antagonists (LTRA) ($p=0.005$) were significantly associated with lower 25$\text{OH}D_3$ levels, but there was no association between anti-reflux therapy and serum 25$\text{OH}D_3$ level (Table 3.3). Moreover, the daily dose of ICS ($r=-0.39$, $p=0.001$) and oral maintenance corticosteroids ($r=-0.43$, $p<0.001$) were inversely related to serum 25$\text{OH}D_3$ levels (Figure 3.8C). Because of relatively small numbers in the study population it was not possible to control for the potential confounding effects of corticosteroids in this work (addressed further in discussion).

Table 3.3. Serum vitamin D levels and medication use in all asthmatics

<table>
<thead>
<tr>
<th>Medications used</th>
<th>Median 25-hydroxyvitamin D levels (IQR)</th>
<th>$p$ value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral CS</td>
<td>Oral CS: 28 (21-33) No Oral CS: 45 (21-61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LTRA</td>
<td>LTRA: 33.7 (27-39) No LTRA: 47 (40-53)</td>
<td>0.005</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Theophylline: 31 (21.5-39.5) No Theophylline: 38.5 (27-59)</td>
<td>0.02</td>
</tr>
<tr>
<td>Anti-reflux medications</td>
<td>Yes: 39 (33-44) No: 44.2 (32-57)</td>
<td>0.60</td>
</tr>
</tbody>
</table>

CS, Corticosteroid; IQR, interquartile range; LTRA, leukotriene receptor antagonist

*The Wilcoxon test was used for median value differences for categorical variables.
Figure 3.8 Relationship between serum vitamin D levels and steroids

(A) Lower serum vitamin D levels were associated with increased oral corticosteroid usage ($p<0.01$) (B) Asthmatics with lower serum vitamin D levels were on higher dose of inhaled corticosteroids as compared to those who had higher serum vitamin D levels. (C) Daily dose of ICS ($r=-0.39$, $p=0.001$) and oral maintenance corticosteroids ($r=-0.43$, $p<0.001$) were inversely related to serum 25(OH)D3 levels Kruskal-Wallis test and Mann Whitney U test, followed by a Bonferroni correction was used to compare differences between groups.
### 3.3.7 Serum vitamin D levels and asthma pathology

Demographic data of the children in whom asthma pathology and serum vitamin D levels were measured are presented in Table 3.4. Most children had normal airway anatomy at flexible bronchoscopy (all except one were normal, one child had tracheobronchomalacia). Macroscopically these children showed evidence of inflammation (hyperaemia), oedema and mucus plugging.

**Table 3.4. Demographic characteristics of STRA children (n=22) in whom asthma pathology was assessed.**

<table>
<thead>
<tr>
<th></th>
<th>STRA children (n=22) in whom asthma pathology was assessed</th>
<th>STRA children (n=14) in whom asthma pathology was not assessed</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>11.5 (9, 14)</td>
<td>12 (10, 14)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>14 (64%)</td>
<td>7 (50%)</td>
<td>NS</td>
</tr>
<tr>
<td>Non-white</td>
<td>8 (36%)</td>
<td>7 (50%)</td>
<td></td>
</tr>
<tr>
<td><strong>FEV₁ (%predicted)</strong></td>
<td>78 (67, 91)</td>
<td>72 (60, 82)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>FVC (%predicted)</strong></td>
<td>90 (77, 101)</td>
<td>86 (74, 95)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Atopic</strong> (%)</td>
<td>19 (86)</td>
<td>13 (92)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IgE (IU/ml)</strong></td>
<td>492 (122, 652)</td>
<td>462 (252, 616)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>BDR</strong> (%)</td>
<td>15 (7, 25)</td>
<td>15 (12, 17)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ACT</strong></td>
<td>12.5 (8.7, 15.2)</td>
<td>11.5 (7.7, 14)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ICS dose</strong> (microgram/day)</td>
<td>1450 (1000- 2000)</td>
<td>1600 (1000- 2400)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median exacerbations</strong></td>
<td>3 (2,4)</td>
<td>3 (2,4)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Definition of abbreviations: FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; BDR= Bronchodilator response; ACT= asthma control test;

Values are given in Median (interquartile range) for continuous variables or as number (%) for binary variables.

+One or more positive allergen-specific IgE responses

*Daily Inhaled Corticosteroid dose

**Median exacerbations in last six months requiring oral steroids

$Rise in FEV₁ post bronchodilator (%)

^Score out of 25

%Beclomethasone equivalent
3.3.7.1 Airway and tissue inflammation

It may be important to know the extent to which inflammation in each compartment, airway lumen (represented by sputum & BAL) and airway wall (represented by EB), are related to serum vitamin D levels in children with STRA. The intra-observer sputum eosinophil variability, percentage of eosinophils counted on 3 separate occasions from 3 different subjects is addressed in chapter 2, table 2.1. The 18 children with STRA had an adequate sample to perform differential cell count in sputum. A high sputum eosinophil count (median 3%, IQR 0.9-10) was seen in children with STRA (12/18 had sputum eosinophil >2.5%). BAL cytology was available for all 22 children with STRA. Similarly, a high BAL eosinophil count was also seen (median BAL eosinophil % of 4.6, IQR 1.2-8.2).

Associations between number of inflammatory cells (eosinophils and neutrophils) and serum vitamin D levels were assessed. Table 3.5 outlines the relationship between serum 25\([\text{OH}]\text{D}_3\) levels and airway inflammation in children with STRA. There was no significant correlation between serum 25\([\text{OH}]\text{D}_3\) levels and eosinophils (Figure 3.9 ) or neutrophils (Figure 3.10) in induced sputum (n=18), BAL (n=22) or EB (n=19). There was also no association between tissue mast cells and serum 25\([\text{OH}]\text{D}_3\).
Table 3.5. Association between serum vitamin D levels and airway inflammation in children with STRA.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Correlation (r)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum Eosinophils</td>
<td>18</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>BAL Eosinophils</td>
<td>22</td>
<td>-0.24</td>
<td>0.6</td>
</tr>
<tr>
<td>Mucosal Eosinophil</td>
<td>19</td>
<td>0.05</td>
<td>0.8</td>
</tr>
<tr>
<td>Sputum Neutrophils</td>
<td>18</td>
<td>-0.2</td>
<td>0.6</td>
</tr>
<tr>
<td>BAL Neutrophils</td>
<td>22</td>
<td>0.17</td>
<td>0.24</td>
</tr>
<tr>
<td>Mucosal Neutrophils</td>
<td>19</td>
<td>0.02</td>
<td>0.97</td>
</tr>
<tr>
<td>Mast cells within the smooth muscle</td>
<td>19</td>
<td>-0.02</td>
<td>0.92</td>
</tr>
<tr>
<td>Mucosal Mast cells</td>
<td>19</td>
<td>-0.18</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Correlation was determined by the Spearman rank correlation coefficient.
Figure 3.9 No relationship between serum 25(OH)D₃ levels and eosinophilic inflammation.

There was no significant correlation between serum 25(OH)D₃ and % sputum eosinophils (r= 0.1, p=0.9) (A), % BAL eosinophils (r= -0.2, p=0.6) (B) and mucosal eosinophils (r= 0.05, p=0.8) (C) Correlation was determined by the Spearman rank correlation coefficient.
Figure 3.10 No relationship between serum 25\([\text{OH}]\text{D}_3\) levels and neutrophilic inflammation.

There was no significant correlation between serum 25\([\text{OH}]\text{D}_3\) and % sputum neutrophils \((r = -0.2, p=0.6)\) (A), % BAL neutrophils \((r=0.1, p=0.2)\) (B) and mucosal neutrophils \((r = 0.2, p=0.9)\) (C) Correlation was determined by the Spearman rank correlation coefficient.
3.3.7.2 Airway Remodelling

EB were deemed evaluable if epithelium, RBM and subepithelium were present with a minimum subepithelial area of 0.2 mm$^2$ and minimum length of RBM of 800 µm. EB were of sufficient quality to quantify airway remodelling in 19 of 21 children with STRA (Table 3.6). Median volume fraction of smooth muscle was 0.16 (range 0.07-0.20). There was a significant negative correlation between serum 25(OH)D$_3$ and volume fraction of ASM ($r=-0.63$, $p=0.007$) (Figure 3.11A). Median RBM thickness in children with STRA was 8.4 (range 7.9-9.7 micrometers). There was no relationship between serum 25(OH)D$_3$ and RBM thickness ($r=-0.12$, $p=0.62$) (Figure 3.11B). Median % epithelial shedding in the biopsies was 98 (range 87.4-99.7)%. There was no significant relationship between epithelial shedding and serum 25(OH)D$_3$ ($r=-0.09$, $p=0.69$) (Figure 3.11C). Smooth muscle proliferation was assessed by quantifying the proportion of PCNA positive smooth muscle cells (Figure 3.12). Median myocyte proliferation was 35.3% (range 25.3-64.3)%. There was no relationship between serum 25(OH)D$_3$ and % of smooth muscle cells positive for PCNA ($r=0.007$, $p=0.07$) (Figure 3.11D).

Table 3.6. Biopsy cell count results, Median (Interquartile range (IQR)).

<table>
<thead>
<tr>
<th>Biopsy Cell Count</th>
<th>STRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with good quality biopsies</td>
<td>19 (out of 21)</td>
</tr>
<tr>
<td>% smooth muscle cells positive for PCNA (IQR)</td>
<td>34.1 (20.8-56.1)</td>
</tr>
<tr>
<td>Epithelial loss (%) median (IQR)</td>
<td>98 (87-100)</td>
</tr>
<tr>
<td>RBM thickness in micrometer median (IQR)</td>
<td>8.4 (7.9-9.7)</td>
</tr>
<tr>
<td>Mast cells in submucosa cells/mm$^2$ median (IQR)</td>
<td>65 (49-106)</td>
</tr>
<tr>
<td>Mast cells in smooth muscle cells/mm$^2$ median (IQR)</td>
<td>11 (4-40)</td>
</tr>
<tr>
<td>Neutrophils in submucosa cells/mm$^2$ median (IQR)</td>
<td>5 (0-12)</td>
</tr>
<tr>
<td>Eosinophils in submucosa cells/mm$^2$ median (IQR)</td>
<td>14 (3-78)</td>
</tr>
</tbody>
</table>
Figure 3.11. Relationship between serum 25[OH]D₃ levels and airway remodelling in children with STRA.

There was a significant negative correlation between serum 25[OH]D₃ and volume fraction of ASM ($r = -0.6, p<0.008$) (A). There was no significant correlation between serum 25[OH]D₃ and RBM thickness (B) or epithelial shedding (C). There was no relationship between serum 25[OH]D₃ and proliferating cell nuclear antigen (PCNA) positive smooth muscle cells (D). Correlation was determined by the Spearman rank correlation coefficient.

Vv (smooth muscle (SM)/SM + submucosa) = Volume fraction of smooth muscle indexed to volume of submucosa tissue

PCNA SM cells/ total SM cells (%) = Positively stained smooth muscle nuclei were counted in every biopsy at x 400 magnification and divided by the total number of smooth muscle nuclei and expressed as a percentage (%).
Figure 3.12 Endobronchial biopsy sections from a child with STRA

Endobronchial biopsies were stained with haematoxylin and eosin (A) and proliferating cell nuclear antigen (PCNA) (B) (magnification x 200). Smooth muscle volume fraction was quantified by overlaying a weibel grid at x 200 magnification over the section and performing point counting. The volume fraction of smooth muscle was indexed to volume of submucosal tissue (C). Smooth muscle proliferation was assessed by quantifying the proportion of PCNA positive smooth muscle cells and dividing by the total number of smooth muscle nuclei, expressed as a percentage (magnification x400) (D). Arrows indicate a positively stained nucleus (dark brown) and a negative nucleus (light blue).
3.3.8 Serum vitamin D levels and disease severity

To assess the association between serum vitamin D levels and severity of disease, linear regression was used for continuous variables and logistic regression for categorical variables (Table 3.7). When a non-normal variable remained skewed even after log transformation, this variable was dichotomized and used in the logistic regression. For both types of regression analyses, 2 models (an unadjusted and a multivariable model were constructed). In Table 3.8 all subjects were classified as vitamin D deficient (<50 nmol/L) or not, and performed a logistic regression. Multivariable analysis was adjusted for confounding factors (age, sex, lung function, body mass index and ethnicity).

Even after adjusting for confounding factors including age, sex, body mass index, FEV$_1$ and ethnicity, there was still a significant relationship between serum vitamin D levels and each of symptoms, exacerbations, inhaled and oral steroid use and positive BDR (Table 3.7).
Table 3.7. Serum vitamin D levels and disease severity in all asthmatics (mild/moderate asthma and severe therapy resistant asthma). (A) Linear regression was used for continuous measures of disease severity (B) Logistic regression was used for categorical variables.

(A)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>β*Beta coefficient [95% confidence interval] (p value)</th>
<th>Unadjusted</th>
<th>Multivariate model^</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>0.18 [0.13 to 0.24] (&lt;0.001)</td>
<td>0.15 [0.09 to 0.20] (&lt; 0.001)</td>
<td></td>
</tr>
<tr>
<td>Daily Inhaled Corticosteroid dose</td>
<td>-0.006 [-0.01 to -0.003] (0.001)</td>
<td>-0.004 [-0.007 to 0.0001] (0.05)</td>
<td></td>
</tr>
</tbody>
</table>

* Beta coefficient is for each nmol/L increase in serum vitamin D levels
^ Multivariate model adjusted for age, sex, BMI, FEV\textsubscript{1} and ethnicity.

ACT= asthma control test

(B)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>*Odds Ratio [95% confidence interval] (p value)</th>
<th>Unadjusted</th>
<th>Multivariate model^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive BDR™</td>
<td>0.91 [0.84 to 0.98] (0.01)</td>
<td>0.86 [0.76 to 0.97] (0.01)</td>
<td></td>
</tr>
<tr>
<td>Oral CS$</td>
<td>0.92 [0.88 to 0.98] (0.004)</td>
<td>0.92 [0.86 to 0.98] (0.006)</td>
<td></td>
</tr>
<tr>
<td>Exacerbation£</td>
<td>0.87 [0.80 to 0.95] (0.001)</td>
<td>0.79 [0.64 to 0.97] (0.02)</td>
<td></td>
</tr>
</tbody>
</table>

* Odds ratio are for each nmol/L increase in serum vitamin D levels
^ Multivariate model adjusted for age, sex, BMI, FEV\textsubscript{1} and ethnicity.
™ Positive Bronchodilator response (BDR) = FEV\textsubscript{1} improvement of at least 12% after inhalation of 1,000 µg of salbutamol
$ CS= Corticosteroid; use of maintenance daily oral corticosteroids
£ Exacerbation = any acute exacerbations in the last 6 months requiring oral steroids

Decimal values were approximated to closed integer for ease of exposition.
Table 3.8. Serum vitamin D status in children with severe therapy resistant asthma (STRA), mild/moderate asthma (MA) and controls.

<table>
<thead>
<tr>
<th>Vitamin D status</th>
<th>Odds Ratio [95% CI] (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not deficient (serum 25[OH]D₃ levels &gt; 50 nmol/L)</td>
<td>Deficient (serum 25[OH]D₃ levels &lt; 50 nmol/L)</td>
</tr>
<tr>
<td>Control (n=24)</td>
<td>15 (63%)</td>
</tr>
<tr>
<td>MA (n=26)</td>
<td>11 (42%)</td>
</tr>
<tr>
<td>STRA (n=36)</td>
<td>2 (6%)</td>
</tr>
</tbody>
</table>

@ Multivariate model adjusted for age, body mass index (BMI), % predicted FEV₁ (forced expiratory volume in 1 second), % predicted FVC (forced vital capacity), ethnicity.

Serum vitamin D levels were significantly lower in ‘non-White’ children than those of ‘White’ ethnic background, (p<0.001 [***] measured by Mann Whitney test) (Figure 3.13). However, within the three groups (severe therapy resistant asthma, mild/moderate asthma and controls) there was no difference in serum vitamin D levels between ‘White’ and ‘non-White’ children (Figure 3.13). Also only looking at ‘white’ children in all groups serum vitamin D levels were significantly lower in ‘white’ children with STRA compared to ‘white’ children with MA and controls (p<0.01 for both) but the numbers are relatively small.
Figure 3.13. (A) Serum vitamin D levels were significantly lower in ‘non-White’ children than those of ‘White’ ethnic background, ($p<0.001 \ [***]$ measured by Mann Whitney test). (B) Within the three groups (severe therapy resistant asthma, moderate asthma and controls) there was no difference in serum vitamin D levels between ‘White’ and ‘non-White’ children (Kruskal-Wallis $p<0.001$ followed by Mann Whitney test for inter-group differences and then a Bonferroni correction for multiple comparison)
3.4 Discussion

3.4.1 Principle findings

The main findings are summarized in text box 3.1. The work of this chapter reports for the first time that children with STRA have significantly lower serum 25(OH)D$_3$ levels than MA. Lower serum 25(OH)D$_3$ levels were associated with worse lung function, poorer asthma control and more steroid use in MA and STRA. Within STRA, low 25(OH)D$_3$ levels were associated with increased ASM mass, but not with other parameters of airway remodelling, nor with airway inflammation despite an association with the degree of aeroallergen sensitisation.

In this group with STRA, a significant negative association was present between volume fraction of ASM and 25(OH)D$_3$ levels. Of note, however, there was no association between RBM thickness or epithelial shedding and vitamin D. Although a negative relationship between ASM mass and lung function has been reported in pediatric difficult asthmatics,$^{116}$ this is the first demonstration of an association between low serum 25(OH)D$_3$, poor lung function and asthma control, increased BDR and ASM mass. It is therefore plausible that the link between ASM mass and lung function in severe asthma may partly be explained by low 25(OH)D$_3$ levels. The association between increased ASM mass and low 25(OH)D$_3$ is supported by independent in vitro studies, which have shown that vitamin D inhibits smooth muscle proliferation.$^{280, 281, 311}$ In contrast to the published in vitro studies, there was no relationship between serum 25(OH)D$_3$ levels and ASM proliferation assessed by PCNA staining. However, all in vitro work is in adult ASM and mechanisms may be different in children. Importantly, ASM mass is still increasing as part of normal growth and development in children,$^{370}$ therefore the influence of superimposed pathological abnormalities are likely to be different to those in adults.
Text box 3.1 Relationship between serum vitamin D and clinical markers of asthma severity

Main findings in children with STRA

- Serum 25[OH]D₃ levels were significantly lower in children with STRA than those with MA, and non-asthmatic controls.
- Most of the children with STRA were vitamin D deficient.
- Serum 25[OH]D₃ levels were inversely related to serum total IgE.
- There was a positive correlation between serum 25[OH]D₃ levels and % predicted FEV₁ and FVC.
- Serum 25[OH]D₃ was inversely associated with % BDR.
- A positive relationship was found between 25[OH]D₃ level and ACT, whereby higher serum 25[OH]D₃ was associated with better asthma control as assessed by ACT.
- Lower serum 25[OH]D₃ levels were associated with increased acute asthma exacerbations in the previous six months.
- Daily prescribed dose of corticosteroids were inversely related to serum 25[OH]D₃ levels.
- There was no significant correlation between serum 25[OH]D₃ levels and eosinophils or neutrophils in induced sputum, BAL or EB.
- Low serum 25[OH]D₃ levels were associated with increased ASM mass, but not with other parameters of airway remodelling.

3.4.2 Strengths and weaknesses

The children with STRA had been carefully assessed such that their basic management had been optimized, and any ‘difficult asthmatics’ (whose asthma was uncontrolled because of modifiable factors such as poor adherence to treatment) had been excluded as far as possible.

Interestingly, there was no association between any of the inflammatory cells quantified (eosinophils, neutrophils or mast cells) and serum 25[OH]D₃ levels. This remained true for both luminal inflammation (BAL and sputum) and tissue...
inflammation (EB). This may be related to relatively small numbers, although this study is comparable in size with other airway biopsy manuscripts. It is also possible that the substantial anti-inflammatory treatment prescribed for these children may have masked any relationship between vitamin D and airway inflammation in STRA.

The cross-sectional design of this study made it impossible to determine whether low 25\([\text{OH}]D_3\) levels result in severe asthma in children, or whether children with severe asthma have low 25\([\text{OH}]D_3\) levels because, for example, they are unable to go outside and exercise normally. In addition, correlating ASM mass with the results of airway challenge would be of interest. However, given the STRA subjects’ disease severity, this was thought to be unsafe and therefore unethical. A further limitation of this study was that BDR and ACT were not performed in normal controls. The small numbers of children with STRA with normal serum vitamin D levels is also a limitation. It could be postulated that STRA, low vitamin D levels, high corticosteroid usage, high ASM mass and bad outcomes all cluster together. Low serum vitamin D levels may contribute to asthma severity in children, or corticosteroid usage might result in low serum 25\([\text{OH}]D_3\) levels; or that asthma severity, steroid usage, and low 25\([\text{OH}]D_3\) levels results independently from another as yet undetermined factor. By definition, children with STRA will always be on more treatment than children with MA. To unpick the interactions between vitamin D and asthma severity/ corticosteroid usage, either a prospective study is needed, enrolling subjects with asthma before initiation of any treatment to see if those who eventually prove to have STRA had lower 25\([\text{OH}]D_3\) levels from the outset; or, more practically, to show that supplementing children with established STRA with vitamin D improves serum vitamin D levels, and with it, leads to better asthma control and fewer exacerbations.
3.4.3 Strength and weaknesses in relation to other studies

Even after adjusting for confounding factors including age, sex, body mass index, FEV$_1$ and ethnicity, a significant relationship between serum vitamin D levels and asthma control, exacerbations, inhaled and oral steroid use and positive BDR remained. Some of the findings concur with reports in children and adults with much less severe asthma. These include the associations found between low 25(OH)D$_3$ levels and asthma control and exacerbations, lower lung function, increased reversibility to bronchodilator and greater anti-inflammatory medication use. However association does not prove causation and reverse causation remains a possibility.

In terms of ethnicity, because numbers are small the children were divided into 'White' and 'non-White' groups, but ethnicity was not further subdivided. There was a non-significant trend for more non-White children in the STRA group (36%) compared to MA (16%) and controls (12%) ($p=0.056$). Children with non-White skin had lower serum vitamin D levels, as reported by others. Moreover, non-White children had more severe asthma compared to White children. However the repeated measures ANOVA results have shown that there is no interaction between ethnicity and disease severity for levels of vitamin D ($p=0.20$). This could be due to the small number of subjects in the study. Importantly, race is a proxy for skin colour, and although skin colour is a proxy for low Vitamin D levels, the conclusions relate to the relationship between low serum Vitamin D levels, whether driven by diet, sunlight, ethnicity or another factor, and asthma severity and pathology.

In recent study, Agrawal and colleagues in a murine model of allergic airways inflammation, noted that vitamin D supplementation reduces eosinophilic inflammation in the BAL as compared to vitamin D deficient group. Although total serum IgE levels and specific IgE to cat, dog, pollen, *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* were inversely related to 25(OH)D$_3$ levels, there was no relationship between 25(OH)D$_3$ and serum, BAL or biopsy eosinophils. This may be because children with STRA are highly atopic, and the majority of STRA patients are vitamin D deficient.
Some, but not all investigators have found correlations between lower 25\(\text{OH}\)D\(_3\) levels and markers of allergy in childhood asthma. An association between 25\(\text{OH}\)D\(_3\) deficiency and increased sensitization to allergens in children, but not in adults was also shown in the National Health and Nutrition Examination survey. This chapter results are in agreement with this report that children with low serum 25\(\text{OH}\)D\(_3\) levels are more likely to have allergic sensitization. Interestingly, there was only an association between sensitization to aeroallergens and 25\(\text{OH}\)D\(_3\) levels, but not to food allergens, and this is also partly in agreement with the work of Sharief et al. They did not find any association between vitamin D and sensitization to milk and egg allergen, however serum vitamin D was inversely associated with peanut sensitization. It could be postulated that vitamin D may have a role in preventing sensitization (tolerance) to aeroallergen (lung) but not food (gut) allergen. Although this chapter does not explain these associations, it is hypothesis generating and suggests an important area to validate and extend studies. No association with airway eosinophilia in this chapter may be related to the substantial anti-inflammatory treatment prescribed for these children and may have masked a relationship between vitamin D and airway inflammation in STRA.

The relationship between ASM and serum vitamin D levels in children with STRA noted in this chapter is supported by two recently published studies (in a mouse model). In both animal studies, vitamin D supplementation attenuated evidence of airway remodelling (reduced ASM mass, subepithelial collagen deposition, epithelial thickening and prevents goblet cell hyperplasia) in murine models of allergic airways disease. The cross-sectional nature of the biopsy data prevents certainty as to whether the relationship between increased ASM mass and vitamin D is a result of severe asthma, or whether the increased ASM mass may have been present before the development of disease and caused the asthma. It is possible that a developmental structural defect of the airway wall, such as ASM hypertrophy in children with STRA results from vitamin D deficiency in-utero, and that may have led to asthma in the first place. It may be that exaggerated ASM hypertrophy is a cause of the asthma, rather than a consequence, as a result of in-utero and post-
natal vitamin D deficiency. The effects of vitamin D deficiency in utero could be in addition to, or independent of, airway remodelling. This is especially important as a randomized controlled trial of vitamin D therapy in children with STRA could potentially reverse the ASM hypertrophy and change the course or natural history of these patients' asthma. However, if vitamin D induces a smooth muscle developmental defect in-utero, then it may prove more challenging to reverse.

3.4.4 Meaning of the results
Having established a link between serum 25[OH]D₃ levels and spirometry and asthma control, and importantly, having now seen a novel link between serum vitamin D levels and asthmatic airway smooth muscle, it is logical to suggest that vitamin D supplementation in children with STRA and low 25[OH]D₃ levels, may be a novel therapeutic target directed against some aspects of remodelling. It is challenging to propound a unifying hypothesis to account for ASM changes being the sole manifestation of vitamin D deficiency. In terms of remodelling, this may be due to a heightened sensitivity of ASM to vitamin D deficiency as compared to other airway wall components. The lack of any association between serum vitamin D and inflammation may in part be due to the high dose of inhaled and oral steroid therapy being prescribed. Although vitamin D deficiency causes a degree of steroid insensitivity, the high ICS doses used in the STRA children may have overcome this. It is possible that in the setting of chronic airway inflammation systemic vitamin D levels may be reduced as a direct result of the inflammatory response, which further confounds the association between vitamin D deficiency and disease severity. However, these ideas remain speculative at the present time and the determination of the exact mechanism between low 25[OH]D₃ and airway inflammation and remodelling in STRA will require intervention studies.

3.4.5 Summary
Children who, as far as could be determined had genuine severe asthma had significantly lower serum vitamin D levels than MA and controls. Lower serum vitamin D levels were associated with worse parameters of asthma severity,
and a contributory mechanism may be via an effect on ASM. As numbers are small, and represent a selected population, the conclusions drawn must be tentative. However, these findings suggest that detecting and treating low serum vitamin D levels in children with STRA may aid in treatment of specific structural airway changes as well as of asthma symptoms and severity.

Reverting back to the hypotheses, the data have shown that the hypothesis tested in this chapter has been proven, albeit in relatively small numbers, a selected population of children who as far as could be determined had genuine severe asthma; they had significantly lower serum vitamin D levels than MA and controls. Lower serum vitamin D levels were associated with worse parameters of asthma severity including lung function and symptoms, and ASM mass. As there were few children with STRA with normal vitamin D levels, any conclusions must be tentative. To understand the underlying mechanisms and elucidate the effect of vitamin D treatment, the next chapter describes in vitro responses of peripheral immune cells in cell culture to glucocorticoids and vitamin D.
Chapter 4 The effects of vitamin D on IL-10 secretion from PBMC in vitro from asthmatic children and controls

4.1 Introduction

The previous chapter describes the relationship between serum vitamin D levels and clinical markers of asthma severity, airway inflammation and remodelling. Children with STRA had significantly lower serum vitamin D levels than MA and controls. Lower serum vitamin D levels were associated with worse parameters of asthma severity. However as discussed, cross-sectional data does not prove causation nor explain mechanisms. The vast majority of studies investigating the potential mechanisms of steroid resistance in asthma (discussed in chapter 1 section 1.3) have been performed in adults, but not yet extensively in children. The well-characterized cohort of children with STRA included here provides the unique opportunity to investigate mechanisms underlying steroid resistance and vitamin D levels in asthmatic children. As discussed in chapter 1 section 1.2 children with STRA had undergone a staged investigation protocol and this protocol, (Figure 1.4) which includes a formal assessment in the home and results in approximately half the referrals being classified as difficult asthmatics, in whom basic management needs to be optimized rather than therapy escalated. The remaining children (characterised as STRA) have persistent symptoms, acute severe exacerbations and/or fixed airflow obstruction unresponsive to high-dose steroid therapy. I performed in vitro experiments in samples from children with STRA to understand responses of PBMC in the cell culture to glucocorticoids and vitamin D.

IL-10 is secreted by many cell types including B cells, mast cells, eosinophils, macrophages, dendritic cells and many T cell subsets such as CD4+ T cells, including CD4+Foxp3 Treg cells. IL-10 is a potent immunoregulatory cytokine and has multiple immunosuppressive functions and anti-inflammatory effects in adult asthma (summarized in Figure 4.1). IL-10 also regulates the function of innate immune cells and inhibits pro-inflammatory cytokine production by both Th1 and Th2 cells (discussed in chapter 1).
Figure 4.1 Functions of IL-10 relevant to asthma. Reproduced from Hawrylowicz and O’Garra

Adult studies have shown that there is an inverse association between IL-10 levels and the incidence and severity of asthma in adults.\textsuperscript{378, 379} Akdis and colleagues showed that there is an increased frequency of IL-4-secreting allergen-specific T cells and a reduced frequency of peripheral IL-10-secreting allergen-specific CD4+ T cells in atopic patients as compared to healthy controls suggesting an imbalance between IL-10-secreting Treg cells and Th2 cells.\textsuperscript{83} Adult asthmatic patients prescribed corticosteroid treatment showed increased mRNA expression for IL-10 in PBMC as compared to the controls.
Importantly, corticosteroids are associated with a dose dependent induction of IL-10 synthesis in healthy and steroid sensitive adult asthmatic adult PBMC in vitro. However, there is a poor response in steroid resistant adult asthmatics, suggesting that IL-10 has an important role in the anti-inflammatory effect of corticosteroids. Hawrylowicz and colleagues suggested a potential role for IL-10 in improving steroid responsiveness in adult asthmatics. Further studies in healthy adult PBMC has shown that the combination of dexamethasone and vitamin D potently induced IL-10 synthesis and importantly to a greater degree than that observed with dexamethasone alone. Moreover in another study, vitamin D was noted to restore the defective steroid-induced IL-10 synthesis both in vitro and ex vivo following vitamin D ingestion (0.5mcg daily for 7 days) in steroid-resistant asthmatic patients. These data from adult studies, using peripheral blood, suggest a potential for improving steroid responsiveness with vitamin D.

It is increasingly recognized that paediatric asthma is different from adult asthma immunologically and this has implications for the development of novel therapies; adult responses cannot be used accurately to predict those of children. Very little is known about the in vitro response of PBMC to vitamin D and corticosteroids in children with STRA.

4.1.1 Hypothesis

In this chapter the hypothesis tested is that CD8-depleted PBMCs, from children with STRA exhibit a reduction in glucocorticoid-induced IL-10 synthesis in cell culture, and this is restored by vitamin D.

4.1.2 Aims

i. To assess the differences in PBMC responses from STRA, MA and control subjects to glucocorticoids

ii. To determine whether addition of vitamin D modulates the effects of glucocorticoids and restores steroid sensitivity in children with STRA.
Some of the findings have been reported in the European Journal of Immunology and Thorax.

4.2 Methods
4.2.1 Subjects
Children aged 5-16 years with STRA (n=19), MA (n=16) and non-asthmatic controls (n=13) were recruited prospectively from RBH. STRA was defined as previously described in chapter 1 section 1.2. Non-asthmatic controls comprised either children with no lower airway disease whose parents had consented for a blood test during an elective surgical procedure (cardiac catheterization n=7) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (n=6) at RBH. Parents of 21 non-asthmatic control children were approached, 13 of these agreed to participate. The most common reason for refusal was procedural and general anaesthesia anxiety (5/12), the remaining three gave no reason for refusal. Parents of 38 MA children were approached, 16 of these agreed to participate and the most common reason for refusal was anxiety/phobia for blood test (14/22), the remaining eight gave no reason for refusal. Parents of all 19 STRA children approached agreed to participate.

4.2.2 Steroid responsiveness
Clinical steroid responsiveness was assessed in children with STRA only, as described in chapter 1 section 1.2.1 after a trial of intra-muscular corticosteroid (triamcinolone) as suggested by Bush et al using lung function response (normalisation or improvement of ≥15% in FEV1), symptom response (normalisation or improvement by ≥ 50% in ACT) and inflammatory response (if paired sputum available - normalisation of sputum eosinophil % <2.5% or if paired sputum samples were not available, normalisation of FeNO50 <24ppb). Non-response (steroid refractory, SR) was classified as no improvement in any of the three domains; partial response (partial responders, PR) was improvement in one or two domains; and complete response (steroid sensitive, SS) was normalisation in all three domains.
4.2.3 Isolation of cell populations from peripheral blood

4.2.4 Isolation of total PBMC

Peripheral blood (10-25 mls, maximum 1ml/kg) was collected from all subjects by venepuncture and placed into the anti-coagulant sodium citrate (Sigma-Aldrich Co. Ltd, Poole, UK) at a ratio of 10:1. Blood was transferred and processed immediately (<1hr) at the MRC Centre for Allergic Mechanisms of Asthma, Department of Asthma, Allergy and Respiratory Science, Guy’s Hospital, London. Blood was diluted 1:1 in Hank’s balanced salt solution (HBSS; Life Technologies, Gaithsburg, MD). This was layered at a ratio of 3:1 onto lymphoprep® density gradient (Axis-Shield, Oslo, Norway) and centrifuged at 800g for 20 minutes at 4ºC. PBMC were removed from the lymphoprep® and plasma interface. They were then washed in HBSS supplemented with 2% foetal bovine serum (FBS; PAA laboratories, Pasching, Austria) (2%FBS/HBSS), at 600g for 10 minutes at 4ºC. PBMC were washed again in HBSS supplemented with 2%FBS/HBSS at 200g for 10 minutes at 4ºC. This cell purification protocol has been previously described.178, 386

4.2.5 Preparation of CD8 depleted PBMC cells

CD8+ T cells were removed from total PBMC by positive selection using Dynabeads (Dynal, Oslo, Norway; typical removal 98.5%). The PBMCs were incubated with CD8 coated Dynabeads® (CD8 beads: 25µl per 10⁷ PBMC) for 20 minutes at 4ºC under continuous rotation. The sample was then placed on a magnet for 2 minutes. The supernatant containing the CD8 depleted cells were transferred to a fresh tube and bead bound cells were discarded. The CD8 depleted cells were then washed twice in 2%FBS/HBSS at 200g for 10 minutes at 4ºC to remove any trace of the Dynabeads®. The typical levels of depletion of CD8T cells were >99% (Figure 4.2). Isolated populations were washed and resuspended at 1×10⁶/ml in RPMI 1640 (Life Sciences, Abingdon, GB) containing 10% heat-inactivated FCS (PAA Laboratories, Oxford, GB), 2 mM L-glutamine (Life Technologies) and 50 mg/ml gentamicin (Sigma-Aldrich).
Figure 4.2 Typical depletion of CD8 T cells isolated by bead-based selection

CD8+ T depleted cells were isolated from PBMCs by bead selection using antibody-coated Dynal®. Purity was assessed by surface staining using CD4-APC, CD8-PE. 10,000 live cells were analysed for fluorescence by FACS. Values shown are indicative of the percentage of positive cells within the relevant quadrant.

4.2.6 CD8 depleted PBMC cell culture

Isolated cells were counted using a haemocytometer with dead cells excluded by the use of Trypan blue staining. Purified cells were resuspended at a concentration of 1x10^6 cells/ml in 10%FCS/RPMI/ L-glutamine/gentamicin. On day 0, CD8 depleted PBMC cells were stimulated with the T cell stimulus plate-bound anti-CD3 (1mg/ml, clone OKT3; purified in-house) and IL-2 (50U/ml; Eurocetus, Harefield, UK). Cells were treated with a titration of dexamethasone (10^-9 to 10^-7 M Dex, Sigma-Aldrich, UK) and/or 10^-7 M 1alpha, 25-dihydroxyvitaminD3 (also known as calcitriol, the active form of vitamin D3; Enzo Life Sciences, USA) on day 0. Cells were then incubated at 37°C/5%CO₂ for 7 days. Feeding occurred on day 3; half the volume of culture medium was removed and replaced with 10%FCS/RPMI with 20U/ml IL-2. Removed supernatant was frozen at -20°C. On day 7, cells underwent a second cycle of stimulation. The rationale for this restimulation is to account for differential cell loss under different culture /drug conditions during the initial 7-day culture period. Prior to restimulation, cell culture supernatant was frozen at -20°C. Cells were washed twice in 2% FCS/HBSS by centrifugation at 200g.
for 10 minutes at 4°C. To exclude dead cells from the analysis 0.4 mcg propidium iodide (PI; Sigma-Aldrich) was added. Prior to restimulation, counting was also performed by flow cytometer analysis, using a FACS Calibur (BD Biosciences). Cells were then resuspended at 1x10^6 cells/ml in 10% FCS/RPMI and restimulated with plate-bound anti-CD3 and IL-2 (50U/ml). On day 9 (48 hrs after restimulation), cell culture supernatant was taken and stored at -20°C to assess cytokines by CBA (chapter 2 section 2.5).

The doses of dexamethasone (10^{-7} M) and 1α,25VitD3 (10^{-7} M) used are based on extensive earlier work in the laboratory in adults,^{162, 171, 178} paediatric (Xystrakis and Payne, unpublished data) and cord blood studies (Hornsby, Rice and Hawrylowicz, unpublished data). Also, dose titrations with paediatric PBMC (n=3 non-asthmatic controls) showed optimal IL-10 secretion with the dose of 10^{-7} M 1α,25VitD3 and 10^{-7} M dexamethasone (Figure 4.3). It would have been ideal to perform a dose response in all the subjects, but this was limited by the small volume of blood permitted by the Ethics committee.
Figure 4.3. The effect of various concentrations of 1α,25VitD₃ and dexamethasone on CD8 depleted PBMC cytokine production.

CD8-depleted PBMC from non-asthmatic controls (n=3) were cultured for 7 days with polyclonal conventional T cell stimulation (soluble anti-CD3 (1mcg/ml) plus neutral conditions (IL-2, 50U/ml) or additionally in the presence of dexamethasone (Dex;10⁻⁷M and/or 1α,25VitD₃ (VitD;10⁻⁸M as indicated). At day 7, cells were re-stimulated for 48 hours with anti-CD3 and IL-2 and supernatants were harvested and analysed for IL-10 content by CBA. The optimal IL-10 secretion was noted with the 1α,25VitD₃ dose of 10⁻⁷M.
4.3 Results
Demographic data of the children studied are presented in Table 4.1.

Table 4.1. Demographic characteristics of subjects
No significant differences between children with MA, STRA, and non-asthmatic controls in age and sex. As expected, children with STRA had lower spirometry, ACT and more exacerbations and were prescribed higher dose of inhaled corticosteroids.

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=19)</th>
<th>MA (n=16)</th>
<th>Controls (n=13)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11 (9-14)</td>
<td>12 (11-13)</td>
<td>10.5 (9-13)</td>
<td>0.2a</td>
</tr>
<tr>
<td>Male</td>
<td>9 (47%)</td>
<td>7 (58%)</td>
<td>8 (61%)</td>
<td>0.1</td>
</tr>
<tr>
<td>%predicted FEV₁</td>
<td>75 (65-86)</td>
<td>89 (84-96)</td>
<td>95 (90-98)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>%predicted FVC</td>
<td>88 (76-96)</td>
<td>102 (96-110)</td>
<td>96 (94-108)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>FEV₁/FVC ratio</td>
<td>73 (68-84)</td>
<td>88 (78-92)</td>
<td>93 (89-97)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>Atopic+ (%)</td>
<td>16 (84%)</td>
<td>12 (75%)</td>
<td>3 (23%)</td>
<td>&lt;0.001c</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>498 (127-650)</td>
<td>690 (280-1240)</td>
<td>12.5 (8-51)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>BDR$%$ (%)</td>
<td>14 (6-17)</td>
<td>4 (3.5-6) (n=4)</td>
<td>N/A</td>
<td>0.08a</td>
</tr>
<tr>
<td>ACT</td>
<td>12 (8-15)</td>
<td>19 (17-21)</td>
<td></td>
<td>&gt;0.001a</td>
</tr>
<tr>
<td>Daily Inhaled Corticosteroid dose ( microgram/day)</td>
<td>1400 (1000-2000)</td>
<td>600 (500-800)</td>
<td>N/A</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>Median exacerbations in last six months requiring oral steroids</td>
<td>3 (2-4)</td>
<td>1 (0-2)</td>
<td>N/A</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>Median serum 25[OH]D₃ (nmol/L)</td>
<td>33 (22-42)</td>
<td>44 (31-61)</td>
<td>54 (46-68)</td>
<td>&lt;0.001a</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>12 (64%)</td>
<td>14 (87%)</td>
<td>11 (85%)</td>
<td>0.1c</td>
</tr>
<tr>
<td>Non-white</td>
<td>7 (36%)</td>
<td>2 (13%)</td>
<td>2 (15%)</td>
<td></td>
</tr>
</tbody>
</table>
FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; BDR = Bronchodilator response; ACT = asthma control test;

Values are given in Median (interquartile range) for continuous variables or as number (%) for binary variables; + One or more positive allergen-specific IgE responses; ² Rise in FEV₁ post bronchodilator (%); ³ Score out of 25; ⁴ Beclomethasone equivalent; ⁵ P value calculated by Kruskal-Wallis test; ⁶ P value calculated by Chi-square test; ⁷ P value calculated by Mann Whitney test.

4.3.1 Serum cytokine levels

All the cytokines (IL-1β, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, TNF-α and IFN-γ) measured by CBA in serum from STRA, MA and non-asthmatic controls were largely undetectable with more than 75% of cytokines below the detection limit. The median serum cytokine levels of all the subjects are shown below in Table 4.2 and correlation with serum vitamin D levels are shown in Table 4.3

Table 4.2 Comparison of serum cytokines in all groups.

Serum cytokines were undetectable in more than 90% of controls and 75% of MA & STRA

<table>
<thead>
<tr>
<th>Serum Cytokine</th>
<th>(N)</th>
<th>STRA</th>
<th>(N)</th>
<th>MA</th>
<th>(N)</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>4/19</td>
<td>u(u-4)</td>
<td>1/16</td>
<td>u(u-2)</td>
<td>0/13</td>
<td>u</td>
</tr>
<tr>
<td>IL-5</td>
<td>1/19</td>
<td>u(u-2)</td>
<td>0/16</td>
<td>u</td>
<td>0/13</td>
<td>u</td>
</tr>
<tr>
<td>IL-6</td>
<td>3/19</td>
<td>u(u-22)</td>
<td>1</td>
<td>u</td>
<td>0/13</td>
<td>u</td>
</tr>
<tr>
<td>IL-8</td>
<td>12/19</td>
<td>3.5(u-22)</td>
<td>8/16</td>
<td>9.5(u-33)</td>
<td>3/13</td>
<td>u(u-25)</td>
</tr>
<tr>
<td>IL-10</td>
<td>18/19</td>
<td>u(u-5)</td>
<td>10/16</td>
<td>2(u-13)</td>
<td>8/13</td>
<td>2(u-4)</td>
</tr>
<tr>
<td>IL-13</td>
<td>5/19</td>
<td>u(u-3)</td>
<td>0/16</td>
<td>u</td>
<td>0/13</td>
<td>u</td>
</tr>
<tr>
<td>IL-17</td>
<td>7/19</td>
<td>u(u-137)</td>
<td>1/16</td>
<td>u(u-52)</td>
<td>0/13</td>
<td>u</td>
</tr>
<tr>
<td>TNF-α</td>
<td>7/19</td>
<td>u(u-59)</td>
<td>6/16</td>
<td>11(u-87)</td>
<td>1/13</td>
<td>u(u-26)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>3/19</td>
<td>u(u-9)</td>
<td>2/16</td>
<td>u(u-2)</td>
<td>0/13</td>
<td>u</td>
</tr>
</tbody>
</table>

N=detectable cytokines, u=undetectable

All values median (range) and pg/ml. The numbers were too low to do any reasonable statistics.
Table 4.3 Relationship between serum vitamin D levels and serum cytokines of those asthmatics with detectable cytokines.

No significant correlation between serum vitamin D levels and serum cytokines.

<table>
<thead>
<tr>
<th>Serum Cytokine</th>
<th>N with detectable cytokines in asthmatics</th>
<th>Serum vitamin D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
</tr>
<tr>
<td>IL-8</td>
<td>20</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-10</td>
<td>28</td>
<td>-0.01</td>
</tr>
<tr>
<td>IL-17</td>
<td>8</td>
<td>-0.6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>13</td>
<td>-0.2</td>
</tr>
</tbody>
</table>

Analysis performed using Spearman correlations.

4.3.2 CD8 depleted PBMC from asthmatic children demonstrate impaired IL-10 secretion.

The relative capacity of CD8 depleted PBMC from non-asthmatic controls, MA and children with STRA to produce cytokines in vitro was first assessed. PBMC were depleted of CD8+ T cells, to provide a CD4+ T cell-enriched cell-culture system. Following polyclonal T cell activation with soluble anti-CD3 (1mcg/ml), induced IL-10 secretion was significantly lower in MA and STRA as compared to non-asthmatic control subjects (p<0.001, Figure 4.4). There was also a non-significant trend for increased IL-13 and IL-17A secretion (Figure 4.4); these responses were more heterogeneous in the group.
Figure 4.4. CD8-depleted PBMC from asthmatics secrete significantly less IL-10 than non-asthmatics, and there is a non-significant trend towards increased IL-17A and IL-13 production.

CD8-depleted PBMC from non-asthmatic controls (n=13), MA (n=16) and STRA (n=19) were cultured for 7 days with polyclonal conventional T cell stimulation (soluble anti-CD3 (1mcg/ml) plus IL-2 (50U/ml). At day 7, cells were re-stimulated for 48 hours with anti-CD3 and IL-2 and supernatants were harvested and analysed for (a) IL-10 (b) IL-17A and (c) IL-13 content by CBA. For some controls (n=4) IL-13 in was below the detection limit, hence not the same number of symbols in each group.

Data are presented as individual donor responses and the bars represent the mean. A Mann Whitney U test was used to compare differences between groups followed by a Bonferroni correction.

***p<0.001.
4.3.3 Dexamethasone inhibits IL-13 secretion, but enhances both IL-10 and IL-17A production in cultures of CD8 depleted PBMC

To investigate in vitro dose response and regulation by corticosteroids of CD8 depleted PBMC cytokine profiles in paediatric asthma, cells were cultured with dexamethasone (10^{-7}M to 10^{-9}M). This significantly promoted IL-10 secretion by CD8 depleted PBMC in all three patient groups studied. IL-10 induction occurred in a dose-dependent manner across the range of dexamethasone doses studied (10^{-7}M to 10^{-9}M) but remained markedly less in asthmatics (MA and STRA) as compared to non-asthmatic control children (p<0.01, as assessed by repeated measures ANOVA) (Figure 4.5). There was no clear difference between children with MA and STRA in IL-10 secretion (Figure 4.5). Additionally, dexamethasone significantly reduced IL-13 secretion by PBMC from children with STRA, which was elevated compared to the other groups studied (Figure 4.5). However, PBMC IL-17A secretion, which was also the highest in STRA was not suppressed by dexamethasone treatment, but was in fact significantly further enhanced (Figure 4.5)
**Figure 4.5** Dexamethasone increases IL-10 secretion, and in STRA inhibits IL-13 secretion, but significantly enhances IL-17A production by PBMCs.

CD8-depleted PBMC from non-asthmatic controls (n=13), MA (n=16) and STRA (n=19) were cultured for 7 days with polyclonal conventional T cell stimulation (soluble anti-CD3 (1mcg/ml) and IL-2 (50U/ml), without or with a titration of dexamethasone (10^{-7} to 10^{-9}M). At day 7, cells were re-stimulated for 48 hours with anti-CD3 and IL-2 alone and supernatants were harvested and analysed for expression of (a) IL-10, and (b) IL-17A and (c) IL-13 by CBA. Data are summarized as mean +/- SD. Mann Whitney U test was used to compare differences across the various conditions.
concentrations of dexamethasone. Repeated measures ANOVA was used to compare differences between the groups.
4.3.4 Vitamin D potentiates steroid-induced IL-10, but not IL-17A in CD8 depleted PBMC cultures

In healthy adults, the active form of vitamin D, 1α,25-dihydroxyvitamin D3 drives IL-10 secretion by CD4+ T cells \textit{in vitro} and additionally potentiates dexamethasone-induced IL-10.\textsuperscript{171, 178} Furthermore, in steroid-refractory asthmatics, oral vitamin D ingestion restores impaired T cell responsiveness to dexamethasone \textit{in vitro} for IL-10 induction.\textsuperscript{163} Conversely, vitamin D is reported to negatively regulate IL-17A expression, both in human lymphocytes \textit{in vitro} and in a number of experimental autoimmune models.\textsuperscript{387, 388} The effects of vitamin D on dexamethasone-induced IL-10 and IL-17A production by PBMC were therefore investigated. In both asthmatic and control individuals, inclusion of vitamin D with dexamethasone potentiated IL-10 secretion, although notably this still remained lower in the MA and STRA patients as compared to controls (Figure 4.6A). Vitamin D did not however further enhance steroid-induced IL-17A expression in STRA (Figure 4.6B) and vitamin D alone without dexamethasone did not significantly alter PBMC secretion of IL-10, IL-13 or IL-17A in any group studied (Figure 4.6). However, there was a trend towards inhibition of IL-17A by vitamin D alone in controls (p=0.06) and MA (p=0.08) but not in STRA (p=0.7). Because of limited sample size it was not possible to assess multiple concentrations of dexamethasone and vitamin D.
Figure 4.6 Vitamin D enhances dexamethasone-induced IL-10 secretion by asthmatic PBMC (A), but does not alter IL-17A (B) or IL-13 secretion (C).

CD8-depleted PBMC were cultured as described in figure 4.5 with or without dexamethasone, 1α,25VitD3 or a combination of both (at 10^{-7}M) and supernatants were harvested and analysed for expression of (a) IL-10, (b) IL-17A and (c) IL-13 by CBA. Data are summarized as mean +/- SD. Mann Whitney U test was used to compare differences across the various treatments. Repeated measures ANOVA was used to compare differences between the groups.

*p<0.05, **p<0.01, ***p<0.001
4.3.5 Effect of dexamethasone and Vitamin D on CD8 depleted PBMC cytokine profiles of STRA - related to clinical steroid responsiveness

Having demonstrated the capacity for vitamin D to enhance steroid-induced IL-10 secretion in STRA by paediatric PBMC *in vitro*. STRA children enrolled in the study were further sub-classified according to their clinical response to a single dose of an intramuscular steroid (triamcinolone).\textsuperscript{134} There is no accepted definition of steroid responsiveness in children; neither the dose, route of administration or duration of steroid therapy is standardized, nor the criteria for positive response. In this study, single dose triamcinolone was chosen to avoid the problems of adherence, maximizing the chance of showing responsiveness and minimizing side effects. This is not an evidence based protocol but is the standard clinical protocol at RBH to assess steroid responsiveness (see chapter 1, section 1.2.1).\textsuperscript{134} The demographic data of the children are presented in table 4.4 Six subjects were classified as complete responder (SS), 7 as partial responders (PR) and six as non-responder (SR) as suggested by Bush et al.\textsuperscript{134} There were no significant differences in lung function, ACT, inflammation in any compartment at baseline between the three groups. As there are very small numbers in each group, data should be interpreted with caution.
Table 4.4 Demographic of children with complete, partial and non-response to intramuscular steroid (triamcinolone).

<table>
<thead>
<tr>
<th></th>
<th>Non-response (n=6)</th>
<th>Partial response (n=7)</th>
<th>Complete response (n=6)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>12 (11,14)</td>
<td>10 (8.7,14)</td>
<td>13.5 (9,14)</td>
<td>NS</td>
</tr>
<tr>
<td>Atopy</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>FeNO&lt;sub&gt;50&lt;/sub&gt;</td>
<td>58 (39, 124)</td>
<td>52 (16, 86)</td>
<td>48 (18, 79)</td>
<td>NS</td>
</tr>
<tr>
<td>ACT</td>
<td>11 (6.7,11.5)</td>
<td>15 (13, 17)</td>
<td>12 (7.5, 15)</td>
<td>NS</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; %</td>
<td>72 (53, 84)</td>
<td>80 (74, 75)</td>
<td>75 (64, 87)</td>
<td>NS</td>
</tr>
<tr>
<td>FVC%</td>
<td>86 (63, 93)</td>
<td>96 (80, 101)</td>
<td>91 (85, 101)</td>
<td>NS</td>
</tr>
<tr>
<td>ICS dose%</td>
<td>1600 (1475, 2000)</td>
<td>1400 (1000, 2400)</td>
<td>1500 (1400, 2000)</td>
<td>NS</td>
</tr>
<tr>
<td>Exacerbations</td>
<td>3.5 (1.7,4)</td>
<td>3 (2.4,5)</td>
<td>3.5 (2.7, 4.7)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum 25[OH]D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>23 (16.5, 35)</td>
<td>38 (15, 43)</td>
<td>31 (24, 44)</td>
<td>NS</td>
</tr>
<tr>
<td>IgE</td>
<td>400 (129, 1389)</td>
<td>458 (122, 640)</td>
<td>276 (44, 2179)</td>
<td>NS</td>
</tr>
<tr>
<td>Blood eosinophils</td>
<td>0.8 (0.2, 1.1)</td>
<td>0.8 (0.4, 1)</td>
<td>0.5 (0.07, 1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Sputum eosinophils</td>
<td>3 (2.5, 10)</td>
<td>3.6 (0.6, 8)</td>
<td>2.8 (0.8, 3)</td>
<td>NS</td>
</tr>
<tr>
<td>BAL eosinophils</td>
<td>2.1 (0.7, 3.2)</td>
<td>3.5 (0.7, 6.4)</td>
<td>2 (0, 5)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Analysis was performed using Kruskal-Wallis with correction for multiple groups. Data are number tested or Median (interquartile range) (IQR), FEV<sub>1</sub> = forced expiratory volume in 1 second; FVC = forced vital capacity; BDR= Bronchodilator response; ACT= asthma control test; FeNO<sub>50</sub> fractional exhaled nitric oxide at 50 ml/sec; Exacerbations in last 6 months needing oral steroid

% Beclometasone equivalent; NS (non-significant) =P>0.05
CD8-depleted PBMC from complete responders (SS, n=6), partial responders (PR, n=7) and non-responders (SR, n=6) were cultured with polyclonal conventional T cell stimulation. (Figure 4.7) Stratification of in vitro responses in this manner revealed that PBMC from the complete responder (SS) group had higher IL-10 secretion as compared to the partial (PR) (p=0.03) or non-responder group (p=0.01) (SR). Dexamethasone induced IL-10 in all three groups, however it only achieved significance in the complete responder (SS) group (Figure 4.7A). The addition of vitamin D with dexamethasone also potentiated IL-10 secretion, however it only achieved significance in the complete responder (SS) group (Figure 4.7A). In contrast, PBMC from non-responders (SR) had higher IL-17A secretion as compared to non-responders (SR) (p=0.008). There was non-significant trend in dexamethasone induced IL-17A secretion in complete responders (p=0.07) and IL-17A was not suppressed by dexamethasone in the partial responder (PR) (p=0.1) or non-responder groups (SR) (p=0.3) (Figure 4.7B). IL-13 secretion was highest in non-responders (SR) as compared to partial (PR) or complete responder (SS) groups (p=0.03). Dexamethasone alone and in combination with vitamin D suppressed IL-13 secretion in the non-responder group (SR) but not in partial (PR) or complete responder (SS) group (Figure 4.7B).

In summary on further stratification of children with STRA, PBMC from steroid non-responders (SR) produced the greatest amounts of both IL-13 and IL-17A in vitro, but whilst IL-13 could be suppressed by dexamethasone, IL-17A was not (Figure 4.7B&C). Children with a complete clinical response (SS) also demonstrated the best response in vitro in terms of IL-10 induction (Figure 4.7A). IL-10 potentiation by vitamin D was highest in SS STRA, and apparent in SR and PR as well. The vitamin D did not profoundly alter IL-13 or IL-17A profiles in any subgroup (Figure 4.7B&C).
Figure 4.7 Effect of dexamethasone and 1α,25VitD3 on PBMC cytokine profiles of STRA asthmatics delineated by steroid-responsiveness.

CD8 depleted PBMC from complete responder (SS, n=6), partial responders (PR, n=7) and non-responder (SR, n=6) STRA were cultured for 7 days with polyclonal conventional T cell stimulation (soluble anti-CD3 (1mcg/ml), IL-2 (50U/ml). Additionally they were treated with or without dexamethasone, 1α,25VitD3 or a combination of both (both at 10^{-7}M). At day 7, cells were re-stimulated for 48 hours with anti-CD3 and IL-2 alone and supernatants were harvested and analysed for expression of (a) IL-10, (b) IL-17A and (c) IL-13 by CBA.
CD8 depleted PBMC from SR children produced the greatest amounts of both IL-13 (C) and IL-17A in vitro (B), but whilst IL-13 could be suppressed by dexamethasone (C), IL-17A could not (B). SS individuals demonstrated the best response in terms of IL-10 induction (A). IL-10 potentiation by 1α,25VitD3 was highest in SS STRA (A) and 1α,25VitD3 did not profoundly alter IL-13 (C) or IL-17A (B) profiles in any subgroup.

Data are summarized as mean +/- SD. *p<0.05, **p<0.01, ***p<0.001 as assessed by Mann Whitney U test was used to compare differences.
Further stratification of IL-10 secretion according to all 4 individual responses (symptom, lung function, FeNO_{50} and sputum eosinophils) after intramuscular steroid (triamcinolone) is described next. PBMC of STRA children who had a positive (SS) and negative response (SR) to lung function (normalization or improvement of more than 15% in FEV_{1}) (Figure 4.8A) showed induction of IL-10 secretion with dexamethasone alone and in combination with dexamethasone and 1α,25VitD3. A similar trend was noted with symptom response (normalisation or improvement by ≥ 50% in ACT) (Figure 4.8B), FeNO_{50} response (normalisation of FeNO_{50} <24 ppb) (Figure 4.8C) and sputum eosinophil response (normalization of sputum eosinophil to <2.5%) (Figure 4.8D). This correlation between clinical steroid response and IL-10 secretion revealed no significant difference between the two groups, however a trend towards higher secretion in non-responders (SR) as compared to responders (SS) was apparent with symptoms and inflammatory response (Figure 4.8). However, as stated above, the numbers are small and the results must be considered preliminary.
Steroid responsiveness was assessed after a trial of intra-muscular triamcinolone injection in four ways, (A) Lung function response (normalisation or improvement of $\geq$15% in FEV$_1$) - responder (SS, n=7), and non-responder (SR, n=12) STRA (B) Symptom response (normalisation or improvement by $\geq$ 50% in ACT) - responder (SS, n=7), and non-responder (SR, n=12) STRA (C) FeNO$_{50}$ response (normalisation of FeNO$_{50}$ <24ppb) - responder (SS, n=10), and non-responder (SR, n=9) STRA (D) Sputum eosinophil response (normalisation of sputum eosinophil % <2.5%) - responder (SS, n=14), and non-responder (SR, n=5) STRA. Data are summarized as mean +/- SD.

Figure 4.8 Similar PBMC IL-10 secretion following dexamethasone and 1α,25VitD3 from SR and SS STRA.
4.3.6 Relationship between serum vitamin D levels and steroid responsiveness

The relationship between serum vitamin D levels and markers of asthma severity are described in chapter 3. This section compares serum vitamin D levels in children with STRA to individual clinical response following intramuscular steroid trial. Serum 25(OH)D$_3$ levels were significantly lower in SR as compared to SS children when steroid responsiveness was assessed using symptom response (Figure 4.9b). However, no difference was noted when steroid responsiveness was assessed using lung function response, FeNO$_{50}$ response or sputum eosinophil response (Figure 4.9a,c,d).
Serum vitamin D levels were higher in responder (SS) STRA as compared to non-responder (SR) STRA, when steroid responsiveness was assessed with symptom response (normalisation or improvement by ≥ 50% in ACT) after a trial of intramuscular corticosteroid (triamcinolone) injection (b). No difference in serum vitamin D levels in SR and SS STRA, when steroid responsiveness was assessed with (a) Lung function response (normalisation or improvement of ≥ 15% in FEV\textsubscript{1}) (c) FeNO\textsubscript{50} response (normalisation of FeNO\textsubscript{50} <24ppb) (d) Sputum eosinophil response (normalisation of sputum eosinophil % <2.5%) Data are summarised as median +/- interquartile range. A Mann Whitney U test was used to compare differences between groups.

This section compares serum vitamin D levels in children with STRA, to combined (all three domains – symptoms, lung function and inflammation) clinical response following intramuscular steroid injection. In this thesis, non-response (SR) was classified as no improvement in any of three domains;
partial response (partial responders, PR) was improvement in one or two domains; and complete response (SS) was normalisation in all three domains (see chapter 1, section 1.2.1). There was a non-significant trend towards lower serum vitamin D levels in non-responders, median 23 nmol/L (16.5, 35) as compared to complete responders, median 31 nmol/L (24, 44) (p=0.4) (Figure 4.10).

![Figure 4.10 Serum vitamin D levels and response to a steroid trial in children with STRA.](image)

No significant differences in serum vitamin D levels between the three groups. However, a trend, towards lower serum vitamin D levels in non-responder (SR) as compared to responder (SR) and partial responder (PR), when steroid responsiveness was assessed in children with STRA using three domains: lung function response (normalisation or improvement of ≥15% in FEV₁), symptom response (normalisation or improvement by ≥ 50% in ACT) and inflammatory response (if paired sputum available - normalisation of sputum eosinophil % <2.5% or if paired sputum not available normalisation of FeNO₅₀ <24ppb). Data are summarised as median +/- interquartile range. A Mann Whitney U test was used to compare differences between groups.

4.4 Discussion
4.4.1 Principle findings
The main findings are summarised in Text box 4.1.
**Text box 4.1 Main findings in children with STRA**

- Serum cytokines were largely undetectable and no correlation between serum vitamin D levels and serum cytokines in those with detectable cytokines.
- IL-10 induction occurred in a dose-dependent manner across the range of dexamethasone doses studied (10⁻⁷M to 10⁻⁹M). IL-10 secretion was significantly lower in MA and STRA as compared to non-asthmatic control subjects in CD8 depleted PBMC cell cultures. There was no clear difference between children with MA and STRA in IL-10 secretion.
- Dexamethasone significantly reduced IL-13 secretion by PBMC from children with STRA, which was elevated compared to the other groups studied. However, PBMC IL-17A secretion, which was also the highest in STRA was not suppressed by dexamethasone treatment, but was in fact significantly further enhanced.
- 1α,25VitD3 alone (without dexamethasone) did not significantly alter PBMC secretion of IL-10, IL-13 or IL-17A in any group studied. Vitamin D treatment showed a non-significant trend for inhibition of IL-17A in the controls (p=0.06) and MA (p=0.08) but not in the STRA (p=0.7)
- In both asthmatic and control individuals, the addition of 1α,25VitD3 to dexamethasone potentiated IL-10 secretion, although notably this still remained lower in the MA and STRA patients as compared to controls. 1α,25VitD3 did not however further enhance steroid-induced IL-17A expression in STRA.
- On further stratification of children with STRA, PBMC from non-responder group (SR) produced the greatest amounts of both IL-13 and IL-17A in vitro, but whilst IL-13 could be suppressed by dexamethasone, IL-17A could not. Children with complete response (SS) demonstrated the best response in terms of IL-10 induction.
- Serum 25(OH)D₃ levels were significantly lower in non-responder group (SR) children with STRA than those with complete response (SS), when steroid responsiveness was assessed using symptom response, but not in any other domain.

The most striking peripheral immunological parameter noted was defective IL-10 protein secretion in PBMC culture from asthmatics. Dexamethasone
significantly promoted IL-10 secretion from cultures of PBMCs in a dose dependent manner. Importantly, inclusion of vitamin D to these cultures enhanced IL-10 responses to dexamethasone. These findings support a potential role for IL-10 in regulating peripheral immune responses in children with STRA and build on independent data from both human studies and animal experimental models. There was a non-significant trend for increased IL-13 and IL-17A secretion from STRA PBMC cultures (Figure 4.4), although these responses were heterogeneous. Dexamethasone significantly reduced IL-13 secretion from children with STRA. Interestingly, IL-17A secretion was not suppressed by dexamethasone treatment but was further enhanced.

Looking at the peripheral culture responses in the asthmatics, there was no significant difference between MA & STRA in IL-10, IL-13 or IL-17A secretion. There was a non-significant trend towards higher secretion of IL-17A and IL-13 in STRA as compared to MA. There was no difference in dexamethasone induced IL-10 secretion alone or in combination with vitamin D between MA & STRA. Dexamethasone significantly reduced IL-13 secretion from children with STRA but not MA. On the other hand IL-17A secretion was enhanced by dexamethasone treatment in STRA but not in MA. There was no statistically significant difference in IL-17A synthesis in MA and STRA cultures with vitamin D treatment alone but a trend towards inhibition of IL-17A in MA but not in STRA was seen. The STRA children were then further delineated according to clinical steroid responsiveness, as assessed by their response to intramuscular triamcinolone. Although, there are very small numbers in each group, an interesting trend was noted while for the first time comparing clinical findings with in vitro work. PBMC from complete responders produced the greatest amounts of IL-10 in vitro. Dexamethasone induced IL-10 secretion from PBMC from non-responders was less than from complete responders. PBMC from non-responders produced the greatest amounts of both IL-13 and IL-17A in vitro, but whilst IL-13 could be suppressed by dexamethasone, IL-17A could not. Inclusion of vitamin D in peripheral culture significantly enhanced IL-10 responses to dexamethasone. IL-10 potentiation by vitamin D treatment was highest in complete responders but apparent in non-responder
(SR) and PR as well. However, vitamin D treatment did not profoundly alter IL-13 or IL-17A profiles in any subgroup.

4.4.2 Strengths and weaknesses
This is the first in vitro study to assess peripheral IL-10 secretion and responses to vitamin D in children with STRA and compare the findings with clinical steroid responsiveness. The strength of the present study is that it assesses a clearly defined and characterized population of children with STRA. The requirement to withdraw only small volumes (1ml/kg) of peripheral blood, after the samples for clinical purposes had been taken, precluded thorough functional and mechanistic experiments. For example in vitro detailed dose response curves, confirmation of the cellular source of IL-10 and the regulatory activity of IL-10-secreting cells, which although reported in adult studies,\textsuperscript{377,383} would be of interest in this paediatric cohort. Correlation of serum vitamin D levels with peripheral blood CD4+ IL-10 and Treg FoxP3 cells by flow cytometry is also desirable. In addition, more patients are needed to delineate any associations between clinical steroid responsiveness and the in vitro IL-10 studies. In general, since many comparisons have been made, those in particular of borderline significance must be considered preliminary and hypothesis-generating, rather than definitive.

4.4.3 Strengths and weaknesses in relation to other studies
Lim et al.\textsuperscript{379} investigated whether the difference in IL-10 production between asthmatic and healthy controls was due to a difference in the distribution of the IL-10 producing haplotypes (GCC and ATA). The GCC haplotype has been associated with higher and the ATA halotype with lower IL-10 production in PBMC. They used sequence-specific oligonucleotide probing to screen for the haplotypes in 241 controls, 82 adults with mild asthma, and 113 adults with severe asthma. The distribution of haplotypes in those with mild asthmatics and controls did not differ. But, fewer severe asthmatic had the high IL10-producing haplotype (GCC) and more had the putative low IL-10 producing haplotype (ATA), suggesting the low IL10-producing haplotype is more likely to be associated with severe asthma in adults.\textsuperscript{379} IL-10 producing haplotypes were not assessed in this chapter but reduced peripheral IL-10
expression noted in asthmatics as compared to controls, was consistent with the adult study.\textsuperscript{379}

Both corticosteroids and vitamin D have been proposed as therapeutic candidates to drive IL-10 induction in asthma,\textsuperscript{92} however in adult asthma, patients who fail to demonstrate a clinical response to steroid treatment also demonstrate an impaired IL-10 response to dexamethasone \textit{in vitro}.\textsuperscript{162} In the present chapter, it has been shown that dexamethasone induces IL-10 secretion in PBMC cultures from paediatric asthmatics and controls. However, this is significantly reduced in STRA and MA compared to non-asthmatic controls. Interestingly, significant dexamethasone-induced IL-10 secretion was observed in our children with STRA, which is in contrast to steroid-refractory adults where no significant induction was observed. Dexamethasone-induced IL-10 in CD8 depleted PBMC from non-responder STRA children was less than that from complete responder STRA children. This was similar to the observations made in steroid-refractory adult asthmatics. It was not possible to perform a direct comparison between this paediatric study and adult study\textsuperscript{163} for various reasons including differences in study populations, no standard for comparison between assay, different timing and culture conditions. However, despite these differences, the fact that many results were similar strengthens the likely significance of the findings.

In adult asthmatics it has been demonstrated that \textit{in vitro} addition of 1\alpha,25VitD3 restored T cell IL-10 responses to dexamethasone in culture.\textsuperscript{163} Also, oral treatment with the active form of vitamin D (0.5 mcg of calcitriol daily for 7 days) restored IL-10 responses to dexamethasone.\textsuperscript{163} Similarly, in this chapter non-asthmatic control children and in MA and STRA, inclusion of vitamin D in culture significantly enhanced IL-10 responses to dexamethasone. However in contrast to the adult studies, IL-10 production achieved with vitamin D from the asthmatics in this cohort remains less than that seen in cultures of cells from non-asthmatic controls and the reason for this difference is currently unclear. Other factors (e.g. genetic) for defective IL-10 may potentially contribute in children, or perhaps duration, route or dose of
vitamin D exposure (in vivo versus in vitro) may be important. However, definitive data from interventional studies is needed to confirm the benefit of supplementation with vitamin D, alone or together with inhaled corticosteroids, as a therapeutic approach to augment IL-10 in paediatric asthma.

Vitamin D alone did not drive significant IL-10 secretion from CD8 depleted PBMC cultures in the present study, however, in adult CD4+ T cell cultures vitamin D increased IL-10 secretion.\textsuperscript{163} This observation made in steroid-refractory adult asthmatics has not been extended into our well-characterised children with STRA. However, the sub-optimal response may be secondary to a sub-optimal dose of vitamin D in children, it could be postulated that children are less sensitive but the plateau being the same (right shift of the dose-response curve). Importantly, several differences in culture conditions and systems may have accounted for this difference.

Conversely, IL-17A secretion in STRA peripheral cultures could not be suppressed by dexamethasone treatment in vitro but in fact was significantly enhanced. This effect was not observed in MA and non-asthmatic controls. This is interesting given the successful use of steroids to control a large proportion of steroid-responsive asthmatic disease and it could be postulated that IL-17A is protective in children with STRA but further studies are needed to clarify this. Moreover, it does confirm, for the first time in humans, findings from an earlier study in a mouse model of asthma, where disease induced by adoptively transferred Th17 cells could not be abrogated by dexamethasone treatment, despite a clear benefit in a parallel Th2-adoptive transfer system.\textsuperscript{389} It may also provide some insight into observations made in adults that “Th2 low” asthma does not respond to high doses of steroid treatment, though importantly Th17 activity was not described in this instance.\textsuperscript{40} The implication of these data is that in addition to a defective anti-inflammatory IL-10 response, the clinical picture characterised as steroid-resistant asthma may actually reflect an aberrant inflammatory IL-17A response to escalating doses of inhaled steroids in these patients. This is, in part, supported by data from adult asthmatics indicating that inhaled steroid dose correlates with IL-17A production by PBMC in vitro.\textsuperscript{35} However, it could be postulated that children
with bad asthma will be on high doses of steroids to control the disease and bad asthma leads to more IL-17A as an attempted compensatory mechanism. The exact role of IL-17A in paediatric asthma is yet to be fully defined, with data arising from murine models indicating that IL-17A from gamma-delta T cells is protective in the resolution of acute airway inflammation. Importantly, the CD8-depleted PBMC cell culture employed here does contain gamma-delta T cells, and they cannot be ruled out as a source of steroid-induced IL-17A. There are thus two totally opposite hypotheses about the role of IL-17A, and from this work it remains difficult to elucidate whether IL-17A was detrimental to disease or had a protective role. Thus the role of IL-17A in the human airway requires further investigation. Notably, inclusion of vitamin D in dexamethasone-treated PBMC cultures did not further modulate IL-17A or IL-13 expression, whilst it did augment IL-10 expression. Vitamin D treatment alone without dexamethasone did not significantly alter PBMC secretion of IL-17A in any group studied. However, vitamin D treatment showed a trend towards inhibition of IL-17A in the controls (p=0.06) and MA (p=0.08) but not in STRA (p=0.7). Previously published data show that vitamin D potently inhibits Th17 cells and IL-17A production. Earlier data form our laboratory (Nanzer et al35 and unpublished data) demonstrate that dexamethasone induces IL-17A synthesis by the CD4+ T cell compartment and that this response is inhibited by vitamin D. It would therefore be important to establish whether the steroid induced IL-17A seen in the paediatric studies derives from the CD4 T cells, or some other cellular source e.g. gamma-delta T cells, neutrophils or other cells that might potentially be less sensitive to vitamin D mediated inhibition.

4.4.4 Meaning of the results
Having established reduced anti-inflammatory IL-10 protein synthesis by peripheral immune cells from children with STRA, and importantly that vitamin D potentiates steroid-induced IL-10 secretion, it is logical to suggest vitamin D plays an important role in children with STRA by improving the efficacy of inhaled and oral steroid therapy. The lack of functional and mechanistic experiments limits the ability to propose a unifying hypothesis, nevertheless,
supports for the need for interventional studies in this important patient cohort on high dose steroid therapy.

4.4.5 Summary

In summary, paediatric asthma is characterized immunologically by defective IL-10 protein expression by peripheral immune cells, amongst other abnormalities. Whilst dexamethasone does induce IL-10 expression by peripheral immune cells \textit{in vitro}, this is impaired in STRA, where it also significantly augments IL-17A secretion. Inclusion of vitamin D in culture enhances dexamethasone-induced IL-10, but not IL-17A and may therefore improve steroid efficacy or allow for reduced steroid doses.

Reverting back to the original hypothesis, the work of this chapter has proven that \textit{in vitro} peripheral blood CD8-depleted PBMCs, from children with STRA exhibited a reduction in glucocorticoid-induced IL-10 synthesis in cell culture, and this is restored by vitamin D.

This chapter reports immunological data on PBMCs, and the relationship with vitamin D, the clinical significance of which is difficult to assess. The next chapter describes the relationship of airway (BAL) cells to the vitamin D axis, thus bringing the immunological work closer to the target organ of interest, namely the airway.
Chapter 5 The effects of vitamin D on IL-10 secretion from BAL cells

5.1 Introduction
In the previous chapter, the cytokine response of PBMC cultures from STRA, MA and non-asthmatic controls to glucocorticoids and vitamin D was described. The most striking findings were significantly reduced IL-10 by peripheral blood cells from asthmatics, and their regulation by dexamethasone and vitamin D and in this chapter these observations are further investigated using cells derived from the target organ (BAL). In this chapter, the relationship between serum vitamin D levels and BAL cells is also described. Some of the findings have been reported in the European Journal of Immunology \textsuperscript{273} and others resubmitted to Thorax after revision.

Two important mechanisms for maintenance of peripheral tolerance are the anti-inflammatory cytokine IL-10 and regulatory T cells, one prominent population being CD4+Foxp3+ Treg.\textsuperscript{68, 179} The role of IL-10 in maintaining immune tolerance in the airways is supported by animal studies.\textsuperscript{68, 92, 390} In models of allergic airways disease a reduced cellular infiltration and inflammation (protective effect) is noted after direct IL-10 instillation into the airways, or airway-targeted IL-10 gene transfer.\textsuperscript{391, 392} Specifically, IL-10 dose-dependently inhibited both neutrophilia and eosinophilia in the BAL fluid and in the histologic evaluation of the lung tissue corroborated the findings in the BAL.

Studies in adult patients have shown that IL-10 mRNA and protein in the BAL and alveolar macrophages of mild asthmatics (n=12) is reduced when compared with healthy controls.\textsuperscript{393} Also, there is an inverse association between BAL IL-10 levels and the incidence and GCC and ATA haplotypes associated with low IL-10 production and severity of asthma in adults.\textsuperscript{378, 379} Importantly, adult peripheral blood culture studies have shown that vitamin D restored the defect in steroid-induced IL-10 synthesis in steroid resistant asthmatics.\textsuperscript{163} However, so far all data is from peripheral blood and very little is known about the \textit{in vitro} response of BAL cells to vitamin D and corticosteroids in children with STRA.
5.1.1 Hypothesis
In this chapter the hypothesis tested is that BAL cells from children with STRA exhibit a reduction in glucocorticoid-induced IL-10 synthesis in cell culture, and this is restored by vitamin D.

5.1.2 Aims
To assess in vitro responses of BAL cells to glucocorticoid and vitamin D in culture

Some of the findings have been reported in the European Journal of Immunology \(^{273}\) and Thorax. \(^{385}\)

5.2 Methods
5.2.1 Subjects
Children aged 5-16 years with STRA (n=16), MA (n=5) and non-asthmatic controls (n=10) were recruited prospectively from the Royal Brompton Hospital, London. Non-asthmatic controls (n=10) comprised either children with no lower airway disease whose parents had consented for a flexible bronchoscopy, BAL and blood test during an elective surgical procedure (cardiac catheterization n=2, persistent ductus arteriosus repair n=1, electrophysiology study n=1) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (recurrent episodes of shortness of breath n=1, barking cough n=2, croup/stridor n=2, haemoptysis n=1) at RBH. The indications for flexible bronchoscopy in non-asthmatic controls are described in table 5.1.
Table 5.1 Clinical Indications of flexible bronchoscopy in non-asthmatic controls

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sex</th>
<th>Atopic status</th>
<th>Indication</th>
<th>Findings at Fibreoptic bronchoscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>Nonatopic</td>
<td>Cardiac catheterization</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>Atopic</td>
<td>Recurrent episodes of shortness of breath</td>
<td>Laryngomalacia, tracheomalacia</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>Nonatopic</td>
<td>Persistent ductus arteriosus repair</td>
<td>Normal</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>Nonatopic</td>
<td>Cardiac catheterization</td>
<td>Normal</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>Nonatopic</td>
<td>Electrophysiology study/change of pacemaker</td>
<td>Normal</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>Atopic</td>
<td>Recurrent croup and barking cough</td>
<td>Tracheomalacia</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>Nonatopic</td>
<td>Hemoptysis</td>
<td>No cause found, normal</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>Nonatopic</td>
<td>Noisy breathing</td>
<td>Pharyngomalacia,</td>
</tr>
<tr>
<td>9</td>
<td>Female</td>
<td>Atopic</td>
<td>Stidor</td>
<td>Enlarged tonsils and adenoids</td>
</tr>
<tr>
<td>10</td>
<td>Male</td>
<td>Nonatopic</td>
<td>Recurrent croup</td>
<td>No cause found, normal</td>
</tr>
</tbody>
</table>

5.2.2 Isolation of cells from BAL fluid
BAL was performed as described in Methods (Chapter 2 Section 3), and whole BAL fluid was transferred immediately (<1hr) to the laboratory. BAL fluid was filtered using a 100µm pore nylon mesh filter, held at a 45-degree angle over a 75mm petri dish (such that an edge of the strainer was touching the petri dish). The resultant filtered BAL fluid was run to the bottom of the mesh filter and into the Petri dish. The mesh filter was discarded and filtered BAL fluid was transferred into a 15ml falcon tube and centrifuged at 200g for 10 minutes at 4 °C. BAL supernatant was removed and stored at -80°C. Cell pellets were resuspended in 1ml FACS Flow containing 1% Mouse Serum to
recover total BAL cells. A 50ul of cell suspension was taken to count cells on a haemocytometer and cell concentration was adjusted to $1 \times 10^6$/ml.

5.2.3 BAL cytology
Quantification of inflammatory cells in BAL was performed by the histopathology department at the RBH as described in chapter 2 section 2.3.1

5.2.4 BAL cell culture
Isolated cells were counted using a haemocytometer with dead cells excluded by the use of Trypan blue (Sigma Aldrich, Dorset, UK) staining. BAL cells were then suspended at $1 \times 10^6$/ml in 10% FCS/RPMI and cultured in a 96 well plate (250,000 cells per well). BAL cells were incubated at 37°C/5%CO$_2$ and were either left unstimulated or were stimulated with lipopolysaccharide (LPS, 50ng/ml; R&D Systems) alone and in combination with $1 \times 10^{-7}$M dexamethasone (Sigma) and/or $1 \times 10^{-7}$M 1 alpha,25-dihydroxyvitaminD3 (Enzo Life Sciences, USA). On day 3, cell culture supernatant was frozen at -80°C and cell pellets were preserved by snap freezing in liquid nitrogen and stored in -80°C for future analysis.

5.2.5 Cytokine analysis by CBA
Cytokines were analysed using the CBA kit (CBA, Human Inflammation kit, BD Biosciences, Pharmingen, San Diego) as described in Chapter 2 Section 2.5. This is a flow cytometry based multiplex array application.

5.2.6 FoxP3 intranuclear analysis by flow cytometry
FoxP3 staining was performed with a PE conjugated FoxP3 antibody and the FoxP3 staining buffer set, (Ebiosciences, San Diego, USA) according to manufacturer’s instructions as described in chapter 2 section 2.6. Calculations were performed with Cell Quest software (Becton Dickinson).

5.3 Results
Demographic data of the children studied are presented in Table 5.2.
Table 5.2. Demographic characteristics of subjects\textsuperscript{o} No significant differences between children with MA, STRA, and non-asthmatic controls in age and sex. Children with STRA had lower spirometry and ACT and more exacerbations and were prescribed higher dose of inhaled corticosteroids.

<table>
<thead>
<tr>
<th></th>
<th>STRA(n=16)</th>
<th>MA(n=5)</th>
<th>Controls(n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>12 (10-14)</td>
<td>11 (8-14)</td>
<td>9 (6.7-12)</td>
<td>NS\textsuperscript{a}</td>
</tr>
<tr>
<td>Male</td>
<td>9 (56%)</td>
<td>2 (40%)</td>
<td>6 (60%)</td>
<td>NS£</td>
</tr>
<tr>
<td>%predicted FEV\textsubscript{1}</td>
<td>76 (58-89)</td>
<td>88 (78-102)</td>
<td>92 (88-95)</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
<tr>
<td>%predicted FVC</td>
<td>89 (76-103)</td>
<td>98 (89-111)</td>
<td>96 (93-102)</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
<tr>
<td>Atopic\textsuperscript{+} (%)</td>
<td>13 (81%)</td>
<td>3 (60%)</td>
<td>2 (20%)</td>
<td>&lt;0.05£</td>
</tr>
<tr>
<td>IgE (IU/ml)</td>
<td>196 (67-587)</td>
<td>25 (8-1176)</td>
<td>14 (7.5-84)</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
<tr>
<td>ACT\textsuperscript{^}</td>
<td>11.5 (7.2-15)</td>
<td>18 (17-23)</td>
<td>N/A</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
<tr>
<td>Daily ICS dose</td>
<td>1450 (1000-2000)</td>
<td>500 (400-700)</td>
<td>N/A</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
<tr>
<td>Exacerbations§</td>
<td>3 (2-4)</td>
<td>2 (0.5-2.5)</td>
<td>N/A</td>
<td>&lt;0.05\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textbf{BAL cytology}

<table>
<thead>
<tr>
<th></th>
<th>STRA(n=16)</th>
<th>MA(n=5)</th>
<th>Controls(n=10)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>84% (64-94)</td>
<td>88% (68-92)</td>
<td>90% (76-94)</td>
<td>NS</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>8.5% (3-14)</td>
<td>4% (1.8-8.2)</td>
<td>5.6% (2.5-12)</td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td>3.5% (0.5-16)</td>
<td>1.5% (0.7-6)</td>
<td>1.2% (0.6-2.0)</td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>4% (0.5-16)</td>
<td>3.2% (2.2-16)</td>
<td>2.8% (1-12)</td>
<td></td>
</tr>
</tbody>
</table>

FEV\textsubscript{1} = forced expiratory volume in 1 second; FVC = forced vital capacity; ACT= asthma control test; ICS=inhaled corticosteroid
\textsuperscript{o} Values are given in Median (interquartile range) for continuous variables or as number (%) for binary variables.
\textsuperscript{+} One or more positive allergen-specific IgE responses
\textsuperscript{^} Score out of 25
\textsuperscript{£} P value calculated by Chi-square test.
\textsuperscript{§} Median exacerbations in last six months requiring oral steroids.

\textsuperscript{a} P value calculated by Kruskal-Wallis test.
\textsuperscript{£} P value calculated by Chi-square test.
\textsuperscript{§} P value calculated by Mann Whitney test.

5.3.1 BAL fluid cytokines

Levels of BAL IL-10 were significantly lower in both MA and STRA as compared with non-asthmatic controls (Figure 5.1A). Other cytokines were also measured, including IL-17A, IL-13, IL-5, IL-6 and IL-8 (Figure 5.1). BAL
IL-1βL-1ls of TNF-α and IFN-γ were undetectable in the majority of children (Table 5.2). BAL IL-17A levels were elevated in two asthmatics compared to controls, obviously this did not reach statistical significance (Figure 5.1B). There was no statistically significant difference between the three groups studied in levels of IL-6 and IL-8 (Figure 5.1E&F). The Th2 cytokines IL-5 and IL-13 and IFN-γ were largely undetectable (Figure 5.1 C & D, and Table 5.3). Stratification of individuals into two groups according to the dichotomous BAL IL-17A distribution identified that those with higher BAL IL-17A (>10pg/ml) demonstrated a non-significant trend for worse lung function (% predicted FEV₁, 71% versus 79%, p=0.09) and use of higher doses of inhaled corticosteroids (ICS dose 1800 mcg/day versus 1300 mcg/day, p=0.09).
Figure 5.1. Reduced IL-10 concentrations in the bronchoalveolar lavage in STRA.

There was no significant difference in other cytokines measured in the BAL fluid.

BAL cytokine levels were measured directly *ex vivo*, with no further *in vitro* manipulation, in all of the three groups as delineated in Table 5.1. Cytokine levels were measured by high-sensitivity cytometric bead array (CBA); data are presented as individual results and the bar represents the median. A Mann Whitney U test was used to compare differences between groups.

*p<0.05, ***p<0.001*
Table 5.3 Undetectable cytokines in the BAL fluids. IL-1β, IL-9, TNF-α and IFN-γ were undetectable in all the three groups

<table>
<thead>
<tr>
<th>BAL cytokines (pg/ml)</th>
<th>STRA (n=16)</th>
<th>MA (n=5)</th>
<th>Controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>u (u-116)</td>
<td>u (u-69)</td>
<td>u (u-41)</td>
</tr>
<tr>
<td>IL-9</td>
<td>u (u-71)</td>
<td>u (u-88)</td>
<td>u (u-75)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>u (u-18)</td>
<td>u (u-15)</td>
<td>u (u-30)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>u (u-19)</td>
<td>u (u-17)</td>
<td>u (u-10)</td>
</tr>
</tbody>
</table>

BAL cytokine levels were measured directly ex vivo, with no further in vitro manipulation in all the three groups. Cytokine levels were measured by high-sensitivity CBA; data are presented as median. A Mann Whitney U test was used to compare differences between groups. U-undetectable

5.3.2 FoxP3 intranuclear staining

Tregs are important in maintaining immune homeostasis in pulmonary health and may exhibit impaired function in asthma\(^6^8,^{80,81}\) (discussed in chapter 1 section 1.1.5). Having noted significantly low IL-10 protein levels in the BAL of children with STRA as compared to controls, the frequency of CD4+FoxP3+ cells in BAL cells was assessed next in this chapter.

There was no difference in the frequency of CD4+FoxP3+ cells in the BAL of STRA (n=11) and non-asthmatic subjects (n=5) measured by Flow cytometry (Figure 5.2). Flow cytometry density plots to demonstrate the gating strategy employed to analyse the frequency of CD4+FoxP3+ cells (Treg cells) in BAL are shown in Figure 5.2A. BAL lymphocytes were identified according to forward and side scatter and then cells were next gated on the basis of positive expression of CD3 and CD4 (R2).

Unfortunately, because of very limited BAL volumes available for experiments, it was only possible to perform FoxP3 intranuclear staining in 11 children with STRA and 5 non-asthmatic controls. The difference in FoxP3 may have been significant if more subjects could have been included (Figure 5.2).
Figure 5.2 Frequency of regulatory T cells (CD4+FoxP3+ cells) in bronchoalveolar lavage (BAL) cells of STRA (n=11) and controls (n=5).

(A) Flow cytometry density plots to demonstrate the gating strategy employed to analyse the frequency of Treg in BAL cells. BAL lymphocytes were identified according to forward and side scatter. Cells were next gated on the basis of positive expression of CD3 and CD4 (R2). Values represent the % of gated cells. Quadrant markers were set according to the isotype control, as shown. (B) There was no difference in the frequency of CD4+FoxP3+ cells in the BAL cells of STRA (n=11) and non-asthmatic controls (n=5). This may be the reflection of small numbers and/or the inevitable lack of true healthy controls. Because of very limited BAL volume available for experiments, it was not possible to perform FoxP3 intranuclear staining in all the subjects.
5.3.3 The effect of vitamin D and dexamethasone on BAL cell cultures

In addition to studying peripheral immune cell responses (described in chapter 4), the cytokine secretion profiles of airway-resident populations were addressed by culture of total BAL cells in children with STRA (n=9) and non-asthmatic controls (n=4). This experiment was only possible in a small subgroup of children with STRA and controls because of limited volume of BAL available. Median BAL cells in STRA comprised of a large proportion of alveolar macrophages (84%), lymphocytes (8.5%), neutrophils (4%) and eosinophils (3.5%) (Table 5.1).

Whole BAL cells were suspended and either left unstimulated or were stimulated with LPS (50ng/ml) alone and in combination with $1 \times 10^{-7}$M dexamethasone and/or $1 \times 10^{-7}$M 1alpha,25-dihydroxyvitaminD3. They were incubated at 37°C/5%CO₂ for 72 hours. Cytokines analyzed by CBA from cell culture supernatant revealed IL-10 secretion from STRA BAL cells was significantly lower as compared to non-asthmatic controls ($p<0.01$, assessed by 2 way Repeated measures ANOVA,) (Figure 5.3A). There was a trend ($p=0.07$) toward reduced IL-17A secretion with 1alpha, 25-dihydroxyvitamin D3 alone (Figure 5.3B), and dexamethasone in combination with 1alpha, 25-dihydroxyvitamin D3 increased IL-10 secretion, although not to a significant degree ($p=0.09$ in controls and $p=0.1$ in STRA)(Figure 5.3A). There was a trend towards reduced IL-13 secretion with dexamethasone alone (Figure 5.3C) but not to a significant degree ($p=0.1$). In fact, IL-13 secretion was very low in both the groups.
Figure 5.3 The effect of vitamin D and dexamethasone on cytokine secretion profiles of total BAL cell cultures, stimulated with LPS, from non-asthmatic controls (n=4) and STRA (n=9).

BAL cell culture with lipopolysaccharide stimulation (LPS) (50ng/ml) revealed no statistically change in IL-10 secretion with vitamin D or dexamethasone treatment (A). However, IL-10 secretion from STRA BAL cells was significantly lower as compared to non-asthmatic controls ($p<0.01$, assessed by repeated measures ANOVA). There was a non-significant trend in cultures containing dexamethasone in combination with 1alpha, 25-dihydroxyvitamin D3 towards increased IL-10 secretion, although not to a significant degree ($p=0.09$ in controls and $p=0.1$ in STRA). BAL cell culture revealed no statistically significant change in IL-17A (B) and IL-13 (C).
secretion. However, trend toward reduced IL-17A secretion with 1alpha, 25-dihydroxyvitamin D3 alone in STRA (B), although not to a significant degree (p = 0.07). IL-13 secretion was very low in both control and STRA (C).

BAL cells were cultured with LPS (50 ng/ml) alone and in combination with $10^{-7}$ M dexamethasone (D; $10^{-7}$ M) and/or 1alpha, 25-dihydroxyvitamin D3 for 72 hours. Supernatants were harvested and analysed for expression of IL-10, IL-13 and IL-17A by cytometric bead assay.
5.3.4 Serum vitamin D status correlates with BAL IL-10 and CD4+FoxP3 cells.

Having demonstrated that vitamin D enhances dexamethasone-induced IL-10 secretion by paediatric PBMC *in vitro* (described in chapter 4), and that reduced levels of IL-10 were detected in the BAL of asthmatic (MA and STRA) as compared to non-asthmatic controls (Figure 5.1), the next investigation was to address whether a correlation existed between systemic (serum) vitamin D status and BAL IL-10 concentrations *in vivo*. There is evidence that serum vitamin D levels in both adults and children are frequently deficient or insufficient in moderate to severe asthma (chapter 1 section 1.4.6) and also I have shown that BAL IL-10 levels correlated with the clinical severity of asthma (Figure 5.1A).

Serum vitamin D (25(OH)D3) was measured in samples taken at the time of bronchoscopy and compared with BAL fluid IL-10. When samples from all groups (STRA, MA and non-asthmatic controls) were combined, this revealed a significant correlation between BAL IL-10 and serum 25(OH)D3 status (Spearman r=0.60, *p*<0.001, Figure 5.4A). However, correlation analyses within each patient group are important to consider but were limited by low numbers of children in whom bronchoscopy was clinically indicated, and should not be over-interpreted. However, they may indicate that in asthmatics the association between serum 25(OH)D3 levels and airway (BAL) IL-10 is more important than in non-asthmatic controls (controls, n=10, Spearman r=0.17, *p*=0.63; MA n=5 Spearman r=0.90, *p*=0.08; STRA Spearman r=0.41, *p*=0.12) (Figure 5.4B). There was no association between serum 25(OH)D3 levels and BAL IL-13 (Spearman r=−0.18, *p*= 0.30) or IL-17A (Spearman r=0.28, *p*=0.24), which supports the earlier *in vitro* peripheral culture observations (Figure 4.4).
Figure 5.4 Relationship between BAL IL-10 levels and serum vitamin D levels.

BAL fluid IL-10 directly correlates with serum vitamin D status when samples from all three groups were combined (Spearman $r=0.63$, $p<0.001$) (B) However, this is not significant when each individual group is correlated. (STRA Spearman $r=0.41$, $p=0.12$; MA $n=5$ Spearman $r=0.90$, $p=0.08$; controls, $n=10$, Spearman $r=0.17$ $p=0.63$)

Cytokine levels in BAL fluid were measured by CBA directly *ex vivo*, with no further *in vitro* manipulation in paediatric non-asthmatic controls ($n=10$), MA ($n=5$) and STRA ($n=16$). These levels were correlated with matched serum 25(OH)D3 concentration. A Spearman rank test was used to assess correlation.
Having demonstrated that serum vitamin D levels are positively associated with BAL IL-10 levels (figure 5.4) and vitamin D in vitro enhances steroid-induced IL-10 expression by CD8 depleted PBMC cells (chapter 4) in culture, I next determined whether serum vitamin D status influences Foxp3 expression in whole BAL cells. A statistically significant correlation between serum vitamin D status, and the frequency of CD4+Foxp3+ T cells in the BAL was observed ($r=0.7$, $p = 0.01$), suggesting an association between vitamin D levels with Foxp3+ Treg (Figure 5.5).

![Figure 5.5 Relationship between BAL CD4+ FoxP3+ cells and serum vitamin D levels.](image)

Positive correlation between serum 25-hydroxyvitamin D3 levels and the percentage of CD4+ FoxP3+ cells in BAL of pediatric asthma patients.

BAL Treg (% of CD4+ cells expressing FoxP3) expression was determined by flow cytometry (as shown in Figure 5.2) in the STRA children. Serum was collected from the same patients and the concentration of the circulating form of vitamin D3, 25-hydroxyvitamin D3, was determined.

$p<0.05$ as determined by the Spearman rank correlation test.
5.3.5 Relationship between airway inflammation and BAL IL-10 in children with STRA.

Having demonstrated reduced IL-10 concentration in BAL supernatant and cell cultures in the children with STRA as compared to the controls and also positive associations between serum vitamin D levels and BAL IL-10 protein levels and CD4+Foxp3+ T cells, correlation between BAL IL-10 levels, LPS induced IL-10 secretion and dexamethasone induced IL-10 secretion and airway inflammation was examined next.

Airway inflammation was assessed in sputum and BAL (lumen compartment) and submucosa (airway wall) (described in chapter 2) and correlated with BAL supernatant IL-10 concentration measured directly ex vivo, with no further in vitro manipulation and LPS induced IL-10 secretion and dexamethasone induced IL-10 secretion in the BAL cultures. As discussed earlier, because of limited volume of BAL available, it was only possible to do BAL cell culture in a small sub-group of children with STRA (n=9).

In children with STRA, no association was noted between inflammatory cells quantified (eosinophils or neutrophils cells) and IL-10 secretion (basal or dexamethasone induced) (Table 5.4). This remained true for both luminal inflammation (sputum and BAL) and tissue inflammation (EB). However, this may be related to small sample size (n=9) and also any possible confounding effects of steroid therapy on any relationship.
Table 5.4 No association between airway inflammation and BAL IL-10, dexamethasone induced IL-10 secretion in BAL cell culture and % BAL CD4+FoxP3 cells in children with STRA.

<table>
<thead>
<tr>
<th></th>
<th>BAL IL-10</th>
<th>LPS induced IL-10 secretion in BAL cell culture</th>
<th>Dexamethasone (1x10^7 M) induced IL-10 secretion in BAL cell culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td><strong>Airway inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Sputum eosinophil</td>
<td>0.42</td>
<td>0.6</td>
<td>-0.13</td>
</tr>
<tr>
<td>% Sputum neutrophil</td>
<td>0.74</td>
<td>0.7</td>
<td>-0.36</td>
</tr>
<tr>
<td>% BAL eosinophil</td>
<td>0.18</td>
<td>0.5</td>
<td>-0.31</td>
</tr>
<tr>
<td>% BAL neutrophil</td>
<td>0.32</td>
<td>0.4</td>
<td>-0.18</td>
</tr>
<tr>
<td>Submucosal eosinophils (/mm^2)</td>
<td>0.25</td>
<td>0.2</td>
<td>-0.30</td>
</tr>
<tr>
<td>Submucosal neutrophils (/mm^2)</td>
<td>0.10</td>
<td>0.4</td>
<td>-0.42</td>
</tr>
</tbody>
</table>

BAL, bronchoalveolar lavage; IL, interleukin;
Analysis performed using Spearman correlations.

5.4 Discussion
5.4.1 Principle findings
The main findings are summarised in Text box 5.1. The aim of this chapter was to investigate cytokine expression profiles in the BAL of children with STRA. The most striking immunological observation was of reduced IL-10 protein expression in the BAL of children with STRA in comparison to non-asthmatic controls. Furthermore, the levels of this anti-inflammatory cytokine, but not with the other cytokines measured in BAL showed a positive correlation with serum vitamin D levels. Moreover, in vitro IL-10 secretion by total BAL cells cultured with LPS was significantly lower in STRA as compared to non-asthmatic controls (p<0.001) These findings suggest a central role for IL-10 in regulating airway immune responses, and provide additional support for
Text box 5.1 Main findings in airway studies in children with STRA

- Levels of BAL IL-10 protein were significantly lower in both MA and STRA as compared with non-asthmatic controls. There was no statistically significant difference between the three groups studied in levels of other cytokines measured. A preliminary observation which requires further study was that BAL IL-17A levels were elevated in some asthmatics compared to controls and had a clear dichotomous distribution.
- There was no difference in the frequency of CD4+FoxP3+ cells in the BAL of STRA (n=11) versus non-asthmatic subjects (n=5) measured by Flow cytometry.
- IL-10 secretion from STRA BAL cells in culture was significantly lower as compared to non-asthmatic controls (p<0.01). There was a trend towards reduced IL-13 secretion with dexamethasone treatment alone (Figure 5.3C) but not to a significant degree. There was also a trend (p-0.07) toward reduced IL-17A secretion with vitamin D treatment.
- There was a significant positive correlation between systemic (serum) vitamin D and BAL IL-10 status but not with BAL IL-13 and IL-17A.
- There was a positive correlation between serum 25-hydroxyvitamin D3 levels and the percentage of CD4+ FoxP3+ cells in BAL of paediatric STRA patients.
- In children with STRA, no association was noted between inflammatory cells quantified (eosinophils or neutrophils cells) and IL-10 secretion (basal or dexamethasone induced) (Table 5.3).

Interventional studies. The other important mechanism for maintenance of peripheral tolerance is Tregs and in this chapter circulating levels of serum vitamin D directly correlated with CD4+FoxP3+ cells in airway lavage.

5.4.2 Strengths and weaknesses

This is the first study to assess the relationship between BAL cells and serum vitamin D in children with STRA. The availability of a limited amount of BAL fluid, after the samples for clinical purposes had been taken, precluded thorough functional and mechanistic experiments, for example in vitro.
confirmation of the regulatory activity of IL-10-secreting cells and source of IL-10 in BAL. More patients are needed to delineate any associations between the groups. Additionally the recruitment of controls and MA for bronchoscopies is difficult. All bronchoscopies performed on children at our centre are performed under general anaesthesia; it is therefore unethical for children to have these performed solely for research purposes. Children having clinically indicated bronchoscopies have respiratory conditions and therefore very rarely are completely healthy controls, albeit they are non-asthmatic.

IL-10 is an important immunomodulatory cytokine and the augmentation or restoration of levels in the asthmatic airway may have important therapeutic potential. Interestingly, in children with STRA we did not find an association between inflammatory cells quantified (eosinophils or neutrophils cells) and IL-10 secretion (basal or dexamethasone induced). This may be related to small n numbers or effects of steroid treatment of the children perturbing the relationship. Also, it was not possible to elucidate an effect of ICS treatment on the positive correlation between serum vitamin D and BAL IL-10 and CD4+FoxP3 cells noted in this chapter.

5.4.3 Strengths and weaknesses in relation to other studies
Defective IL-10 expression in asthmatics (MA and STRA) is consistent with the defective IL-10 expression observed in adult asthma.\textsuperscript{162, 163, 379, 394} A substantial reduction in IL-10 mRNA and protein was reported in the BAL of adult patients with asthma as compared to control healthy subjects.\textsuperscript{393} IL-10 gene promoter polymorphisms resulting in reduced IL-10 expression are associated with more severe disease in adult asthmatics.\textsuperscript{379} Airway (sputum) IL-10 levels were reduced in mild-moderate adult asthmatics as compared to controls.\textsuperscript{394} Mild asthmatics were not included in our study but children with moderate asthma had lower BAL IL-10 as compared to the non-asthmatic controls. Sputum cytokines were not quantified in this study but looking at the BAL, similar results were noted.
Both corticosteroids and vitamin D have been proposed as therapeutic candidates to drive IL-10 induction in asthma, however in adult asthma, patients who fail to demonstrate a clinical response to steroid treatment also demonstrate an impaired peripheral IL-10 response to dexamethasone *in vitro*. Whilst dexamethasone and vitamin D did not drive significant IL-10 expression by total BAL cells in the present study, as a group children with STRA had significantly lower IL-10 secretion compared to the controls. Small numbers, different cell type (BAL had predominant macrophages), or perhaps duration or route or dose of vitamin D and steroid exposure may be important and further studies are required to address these issues.

Here it has been clearly demonstrated that serum vitamin D directly correlates with IL-10 levels in BAL and this may be evidence that *in vivo* vitamin D acts as a cofactor for IL-10 expression. It would have been interesting to correlate BAL IL-10 levels with BAL vitamin D levels. However, reliable and readily accessible assays for the measurement of vitamin D levels in the BAL are not yet available and I was therefore unable to assess this (discussed further in chapter 7 section 7.4). However, definitive data from interventional studies is needed to confirm the benefit of supplementation or treatment with vitamin D, alone or together with corticosteroids, as a therapeutic approach to augment airway IL-10 in paediatric asthma.

The Th2-associated cytokines in the BAL, for example IL-5 and IL-13 were largely undetectable. Although not in agreement with early findings in adult asthmatics this is consistent with observations made in similarly-characterised paediatric asthmatics, where Th2 cytokines were not prevalent and could only be detected in a small subgroup of individuals, and is in agreement with more recent findings in adults that some asthma is not characterised by Th2 activity.

The exact role of IL-17A in asthma is also yet to be fully defined and from this chapter it was difficult to elucidate whether IL-17A caused disease or had a protective role in children with STRA. The implication of these data is that in addition to a defective anti-inflammatory IL-10 response, the clinical picture
characterised as steroid-resistant asthma may indeed actually reflect an aberrant inflammatory IL-17A response to escalating doses of inhaled steroids in these patients. This is, in part, supported by the observation made here that those with higher BAL IL-17A levels (>10pg/ml) tended to be receiving higher doses of inhaled corticosteroids – though this did not achieve significance given the sample size (p=0.09), and is additionally supported by data from adult asthmatics indicating that inhaled steroid dose correlates with IL-17A production by PBMC in vitro. However, as discussed in chapter 4, section 4.4.3) IL-17A could have a protective role and that children are prescribed high doses of steroids if they have severe disease and severe asthma could lead to more IL-17A as an attempted compensatory mechanism. A protective role of IL-17A is supported by data from murine models. It is important to emphasize that results of this study are hypothesis generating and need to be tested further in children with STRA.

An independent study in BAL and peripheral blood in children demonstrated reduced Foxp3 mRNA in the BAL cells (but not peripheral blood) in the moderate to severe asthmatic subjects in comparison to children with cough but not asthma. It also demonstrated that in the asthmatic children BAL, but not blood, CD4+ CD25\textsuperscript{high} T cells were decreased in number; however, this was restored following inhalation of corticosteroids. In this study, there was no difference in BAL CD4+FoxP3 cells between STRA and non-asthmatic controls. However, a number of differences exist between these two studies that may account for this, including sample size, the patient cohorts studied, and the antigenic markers used to define Treg.

### 5.4.4 Meaning of the results

Having established reduced anti-inflammatory IL-10 protein in children with STRA by peripheral immune cells (discussed in chapter 4) and BAL IL-10 levels and importantly, a link between serum vitamin D levels and BAL IL-10 and Treg cells (FoxP3), it is logical to suggest vitamin D plays a critical role in children with STRA and may improve steroid efficacy. However, it was not possible to confirm if IL-10 and Tregs are both associated cofactors modulated by vitamin D and the lack of functional and mechanistic
experiments limits the ability to propose unifying hypothesis. Nevertheless, these data support the need for interventional studies in this important patient cohort.

5.4.5 Summary.
In summary, paediatric STRA is (in part) characterized immunologically by defective IL-10 protein expression in BAL cells, and systemic vitamin D status (as measured by serum 25(OH)D3 levels) positively correlates with BAL IL-10 and CD4+FoxP3 cells. Both of these parameters are believed to represent important tolerogenic mechanisms to control inappropriate inflammation both peripherally and in the airways.

Reverting back to the original hypothesis, it was not possible to prove that in vitro addition of vitamin D to BAL cells improves steroid responsiveness in children with STRA. This may relate to the small numbers and limited sample size. In this chapter BAL cells from children with STRA did not exhibit a reduction in glucocorticoid-induced IL-10 synthesis in cell culture, and was not restored by vitamin D. However, this chapter does reinforce the importance of studying the vitamin D axis and immunology locally rather than just relying on peripheral blood.

The vitamin D axis not only includes vitamin D but also incorporates VDBP and the vitamin D receptor. The next chapter takes forward these observations by describing the expression of VDBP in children with STRA, with the aim of gathering further data on potential mechanisms whereby Vitamin D may influence the airway immunological milieu.
Chapter 6. Relationship between Vitamin D binding protein and asthma severity in children

6.1 Introduction

In the previous chapters it has been shown that serum vitamin D levels are associated with asthma severity, asthma control, exacerbations, function (FEV₁, FVC and BDR) and remodelling (ASM mass). Also, in the previous chapters vitamin D and dexamethasone were shown to regulate the synthesis of the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines (e.g. IL-13, IL-17A) in PBMC and BAL cell cultures. These data were complimented by evidence for a positive correlation between BAL IL-10 concentration and the frequency of CD4+Foxp3+ T cells with the systemic (serum) vitamin D status of the host. However, the vitamin D axis not only includes vitamin D but also incorporates VDBP (Figure 1.8), the vitamin D receptor and the various enzymes involved in the generation of active vitamin D, 1α,25VitD3, as well as its inactivation.

IL-10 protein concentration in BAL cells culture from children with MA & STRA was also reduced as compared to controls and importantly BAL IL-10 correlated with serum vitamin D levels. Also, serum vitamin D was positively associated with CD4+ FoxP3 cells. Moreover, in vitro IL-10 secretion by total BAL cells cultured with LPS was significantly lower in STRA as compared to non-asthmatic controls, however small numbers made statistical comparison difficult in the BAL cultures.

The vitamin D axis incorporates VDBP and the vitamin D receptor. VDBP is a glycosylated α-globulin, with 458 amino acids folded into a disulfide-bonded, triple-domain structure (chapter 1, figure 1.8). Most of the vitamin D in the circulation, both 25(OH)D3 and 1α,25VitD3, is bound to VDBP with high affinity. Vitamin D is taken up into cells by diffusion of unbound vitamin D across cell membranes and by endocytosis of that bound to VDBP. Circulating vitamin D is also bound to albumin. Since the affinity of albumin for vitamin D is lower than that of VDBP the vast majority is bound to VDBP.
The relationship of VDBP and vitamin D concentrations is not yet clear. Low vitamin D concentrations were reported in the VDBP knockout mouse model, but did not appear to influence levels of vitamin D that are free to enter cells and tissues.\textsuperscript{301}

As discussed in chapter 1 section 1.4.9, VDBP is a multifunctional protein. Its major functions include binding, solubilization and serum transport of vitamin D. VDBP circulates in the plasma at concentrations 20-fold higher than the total amount of vitamin D metabolites. The purpose of this large molar excess, which is unusual among the carrier proteins of other hormones and vitamins, might relate to the immunomodulatory functions of VDBP.\textsuperscript{395, 302, 303, 299, 302, 304} VDBP is expressed in the plasma membrane of neutrophils,\textsuperscript{305, 306} contributes to macrophage activation,\textsuperscript{303} augments monocyte and neutrophil chemotaxis to C5-derived peptides\textsuperscript{307, 308} and acts as a scavenger protein to clear extracellular G-actin released from necrotic cells.\textsuperscript{309, 310} The anti-inflammatory role of VDBP, together with the relationship with serum vitamin D, may therefore be important in children with asthma. It could be hypothesised that airway inflammation may affect BAL VDBP levels by influencing BAL vitamin D levels. VDBP is present in many body fluids, including serum, BAL, peritoneal fluid\textsuperscript{396} and cerebrospinal fluid,\textsuperscript{397} and is also found on the surface of many cell types including human neutrophils.\textsuperscript{306} This also implies widespread functionality of the protein, consistent with its effects on the innate immunity. The functions of VDBP are summarised in table 6.1.
Table 6.1 The functions of VDBP. Reproduced and modified from ‘The vitamin D axis in the lung: a key role for vitamin D-binding protein’ Chishimba et al.\(^{299}\)

<table>
<thead>
<tr>
<th>Function</th>
<th>Significance to lung</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D transportation(^{299, 300})</td>
<td>Potential influence of levels of vitamin D on host defence and regulation of cell proliferation</td>
<td>Delivery of vitamin D to lung parenchyma and to alveolar macrophages</td>
</tr>
<tr>
<td>Actin binding(^{398})</td>
<td>Prevents systemic vascular occlusion following cellular damage. Potential relevance lung injury and sepsis</td>
<td>Prevents formation of F-actin networks in the systemic circulation. The ability of VDBP to bind actin blocks the formation of F-actin networks that might otherwise occlude the vasculature following cellular damage.</td>
</tr>
<tr>
<td>Neutrophil chemotaxis(^{307, 308, 399})</td>
<td>Neutrophil recruitment is a key part of host defence. Neutrophilic inflammation is a recognised part of adult asthma pathogenesis.</td>
<td>Enhances chemotactic effect of complement-derived peptides. DiMartino et al.(^{305}) demonstrated VDBP-binding sites are upregulated on activated neutrophils,(^{305}) suggesting that changes in local VDBP circulating concentration might occur in inflammatory conditions like asthma. VDBP enhances the chemotactic effect of complement-derived peptides (C5a).(^{399})</td>
</tr>
<tr>
<td>Macrophage activation(^{363})</td>
<td>Macrophage activity is a key part of host defence</td>
<td>VDBP is converted to macrophage-activating factor by enzymes derived from lymphocytes</td>
</tr>
</tbody>
</table>

Metcalf et al.\(^{303}\) suggested that VDBP is converted to macrophage-activating factor by the action of either β-galactosidase from B lymphocytes or sialidase from T lymphocytes on carbohydrate side chains of the protein.\(^{363}\)
Wood and her colleagues recently noted that circulating VDBP levels inversely correlate with FEV₁ in an adult cohort with COPD, and that sputum VDBP contributes to macrophage activation. VDBP is also found in the BAL fluid in mouse models of allergic airways disease, but there have been no paediatric reports comparing VDBP levels of healthy human subjects and those with asthma. Despite the wealth of serum vitamin D studies, there has been relatively little work concerning the role of VDBP in children with asthma.

6.1.1 Hypothesis
In this chapter the hypothesis tested is that children with STRA have higher serum and BAL VDBP levels compared to MA and non-asthmatic controls, and serum and BAL VDBP levels are associated with worse asthma control including lung function, symptoms and steroid usage.

6.1.2 Aims
i. To compare serum and BAL VDBP levels in children with STRA, MA and non-asthmatic controls.
ii. To describe the relationship between serum and BAL VDBP and clinical parameters of asthma control including lung function, symptoms and steroid use.

Some of these finding have already been published.

6.2 Methods
6.2.1 Subjects
Fifteen children with STRA, 7 with MA and 6 non-asthmatic controls were recruited prospectively from the Royal Brompton Hospital, London. STRA was defined as previously in chapter 1 and Children with MA were well controlled on lower dose (<800mcg/day) ICS also as previously defined in chapter 2. Six non-asthmatic controls comprised either children with no respiratory disease whose parents had consented for blood tests during an elective surgical procedure (n=2) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (n=4).
6.2.2 Measurement of Serum 25-hydroxyvitamin D levels

Serum 25-hydroxyvitamin D was measured using a 2 dimensional high performance liquid chromatography system - tandem mass spectrometry (2D LC-MS-MS) as previously described in chapter 2.

6.2.3 Measurement of VDBP levels in the serum and BAL and culture supernatants

VDBP in BAL, serum and cell culture supernatant was measured by Enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s manual (Immunodiagnostik, Bensheim, Germany). The wells of the microliter plate were coated with polyclonal anti-VDBP antibodies. In a first incubation step, the VDBP in the samples is bound to the coated polyclonal rabbit antibodies and in a second incubation step, a polyclonal peroxidase-labeled rabbit-anti-VDBP antibody is added. It is then incubated with the substrate, tetramethylbenzidine. An acidic stopping solution is then added, which converts colour to yellow. The intensity of the yellow colour is directly proportional to the VDBP concentration in the sample and absorption is determined immediately with an ELISA reader at 450 nm against 620 nm as reference. Lower limit of detection of VDBP is 1.2 ng/ml and the upper limit is 600 ng/ml.

Test steps
1. The pre-coated microtiter plate (PLATE) is washed five times with 250 microlitres diluted wash buffer. After the final washing step, the inverted PLATE is firmly tapped on absorbent paper to remove excess solution.
2. Tests were always carried out in duplicate
3. One hundred microliters of Standard, Controls and the pre-diluted samples are then added into the respective well.
4. PLATE is then incubated for 1 hour shaking on a horizontal mixer at room temperature and then the contents of the PLATE are decanted and the wells are washed five times with 250 microlitres diluted wash buffer.
5. One hundred microliters of diluted conjugate, Peroxidase-labelled antibody is then added into the wells and incubated for 1 hour shaking on a horizontal
mixer at room temperature. The contents of the PLATE are decanted and the wells are washed five times with 250 microlitres diluted wash buffer.

6. One hundred microliters of diluted conjugate, Peroxidase-labelled antibody is then added to all the wells and incubated.

7. One hundred microliters of substrate, Tetramethylbenzidine is then added to all the wells and incubated for 10-20 minutes at room temperature.

8. Reaction is stopped with stop solution (acidic) and absorption is determined immediately with an ELISA reader at 450 nm against 620 nm as reference.

6.2.4 BAL cell cultures

BAL cells (1 x 10^6/ml in RPMI/10% foetal calf serum), were cultured in 96 well plates for 72 hours at 37°C/5%CO₂, in the presence of lipopolysaccharide (LPS, 50ng/ml, Sigma-Aldrich) with or without dexamethasone (10⁻⁷M, Sigma-Aldrich) as previously described in chapter 2 for 72 hours. Supernatants were harvested and analysed for expression of VDBP.

The dose of dexamethasone (10⁻⁷M) is based on PBMC culture results (chapter 4) and extensive earlier PBMC work in the laboratory in adults⁶,¹⁶¹ and children. It would have been ideal to perform a dose response in all the subjects but was limited by the small volume of BAL.

6.3 Results
6.3.1 Subjects

Demographic data of the children studied are presented in Table 6.2. There was no significant difference between children with MA, STRA, and non-asthmatic controls in age and sex. Children with STRA had lower FEV₁, FVC and asthma control scores, and by definition were prescribed a higher dose of ICS.
Table 6.2. Demographic characteristics of subjects

<table>
<thead>
<tr>
<th></th>
<th>STRA (n=15)</th>
<th>MA (n=7)</th>
<th>Controls (n=6)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11 (9-13.7)</td>
<td>11 (10-13)</td>
<td>13 (6.7,14.2)</td>
<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Male</td>
<td>7 (47%)</td>
<td>5 (71%)</td>
<td>3 (50%)</td>
<td>0.5&lt;sup&gt;£&lt;/sup&gt;</td>
</tr>
<tr>
<td>FEV&lt;sub&gt;1&lt;/sub&gt; (%predicted)</td>
<td>76 (63, 85)</td>
<td>95 (84, 95)</td>
<td>93 (89, 95)</td>
<td>0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FVC (%predicted)</td>
<td>86 (75, 95)</td>
<td>103 (86,110)</td>
<td>94 (93,103)</td>
<td>0.03&lt;sup&gt;£&lt;/sup&gt;</td>
</tr>
<tr>
<td>ACT</td>
<td>11.5 (7.3, 15)</td>
<td>18 (15, 23)</td>
<td>-</td>
<td>&lt;0.001&lt;sup&gt;£&lt;/sup&gt;</td>
</tr>
<tr>
<td>Daily Inhaled Corticosteroid dose % (microgram/day)</td>
<td>1300 (1000-1900)</td>
<td>600 (400-800)</td>
<td>-</td>
<td>&lt;0.001&lt;sup&gt;£&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

FEV<sub>1</sub> = forced expiratory volume in 1 second; FVC = forced vital capacity; ACT = asthma control test;

* Values are given in Median (interquartile range) for continuous variables or as number (%) for binary variables.
<sup>^</sup> Score out of 25
<sup>%</sup> Beclomethasone equivalent
<sup>a</sup> P value calculated by Kruskal- Wallis test.
<sup>£</sup> P value calculated by Chi-square test.
<sup>£</sup> P value calculated by Mann Whitney test.

6.3.2 VDBP levels in asthmatics and control

VDBP levels were measured by ELISA (section 6.2.3) in serum and BAL of children with STRA, MA and non-asthmatic controls. Children with STRA had significantly higher levels of VDBP in BAL, but not serum as compared to MA (p<0.05) and control individuals (p<0.01) (Figure 6.1). Interestingly, there was no correlation between BAL VDBP and serum VDBP (Spearman r =-0.19, p=0.3). (Figure 6.2)
Figure 6.1 BAL and Serum VDBP in STRA, MA and controls.

(A) BAL VDBP levels were higher in STRA than MA (p<0.05) and controls (p<0.01), (B) however, no difference in serum VDBP existed between the three groups (p=0.8).

Kruskal-Wallis test was used to assess significance
Figure 6.2 Serum and BAL VDBP. No correlation between VDBP in BAL and serum VDBP ($r=-0.199$, $p=0.3$).
Correlation was determined by the Spearman rank correlation coefficient.

6.3.3 VDBP levels and markers of asthma control
There was a negative association between concentration of VDBP in BAL, but not serum, with asthma control (assessed by ACT; BAL VDBP Spearman $r=-0.5$, $p=0.01$; serum VDBP Spearman $r=0.2$, $p=0.3$) as well as spirometry (%FEV$_1$ (BAL VDBP Spearman $r=-0.4$, $p=0.01$; serum VDBP Spearman $r=-0.3$, $p=0.2$)) and a positive association with inhaled corticosteroid usage (BAL VDBP Spearman $r=0.6$, $p=0.002$; serum VDBP Spearman $r=-0.3$, $p=0.2$).
(Figure 6.3)
Figure 6.3 Relationship between BAL and serum VDBP and asthma control, FEV₁, and ICS usage.

(A) Negative association between BAL VDBP levels and asthma control (assessed by ACT, \( r = -0.5, p = 0.01 \)). (B) Inverse association between BAL VDBP and % predicted FEV₁ (\( r = -0.4, p = 0.01 \)). (C) Positive association between BAL VDBP levels and inhaled corticosteroid (ICS) usage (\( r = 0.6, p = 0.002 \)). (D-F) No association between serum VDBP and asthma control (\( r = 0.2, p = 0.3 \)), FEV₁ (\( r = 0.3, p = 0.2 \)) and ICS usage (\( r = -0.3, p = 0.2 \)).

6.3.4 VDBP levels and serum vitamin D levels

There was no association between circulating vitamin D level and either BAL or serum VDBP concentration (BAL VDBP Spearman \( r = -0.3, p = 0.1 \); serum VDBP Spearman \( r = 0.1, p = 0.6 \)) (Figure 6.4). In these patients, serum 25-hydroxyvitamin D levels were inversely correlated with prescribed dose of ICS (\( r = -0.43, p = 0.03 \)) and positively associated with asthma control (measured by
ACT, \( r=0.62, p<0.01 \). However, serum 25-hydroxyvitamin D levels were not associated with spirometry (% \( \text{FEV}_1 \)) \( (r=0.27, p=0.16) \). The relationship between serum vitamin D levels and markers of asthma severity is discussed in chapter 3.

Figure 6.4. Serum vitamin D and VDBP in the serum and BAL.
No association between serum 25-hydroxyvitamin D levels (25 \([\text{OH}]_3 \text{D}_3\)) and BAL vitamin D binding protein (VDBP) \( (r=-0.3, p=0.1) \) and serum VDBP \( (r=0.1, p=0.6) \). Correlation was determined by the Spearman rank correlation coefficient.

6.3.5 Effect of dexamethasone on VDBP production
As expected, children with STRA were on higher doses of inhaled corticosteroids than MA (Table 6.2) and non-asthmatic controls received no corticosteroids. Dexamethasone has previously been reported to increase VDBP gene transcription approximately two-fold in a cultured human hepatoma cell line \(^{401}\) and to increase airway VDBP protein levels in a mouse asthma model.\(^{400}\) Therefore, the effect of dexamethasone on VDBP was
investigated.

The total BAL cells were cultured and stimulated with LPS, with or without additional dexamethasone for 72 hours (n=7). The cell culture supernatant was harvested and analysed for VDBP levels by ELISA.

*In vitro*, the corticosteroid dexamethasone did not promote VDBP synthesis by total BAL cells (paired t test $p = 0.8$) (Figure 6.5). Because of limited sample size it was only possible to measure VDBP in the supernatant of BAL cultures from 7 STRA children.
Figure 6.5 The effect of dexamethasone on bronchoalveolar lavage (BAL) cells of children with STRA (n=7).

No difference in VDBP production ($p=0.8$ as assessed by Mann Whitney test).

BAL cells were cultured in 96-well plate (250,000 cells per well) and exposed to lipopolysaccharide (LPS, 50ng/ml) alone and in combination with $10^{-7}$M dexamethasone (D; $10^{-7}$ M) for 72 hours. Supernatants were harvested and analysed for expression of VDBP.

6.3.6 Relationship between BAL VDBP levels and airway inflammation and remodelling

It is logical to try to determine whether inflammation in each compartment is correlated with BAL VDBP. Therefore, analysis was performed to investigate the relationship between the BAL VDBP and markers of airway inflammation, both luminal (represented by sputum and BAL) and mucosal (represented by EB). There was no association between BAL VDBP and luminal inflammation (BAL neutrophils $r=0.048$, $p=0.8$; BAL eosinophils $r=0.15$, $p=0.5$; BAL macrophages $r=-0.01$, $p=0.9$) or tissue inflammation (submucosal neutrophils $r=0.08$, $p=0.7$; submucosal eosinophils $r=-0.3$, $p=0.2$) (Table 6.3)

In this thesis, serum vitamin D is positively associated with ASM but not with RBM thickness or epithelial shedding. The association between serum vitamin D and airway remodelling (ASM mass) in children with STRA is supported by
in vitro and animal work (discussed in chapter 1 section 1.4.10). As BAL (local) VDBP, but not systemic (serum) VDBP is raised in children with STRA and is associated with disease severity (addressed earlier in this chapter), analysis was performed to investigate the relationship between the BAL VDBP and markers of airway remodelling in EB of children with STRA (assessed by RBM thickness, ASM mass and epithelial shedding. There was no association between BAL VDBP and RBM thickness (r 0.18, p 0.2), ASM mass (r -0.14, p 0.5) or epithelial shedding (r 0.12, p 0.9) (Table 6.4)

Table 6.3 No relationship between BAL VDBP and airway inflammation

<table>
<thead>
<tr>
<th>Marker of inflammation</th>
<th>Spearman r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum neutrophils</td>
<td>0.28</td>
<td>0.1</td>
</tr>
<tr>
<td>Sputum eosinophils</td>
<td>-0.20</td>
<td>0.5</td>
</tr>
<tr>
<td>BAL neutrophils</td>
<td>0.048</td>
<td>0.8</td>
</tr>
<tr>
<td>BAL eosinophils</td>
<td>-0.15</td>
<td>0.5</td>
</tr>
<tr>
<td>BAL macrophages</td>
<td>-0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>Submucosal neutrophils</td>
<td>0.08</td>
<td>0.7</td>
</tr>
<tr>
<td>Submucosal eosinophils</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Table 6.4 No relationship between BAL VDBP and airway remodelling

<table>
<thead>
<tr>
<th>Marker of inflammation</th>
<th>Spearman r</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBM thickness</td>
<td>0.18</td>
<td>0.2</td>
</tr>
<tr>
<td>ASM mass (Vv)</td>
<td>-0.14</td>
<td>0.5</td>
</tr>
<tr>
<td>Epithelial shedding</td>
<td>0.12</td>
<td>0.6</td>
</tr>
<tr>
<td>PCNA</td>
<td>-0.04</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Vv, Volume faction of smooth muscle indexed to volume of submucosa tissue = smooth muscle (SM)/SM+submucosa

PCNA, positive cell nuclear antigen positive smooth muscle cells = PCNA SM cells/SM cells (%)
6.4 Discussion

6.4.1 Principle findings

The work of this chapter has shown for the first time that the local (BAL), but not systemic (serum), levels of VDBP are significantly higher in children with STRA as compared to the MA and non-asthmatic controls and that BAL VDBP levels inversely correlate with asthma severity, as assessed by ACT, spirometry, and inhaled corticosteroid usage. Serum VDBP status did not correlate with any of these parameters of asthma control nor did it correlate with serum vitamin D levels or BAL VDBP levels.

6.4.2 Strengths and weaknesses

These data highlight the potential importance of the vitamin D axis, beyond vitamin D itself, in children with asthma. The cross-sectional design of this study cannot attribute causality, but strongly suggests that further study of local (pulmonary) vitamin D axis as a potential biological modulator is warranted. Functional work to clarify how VDBP may contribute to asthma severity should be a key part of any future investigation of the vitamin D axis, as well as a study of the mechanisms of any beneficial effects of supplementation of vitamin D in lung disease.

Dexamethasone has previously been reported to increase VDBP gene transcription approximately two-fold in a cultured human hepatoma cell line \(^{401}\) and to increase airway (BAL) VDBP protein levels in a mouse asthma model \(^{400}\) suggesting a potential iatrogenic cause of these cross-sectional observations. The current findings indicate no modulation of VDBP expression by total BAL cell culture of 7 STRA children exposed to dexamethasone \textit{in vitro}. However, it was not possible to exclude a dose effect because of limited sample size so an \textit{in vitro} dose titration with dexamethasone was not performed. Another limitation of this work is that the effects of dexamethasone were not studied in MA and controls and importantly, it is not known which cells are producing VDBP in the airways of children with STRA. This limits any firm conclusions.
Interestingly, in children with STRA no association between inflammatory cells quantified (eosinophils or neutrophils cells) and BAL VDBP levels was noted and this may be related to relatively small numbers. However, it is also possible that the substantial anti-inflammatory treatment prescribed for these children may have masked a relationship between VDBP and airway inflammation in STRA.

A weak but significant positive association (Spearman r=0.29, p<0.01) between serum VDBP and serum vitamin D levels has been reported in 469 adults. Interestingly, there was no association between serum VDBP and serum vitamin D levels in the current chapter, and this may be related to small numbers. Importantly, there was also no association between BAL VDBP and serum VDBP and this stresses the importance of studying the vitamin D axis closer to the site of disease (locally) rather than just in peripheral blood.

6.4.3 Possible mechanisms by which excessive VDBP in the airway may limit vitamin D bioactivity with potential immunological consequences

The vast majority of circulating vitamin D, both 25(OH)D3 and 1α,25VitD3, is bound to VDBP, which circulates at levels that far exceed those of its ligand. Cellular uptake of vitamin D can occur via both VDBP-independent and -dependent pathways, although the biological significance of signaling via these different pathways is not known. In vitro addition of VDBP to monocyte cultures impairs vitamin D responsiveness presumably by limiting free diffusion of unbound vitamin D into the cells. This may represent one mechanism by which excessive VDBP in the airway may limit vitamin D bioactivity with potential immunological consequences.

An alternative explanation may relate to the effects of VDBP on innate immune cell function. VDBP augments the chemotactic effect of complement derived peptides (C5a) and together with macrophage activating factor drives macrophage activation in vitro towards a highly phagocytic phenotype with increased superoxide generation. Alveolar macrophages are almost
invariably the most abundant cell type in the airway lumen, and exhibit phenotypic changes in asthma. In health, local signals may restrain these cells, making them relatively unresponsive to inflammatory stimuli, believed to be important for the prevention of airway hyperreactivity. It is plausible that in asthma excessive airway VDBP drives alveolar macrophages into a more inflammatory state, diverting them from their tolerogenic role. The final possible interpretation of these data could simply be that raised airway VDBP is a marker /mediator of steroid resistance. BAL VDBP levels were positively associated with prescribed ICS dose and it would have been interesting to assess relationship between BAL VDBP and steroid response. However, sample size was too small to assess the relationship between BAL VDBP and steroid response in children with STRA and this hypothesis still needs to be proven in larger cohort.

6.4.4 Meaning of the results
Having established a link between local (BAL) VDBP and asthma control, spirometry and prescribed inhaled corticosteroid dose in this chapter, it is logical to support further functional work in investigating BAL VDBP as a biological mechanism or marker of disease severity and/or steroid resistance and how it relates to vitamin D axis, and supplementation of vitamin D. This chapter also emphasizes the importance of studying mechanisms in the hope of finding therapeutic interventions locally (BAL), not just systemically (peripheral blood) in children with STRA. For example from this work totally different conclusions would have been reached if BAL VDBP was not studied.

6.4.5 Summary
The original hypothesis, that children with STRA have higher levels of VDBP in serum and BAL than non-asthmatic controls and VDBP levels are associated with clinical parameters of asthma control, has not been confirmed. In this cross-sectional study, only BAL VDBP levels were elevated in children with STRA, and inversely correlated with asthma control, spirometry and corticosteroid usage. Since the study was cross-sectional, any conclusions must be tentative but nevertheless, this chapter highlights the importance of (a) evaluating not just vitamin D but also other elements of
Vitamin D pathways; and (b) studying these pathways locally within the airway as far as possible, and not relying on peripheral blood. This is discussed in more depth in the next chapter, which also discusses the overall meaning of the study, the strengths and limitations, and what avenues for further work have been opened up.
Chapter 7 Discussion

7.1 Main Findings
The main findings reported in this thesis are as follows;

(1) Children with STRA have significantly lower serum vitamin D levels than MA and controls. Lower serum vitamin D levels were associated with worse lung function, poorer asthma control and more inhaled steroid use in MA and STRA. Importantly, within the STRA group, low serum vitamin D levels were associated with increased ASM mass.

(2) Reduced IL-10 protein concentration in PBMC cultures and BAL supernatant was found in children with asthma. Moreover, in vitro dexamethasone induced IL-10 secretion in the peripheral immune cell cultures was significantly lower in both STRA and MA compared to non-asthmatic controls. The addition of vitamin D to PBMC cultures enhanced IL-10 responses to dexamethasone.

(3) In children with STRA, BAL fluid IL-10 levels were significantly lower than in MA and non-asthmatic controls. Levels of serum vitamin D directly correlated with IL-10 levels and CD4+FoxP3+ cells in the BAL.

(4) BAL, but not serum levels of VDBP were significantly higher in STRA, and BAL VDBP levels inversely correlated with asthma severity, as assessed by ACT, spirometry, and inhaled corticosteroid usage.

7.2 Original hypotheses
The hypotheses underpinning this work were as follows
1. Children with STRA have lower serum vitamin D levels than MA and non-asthmatic controls, and these are associated with worse clinical parameters of asthma control including lung function and symptoms, and worse pathological markers of asthma severity including airway remodelling.

2. In vitro, steroid responsiveness of PBMCs from children with STRA can be improved by addition of Vitamin D.
7.2.1 Hypothesis 1

The first part of the hypothesis was proven: children with STRA had a higher prevalence of vitamin D deficiency even after adjusting for confounding factors (age, sex, % predicted FEV\textsubscript{1} & FVC, body mass index and ethnicity) by multivariate analysis. There was no significant impact of season of sample collection on serum vitamin D level in the three groups. The cross-sectional design of this thesis made it impossible to determine whether low vitamin D levels result in severe asthma in children, or whether children with severe asthma have low vitamin D levels because, for example, they are unable to go outside and exercise normally. Furthermore, because by definition, STRA children were prescribed more medication than MA, it is virtually impossible to determine whether this was related to treatment or asthma severity, and also whether any of the subsequent associations described here were related to treatment or vitamin D levels. There were insufficient STRA children who were vitamin D sufficient for the interactions between STRA, steroid treatment and vitamin D levels to be unpicked.

The second part of this hypothesis was that lower serum vitamin D levels in children with asthma are associated with worse clinical parameters of asthma control and this was partially confirmed. This study has shown that even after adjusting for confounding factors including age, sex, body mass index, FEV\textsubscript{1} and ethnicity, a significant relationship between serum vitamin D levels and asthma control, exacerbations, inhaled and oral steroid use and positive BDR remained. Importantly, within STRA, low vitamin D levels were associated with increased ASM mass, but not with other parameters of airway remodelling. Surprisingly, there was no association between any of the inflammatory cells quantified (eosinophils, neutrophils or mast cells) and serum vitamin D levels. This remained true for both luminal inflammation (BAL and sputum) and tissue inflammation (EB). This may be related to the relatively small numbers, although this study is comparable in size with other airway biopsy studies. It is also possible that the substantial anti-inflammatory treatment prescribed for these children may have masked a relationship between vitamin D and airway inflammation in STRA. So, for example, the
closest relationship between induced sputum eosinophils and FeNO is in steroid naïve asthmatics. The negative association between serum vitamin D levels and ASM mass noted in this thesis may be consequence of confounding factors but with relatively small numbers it was not possible to do multivariate analysis. Ideally it would have been useful to repeat the flexible bronchoscopy 4 weeks later with stage 3 investigations, but this was not thought to be ethical. The cross-sectional nature of the biopsy data prevents certainty as to whether the relationship between increased ASM mass and vitamin D is a result of severe asthma, or whether the increased ASM mass may have been present before the development of disease and caused the asthma, although the findings from the biopsy studies of others militate against this second explanation.

7.2.2 Hypothesis 2
The second hypothesis in this thesis was that in vitro, steroid responsiveness of peripheral immune cells from children with STRA can be improved by addition of vitamin D. This study has shown that children with STRA have reduced IL-10 protein concentration in both PBMC cell cultures and BAL supernatant. Addition of vitamin D to PBMC cell culture enhances dexamethasone induced IL-10 secretion. These data were complimented by evidence for a positive correlation between BAL IL-10 and the percentage of CD4+Foxp3+ T cells with the systemic (serum) vitamin D status of the host. As discussed in chapter 1 section 1.3.3, reduced IL-10 protein levels and Tregs may be related to steroid resistance. Vitamin D may therefore improve steroid responsiveness in children with STRA. However, there may also be additional uninvestigated mechanisms of steroid resistance in the STRA group. The lack of functional and mechanistic experiments (for example how steroids and vitamin D interact at a molecular level) limits the ability to confirm that these mechanisms are of in vivo clinical significance. Nevertheless, the data supports the need for clinical and immunological data from interventional studies in this important patient cohort to confirm the benefit of supplementation with vitamin D.
7.3 Strengths of the study

This is the most extensive study of vitamin D and immune-pathophysiology in children with STRA. A major strength of this study is the very detailed evaluation of potentially reversible factors to ensure as far as possible that the children had truly severe therapy resistant disease. The detailed multidisciplinary assessment to ensure, as far as possible, that basic management is correct is one of the novel features of this study. Adherence was assessed by checking prescription pick up. Paediatric respiratory nurses checked the location and availability of medications in the home and school.\textsuperscript{136} As a part of the clinically indicated stage 1 difficult asthma investigations (see chapter 1, Text box 1.3) the paediatric respiratory nurses also checked for allergen and environmental tobacco smoke exposure in the home and asked the children and family to fill in psychosocial questionnaires, and referred those with significant morbidity for professional help. All these assessments had to be completed prior to invasive assessments (stage 2 & 3 investigations, details in chapter 1). This is really important, because for example if apparent steroid resistance is caused by the steroids not being inhaled, vitamin D related mechanisms are unlikely to be relevant. It should be noted that no assessment is perfect, and there may still be some children in the group who, for example, had undetected poor adherence. The result of this would have been to reduce the chances of finding differences in STRA children, because the group would have been diluted by non-STRA subjects.

Airway inflammation was assessed extensively in children with STRA both non-invasively and invasively with a range of techniques, including induced sputum, BAL and EB. Tissue sections were assessed for markers of remodelling and inflammatory cells. Importantly, the measurements of airway inflammation and remodelling were performed prior to the steroid trial. Rather than an oral steroid trial that relies on adherence from the patient, the steroid trial was given as an intramuscular injection to eliminate adherence from the equation. All but the invasive measurements were repeated 4 weeks after the steroid dose, and used to assess steroid responsiveness (ideally bronchoscopy would have been repeated, but this was not thought to be ethical). A true definition of steroid responsiveness is not known in children,
so rather than relying on the measurement of change in just FEV\(_1\) which can be insensitive in children with severe asthma,\(^{406}\) the response was measured in four different domains.\(^{134}\) Cytokines were measured in BAL and serum as a first step in determining the relationship between vitamin D and airway inflammation and remodelling. An additional strength of the study is the best use of these valuable and limited samples collected from an uncommon group of children who are relatively very difficult to study. However, there are inevitable inherent limitations, which are discussed in the next section.

### 7.4 Limitations of this study

It is important to recognise that, in establishing the link between vitamin D and asthma, there are several challenges. In spite of a wealth of data showing epidemiological associations, we still have a poor understanding of the potential mechanisms due to the complex nature of the pathways involved, the widespread expression of the vitamin D receptor across different cell types, the capacity to synthesize the active form of vitamin D locally and the genetic basis of inter-individual variability in vitamin D status. The majority of the body’s vitamin D is produced by exposure to UV-B radiation and UV-B radiation is also known to be an important immunomodulator and can exert its effects through several non-vitamin D dependent pathways.\(^{407}\) It is possible that serum vitamin D levels are simply a surrogate marker for UV-B exposure and vitamin D is not a causative pathway for disease outcomes. In the background of asthma, the potential relationship with vitamin D deficiency is further confounded by the strong association between physical activity, which is linked to an individual’s level of sun exposure\(^{229}\) and disease severity, which means that it is unclear if vitamin D is simply an indirect marker of physical activity or disease severity and, therefore, is an innocent spectator in disease pathogenesis. However, the experimental work of this thesis and independent adult and animal studies gives biological credibility to the effects of vitamin D being important. Also, whether there is a direct effect of inflammation in asthma on circulating vitamin D levels is not known, it is possible that in the setting of chronic airway inflammation systemic vitamin D levels may be reduced as a direct result of the inflammatory response itself,\(^{230}\) which further confounds the positive association between vitamin D
insufficiency and disease severity.\textsuperscript{230} Therefore the cross-sectional design of this thesis made it impossible to determine whether low serum vitamin D levels result in severe asthma in children, or whether children with severe asthma have low serum vitamin D levels because, for example, they are unable to go outside and exercise normally. The level of physical activity and sunshine exposure were not recorded in this thesis, and this is acknowledged as a weakness, which therefore limits the ability to investigate for reverse causation. Also, it was not possible to adjust for potentially important factors such as the association between atopy and serum vitamin D with relatively small n numbers and non-normal distribution of data. Association does not prove causation and other explanations remain a possibility. Low serum vitamin D levels may contribute to asthma severity in children, or corticosteroid usage might result in low serum vitamin D levels\textsuperscript{220}; or asthma severity, steroid usage, and low vitamin D levels may be caused independently by another as yet undetermined factor. By definition, children with STRA will always be on more treatment than children with MA, and this is inevitable. To unpick the interactions between vitamin D, asthma severity and corticosteroid usage, either a prospective study is needed, enrolling subjects with asthma before initiation of any treatment to see if those who eventually prove to have STRA had lower vitamin D levels from the outset; or, more practically, to show that supplementing children with established STRA with vitamin D improves asthma control and reduces exacerbations. Another limitation is the poor matching of BMI and race/ethnicity in STRA children with non-asthmatic controls.

The assessment of bronchial responsiveness consisted of acute BDR rather than a formal airway challenge, which is often performed in adult studies. Ideally, both direct and indirect airway challenges would have been performed. Airway hyperresponsiveness assessment was considered not to be ethical as it is potentially dangerous in childhood STRA. A further limitation of this study was that BDR and ACT scores were not performed in normal controls. There was a lack of STRA children with normal vitamin D levels and thus it could be postulated that STRA, low vitamin D levels, high corticosteroid usage, high ASM mass and bad outcomes all cluster together and vitamin D
is not a causal factor. A steroid trial was not performed in MA and in fact it would be unethical to perform a high dose steroid response study in a group of mild-moderate asthmatics.

There was no association between airway inflammation and serum vitamin D levels and this may be related to relatively small numbers or the substantial anti-inflammatory therapy prescribed to the STRA children. It is also possible that airway inflammation would correlate with pulmonary vitamin D levels, but we were unable to quantify this in BAL. An attempt was made to measure vitamin D levels in the airway (BAL) with the help of senior clinical biochemists at Homerton Hospital (Dr Peter Timms) and RBH (Dr Jackie Donovan). However, in preliminary work, vitamin D could not be reliably detected in unconcentrated BAL fluid. The presence of vitamin D in the BAL was noted using a 2 dimensional high performance liquid chromatography system - tandem mass spectrometry (2D LC-MS-MS) but was below the limit of detection of 3nmol/L. Unfortunately, not enough resources and in particular spare BAL samples were available for this study to develop efficient BAL concentration and purification techniques. The inability to measure BAL vitamin D levels remains a limitation of this work.

A considerable effort was made to assess the patients before subjecting them to more invasive investigations, but even with this adherence could not be completely assured in the STRA group. Ideally directly observed therapy, or smart inhalers that automatically record when medication is taken would have been employed but unfortunately such resources were not available for this study. Allergen exposure is linked to poor asthma control and exacerbations, therefore a respiratory nurse assessed allergen exposure subjectively at the home visit but environmental sampling was not performed.

Additionally the recruitment of controls and MA for bronchoscopies is difficult. All bronchoscopies undertaken at our centre are performed under general anaesthesia; it is therefore unethical for children to have these performed solely for research purposes. Children having clinically indicated bronchoscopies by definition have respiratory conditions and therefore very
rarely are completely healthy controls, albeit they are non-asthmatic. Similarly, bronchoscopy is only justified in a mild-moderate asthmatic if they have another condition. The ideal study would have involved recruiting totally healthy children, and well-controlled mild asthmatics, but this was not ethically feasible. However, we have previously found differences between asthmatics and these best possible controls, and again in this study, despite the limitations, differences were apparent between children with STRA and controls.

There is emerging evidence that low serum vitamin D levels are associated with more viral respiratory infections, especially among patients with asthma, and with increasing asthma severity. Importantly, viruses often trigger exacerbations in STRA, and are thought to reduce steroid sensitivity in adult asthma. In the present study it was not possible to study this association because as far as possible, for safety reasons, bronchoscopies in STRA had to be performed only when the children were free of a clinically diagnosed viral infection in the 2 weeks prior to bronchoscopy.

The requirement to draw only small volumes of peripheral blood (unavoidable as rightly limited by ethics committee), after the samples for clinical purposes had been taken, precluded thorough functional and mechanistic experiments, for example in vitro confirmation of the cellular source of IL-10 and the regulatory activity of IL-10-secreting cells. It was not possible to assess dose titrations with multiple concentrations of dexamethasone and vitamin D. Therefore, more patients are needed to delineate any associations between steroid responsiveness and the IL-10 studies.

This work examined peripheral cytokine production by CD8-depleted PBMC cell cultures following activation by a T cell specific stimulus. PBMCs include CD8 T cells, CD4 T cells, B cells, monocytes, dendritic cells, natural killer cells, gamma-delta T cells, and innate lymphoid cells. Thus, the cell preparation used for the study cannot be defined as pure CD4+ T cells. However evidence from adult studies suggests that CD4+ T cells are an important source of IL-10 under comparable culture conditions.
the percentage of all the populations prior to activation and after the 9 days in culture for each group was not assessed and remains a limitation of the *in vitro* work. It was not possible to exclude *in vitro* effect of dexamethasone and vitamin D on cells other than on T cells.

The observed difference in median values of BAL fluid IL-10 between the three groups is significant although small and may be difficult to associate with meaningful clinical functional differences. On the other hand the observed difference between BAL IL-17 levels are higher, but were not found to be statistically significant. This may be related to relatively low patient numbers. Although a strict protocol for the BAL (including volume and number of aliquots instilled) was followed in all the children, the effect of dilution cannot be excluded. In this thesis, vitamin D, VDBP, and cytokines levels were not measured in sputum supernatant of the children with STRA. It was also not possible to assess the source of VDBP detected in the BAL or perform functional work to clarify whether, and if so, how VDBP contributes to asthma severity or steroid resistance.

The subgroup analyses were often underpowered (for example when steroid response was assessed). In this thesis, it was not possible to determine the stability of the clinical measurements and vitamin D status to assess the significance of any changes reported. It would also have been ideal to repeat the bronchoscopy 4 weeks after the steroid injection to assess change in the vitamin D status, BAL and biopsy inflammation and remodelling to be assessed following the steroid trial. It is however unethical to repeatedly perform bronchoscopy in children, as it is an invasive procedure requiring a GA.

Finally, this thesis has generated a large number of data points, meaning that a very large number of comparisons have been made. It is important to distinguish findings from pre-test hypotheses and pre-set analyses, from those, which appeared significant in *post-hoc* reviews. *Post-hoc* results will need testing in a prospective cohort.
7.5 **Strengths and weaknesses in relation to other studies**

Some of the findings of this thesis concur with previous reports both in children and adults with much less severe asthma. These include the associations found between low serum vitamin D levels and lower lung function, increased reversibility to bronchodilator and greater anti-inflammatory medication (ICS and oral steroid) usage.

The strong relationship between low serum vitamin D levels and poor asthma control (assessed by ACT) and frequent exacerbations noted in this thesis is supported by previous reports. The association between vitamin D deficiency and asthma exacerbations was first noted by Brehm et al, in an investigation of data gathered in 1024 children of the Childhood Asthma Management Program, a randomized controlled trial of comparison of inhaled budesonide, inhaled nedocromil and placebo in children with mild-moderate asthma. They noted that vitamin D insufficiency (≤75 nmol/L) is related to an increased risk of asthma exacerbations. In another study, Searing et al showed an inverse correlation between serum vitamin D levels and use of corticosteroids in a cross-sectional study of 100 asthmatic children. They also showed that vitamin D augmented glucocorticoid induced IL-10 synthesis in PBMC cell cultures. Chinellato et al have also recently reported an association between lower vitamin D levels and poor asthma control in 75 MA children aged 5-11 years. In another study, 48 asthmatic children on inhaled corticosteroids were randomized to either inhaled budesonide and 500 IU of cholecalciferol per day or inhaled budesonide and placebo for 6 months. Significantly fewer children assigned to the vitamin D arm had an asthma exacerbation, despite the fact that there were no significant differences in the levels of vitamin D achieved in the two groups after 6 months. However, in this relatively small study there was no definition of what represented an asthma exacerbation. There is convincing data that many of the mechanisms modulated (summarized in Figure 7.1) by vitamin D are likely to prevent disease exacerbation, however larger clinical trials of sufficient duration are needed to determine whether vitamin D supplementation affects markers of disease severity.
Vitamin D-binding protein [DBP, also known as group-specific component (Gc)] is the major transport protein for vitamin D metabolites (including 25OHD), although other proteins, such as albumin, also participate greatly in transport. DBP has not previously been implicated in asthma and allergies, although it is known that DBP has immune regulating functions, related to macrophage activation and neutrophil chemotaxis [39]. Recently, Lee et al. [40] showed that DBP concentrations in bronchoalveolar fluid were significantly elevated in 67 asthmatic patients compared with 22 controls. Furthermore, in a murine model, they showed that DBP mRNA and protein levels were elevated in ovalbumin-sensitized/challenged mice than in sham-treated mice, and that treatment with an anti-DBP antibody dose-dependently reduced the airway hyper-reactivity, airway inflammation, and levels of eotaxin, IL-4, IL-5, and IL-13, and IFN-γ.

Whereas the implications of these results with regard to vitamin D deficiency are unclear at the moment, it is known that polymorphisms in the gene for DBP (Gc) are determinants of vitamin D status [41,42]. Finally, it has been shown that DBP can regulate the bioavailability of 25OHD [43]. Further studies are needed to fully understand the relationship between circulating vitamin D and DBP, and/or polymorphisms of Gc on asthma and allergies.

CONCLUSION AND FUTURE DIRECTIONS
In the 2 years since the last review of this topic in this journal [12], the number of studies that has investigated the relationship between vitamin D and asthma and allergies has increased tremendously. An increasing number of studies have measured circulating 25OHD as a determinant of vitamin D status. However, several issues regarding the determination of vitamin D status need to be elucidated. Firstly, the level of 25OHD that determines optimal vitamin D status for asthma and allergies, and for overall health, remains elusive. Whereas the Institute of Medicine (IOM) [44] has recently recommended that a 25OHD level of 50 nmol/l (20 ng/ml) should be considered Adequate vitamin D status in asthmatics ∞

Binding to VDR
Vitamin D-VDR-RXR complex binds to VDREs in gene promoters

Decreased airway smooth muscle proliferation
Decreased airway inflammation
Improved handling of respiratory infections
Decrease airway responsiveness
Prevention of airway remodeling
Decrease severity
Possibly, increased clearance of infections
Faster response to medications, particularly steroids
Reversal of steroid resistance
Improvement in IL10 secretion

Increase in Treg cells
Th1-Th2 balance
Suppress Th17
Upregulation of IL10

FIGURE 2. Mechanisms of vitamin D in decreasing the risk for asthma exacerbations. Many of the mechanisms that are mediated by vitamin D to prevent asthma are likely operating to prevent disease exacerbations. In addition to the effects of vitamin D in improving handling of respiratory infections, improve immune cell function, decreasing inflammation, prevention of airway smooth muscle over proliferation, vitamin D has also been shown to improve the response to steroids in asthma by reversing the steroid resistant state through up-regulation of IL-10 production. Modified extensively from previous work in ref. [13].
Two recently published animal (mouse) studies support the findings of this thesis. In the first study, Lai et al.\textsuperscript{316} showed that in a mouse model of allergic airway inflammation, vitamin D treatment attenuated established structural changes of the airways. Importantly, they showed that vitamin D treatment could efficiently reduce ASM mass, subepithelial collagen deposition, epithelial thickening and prevent goblet cell hyperplasia.\textsuperscript{316} In the second study, Agrawal and colleagues\textsuperscript{317} examined the effects of dietary supplementation with vitamin D in a murine model of allergic airway inflammation. By dietary intervention, mice were made vitamin D deficient, vitamin D sufficient or further supplemented with vitamin D. Serum vitamin D levels were measured at 6 and 13 weeks of age and the values were similar to levels measured in humans\textsuperscript{317} with vitamin D deficiency (15 nmol/L), sufficiency (78 nmol/L), and in the third group of mice (supplemented group) had serum vitamin D levels of 163 nmol/L. At 6 and 8 weeks of age (adulthood) the mice were sensitized to ovalbumin and lung function (airway hyperresponsiveness, whole body plethysmography and airway resistance) was measured. The lungs were then lavaged to obtain BAL for quantification of inflammatory cells and cytokines, lung tissue was isolated and fixed for histological assessment and blood taken for circulating Tregs. As expected, sensitization to ovalbumin significantly increased airway hyperresponsiveness, however in vitamin D deficient mice it was significantly higher and in vitamin D supplemented mice airway hyperresponsiveness was significantly lower. Importantly, airway hyperresponsiveness was significantly higher in vitamin D supplemented as compared to non-sensitized mice, suggesting that vitamin D could only partially attenuate changes due to allergen sensitization or alternatively, vitamin D supplementation was inadequate.\textsuperscript{318} Lung histopathology showed the expected evidence of airway remodelling in sensitized mice with increased collagen deposition and enhanced mucus production. Evidence of more severe airway remodelling (marked epithelial cell hypertrophy indicated by an increase in epithelial cell height, airway occlusion, and mucus staining and collagen deposition) was noted in the vitamin D deficient group as compared to vitamin D sufficient group. Importantly, the lungs of vitamin D supplemented mice exhibited the
mildest features but still had evidence of airway remodelling. As expected, ovalbumin sensitization increased eosinophils and IL-5, IL-13 and IL-6 in the BAL fluid; they were highest in vitamin D deficient group, and significantly reduced (but not to levels measured in non-sensitized mice) in vitamin D supplemented mice. Importantly, levels of IL-10 protein in the BAL and Treg cells in the blood decreased with sensitization, except when the mice were supplemented with vitamin D. For example, in the blood of vitamin D sufficient mice, Treg cells (CD4+CD25+T cells) comprised of 3.4%, decreasing to 2.2% in deficiency and increasing to 5.5% in the mice with supplemented levels of vitamin D. Additionally, for all of the markers that changed with serum vitamin D levels, there was a clear dose-dependent effect of vitamin D. Vitamin D deficiency was associated with BAL eosinophilia and the percentage of eosinophils in the BAL were significantly reduced in the vitamin D supplemented group. The results of this thesis are partly in agreement with the mouse work of Agrawal and colleagues (discussed above), who found that reduced levels of serum vitamin D are associated with positive BDR, low BAL IL-10 protein and Tregs and worse airway remodelling. However, no association was found herein between vitamin D and Th2 cytokines and eosinophilia, which may be related to disease severity or substantial anti-inflammatory treatment prescribed for these children.

Total serum IgE levels and specific IgE to cat, dog, pollen, *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* were inversely related to vitamin D levels in the patients reported here. Some, but not all investigators have found correlations between lower vitamin D levels and markers of allergy in childhood asthma. A U-shaped association between low and high vitamin D levels and serum total IgE levels has been demonstrated by Hypponen et al. Recently Sharief et al. noted an interesting association between vitamin D deficiency and increased sensitization to aero- and food allergens in children (n=3136), but not in adults (n=3454). In particular, serum vitamin D values of less than 15 ng/mL (36 nmol/L) were associated with peanut (odds ratio, 2.39), ragweed (odds ratio, 1.83), and oak (odds ratio, 4.75) allergy. An association between serum vitamin D levels and atopy in children but not adults, points to the potential plasticity of vitamin D mediated responses in
children, as atopy markers may be more sensitive to serum vitamin D levels in children as compared to adults.

High BMI has been linked to vitamin D deficiency\textsuperscript{411} and steroid resistance in adults\textsuperscript{36, 37} however, there was no link between BMI and vitamin D deficiency in the results from the present study, but this may be because of the small sample size and the BMI (median 19.5 Kg/m\textsuperscript{2}, interquartile range 16-24) of children was not high enough to find a significant difference. The study design precluded assessing this possible effect.

Recently Zosky and coworkers\textsuperscript{412} provided important evidence of the role of vitamin D in reducing lung function in a murine model. Female mice were made vitamin D deficient or sufficient by dietary manipulation and then mated with vitamin D sufficient males. Lung volumes and function were studied in offspring by plethysmography, forced oscillometry, thoracic gas volume and lung mechanics were determined. Importantly, lung structure was assessed histologically including quantitating alveolar numbers.\textsuperscript{412} They noted that the offspring from vitamin D deficient mothers had reduced lung function and volume and also a borderline reduced number of alveoli compared with the offspring from vitamin D sufficient mothers. There was no difference in the somatic growth. Zosky and coworkers\textsuperscript{412} claimed that this study provides the first direct mechanistic evidence for linking vitamin D deficiency and lung development. The reduced alveolar numbers could lead to reduced alveolar tethering points and potentially airway instability.\textsuperscript{413} Also, this work provides a relatively poor physiologic link to asthma, which is primarily thought of as a disease of the airways and bronchial smooth muscle, not of reduced lung volumes or reduced alveoli (alveolar simplification) as seen by Zosky and colleagues.\textsuperscript{374, 412} It could be postulated that the vitamin D pathway plays an important role in \textit{in utero} pulmonary development and maternal vitamin D deficiency contributes to the structural defects of the airway wall in children with STRA.

Eosinophils are major effector cells in the pathophysiology of allergic asthma and in response to allergic stimuli, eosinophils migrate from circulation the site
of inflammation where they secrete a range of cytokines, chemokines and mediators. Recently Hiraguchi et al\textsuperscript{414} investigated the direct effect of vitamin D on eosinophils in peripheral blood from healthy adults. They noted that \textit{in vitro} eosinophils express vitamin D receptor and vitamin D prolonged eosinophil survival and upregulated eosinophil surface expression of the C-X-C chemokine receptor 4 (CXCR 4) (the putative inhibitory chemokine receptor for eosinophils) as compared to the non-treated cells. Also, vitamin D counteracted the suppressive effect of IL-5 on CXCR4 while sharing the survival enhancing effect.\textsuperscript{414} Steroids have also been shown to upregulate CXCR4 expression in eosinophils.\textsuperscript{415} Thus, it has been postulated that vitamin D may synergistically promote the anti-inflammatory action of steroids by recruiting eosinophils away from the site of inflammation via CXCR4 upregulation.\textsuperscript{188, 414, 415} Airway eosinophilia predominates in childhood STRA\textsuperscript{119} and the majority of children with STRA are vitamin D deficient. Surprisingly, there was no relationship between serum vitamin D and serum, BAL or biopsy eosinophils in this thesis. This may be because most children with STRA are highly atopic, and the majority of STRA patients are vitamin D deficient. This may also be related to substantial anti-inflammatory treatment prescribed for these children or perhaps the local (BAL & mucosal) inflammation is related to the local (pulmonary) vitamin D axis rather than systemic (serum) vitamin D levels. This hypothesis needs to be tested in a larger cohort of children with STRA.

Chi et al\textsuperscript{416} in a recent study investigated effects of vitamin D in cord blood on immune function parameters. They used participants who were recruited as part of a prospective birth-cohort study designed to evaluate the environmental and immunologic causes of asthma in children. In the subset of participants at the Boston site, regulatory T-cells (CD4+CD25+FoxP3) were also quantified in 55 cord blood samples. The cord blood vitamin D levels were inversely related to the proportion of CD4+CD25+, and CD4+CD25+FoxP3 Treg cells. These results are different from the positive relationship between serum vitamin D and BAL CD4+FoxP3 cells found in children with STRA and in adults with moderate to severe asthma.\textsuperscript{181} These differences may be related to the distinct effects and requirement of vitamin D
in the prenatal period and in childhood. Chambers et al.\textsuperscript{181} have recently shown in a cross sectional study that the frequency of circulating CD4+FoxP3 Treg cells in peripheral blood is significantly lower in steroid resistant than in steroid sensitive adult asthmatics with comparable disease severity. Moreover, the majority of adults with severe asthma had vitamin D deficiency and importantly serum vitamin D levels strongly correlated with Foxp3\textsuperscript{+} Treg cell numbers in the peripheral blood. These data support the work of this thesis and suggest a strong positive correlation between systemic vitamin D status and circulating Tregs\textsuperscript{181} and local (BAL) Tregs. These paediatric and adult studies provide important \textit{in vivo} validation of previous \textit{in vitro} studies with human cells and evidence from animal models\textsuperscript{179, 417} suggesting that the vitamin D pathway plays an important role in enhancing the frequency of the CD4\textsuperscript{+}Foxp3\textsuperscript{+} Treg cell compartment in humans.

In an adult study, Xystrakis and colleagues\textsuperscript{163} obtained peripheral blood CD4+T cells from steroid sensitive and resistant asthmatics. They noted that the addition of vitamin D to peripheral cultures from steroid resistant asthmatics restored IL-10 secretion in response to dexamethasone to levels seen in steroid sensitive asthmatics. More importantly, they also showed that oral treatment with vitamin D (0.5mcg calcitrol once daily for 7 days) in steroid resistant asthmatics restored IL-10 responses to dexamethasone.\textsuperscript{163} In this thesis, an inverse association between serum vitamin D levels and the use of inhaled and oral corticosteroids has been shown in children with STRA. Children with the lowest serum vitamin D levels were prescribed significantly higher doses of ICS to control their symptoms. It could be postulated that lower vitamin D levels contribute to increasing asthma severity resulting in a concomitant need to escalate pharmacologic intervention. Reduced IL-10 protein expression found in the supernatant of peripheral cultures from paediatric asthmatics (MA and STRA) in this study is consistent with the defective IL-10 expression observed in adult asthma.\textsuperscript{379, 394} By definition children with STRA are steroid resistant and it was interesting to note that there was no significant difference in IL-10 secretion by PBMC between MA and STRA. In the present study a significant dexamethasone-induced IL-10 response was found in STRA, however in the adults\textsuperscript{163} responses were
lacking in steroid resistant asthmatics. In this thesis, addition of vitamin D in peripheral culture significantly enhanced IL-10 responses to dexamethasone, similar to the adult study. Importantly, in the present study, STRA children were further sub-classified according to their clinical response to a single dose of an intramuscular steroid (triamcinolone), as suggested by Bush et al\textsuperscript{134} using lung function response, symptom response and inflammatory response. Non-responders (SR) were classified as no improvement in any of the three domains; partial response (partial responders, PR) was improvement in one or two domains; and complete response (SS) was normalisation in all three domains.\textsuperscript{134} Stratification of in vitro responses in this manner revealed that IL-10 potentiation by vitamin D was highest in SS STRA, but still apparent in SR and PR as well. Xystrakis and colleagues noted an increase in IL-10 secretion with a combination of vitamin D and dexamethasone in culture of peripheral immune cells of steroid resistant asthmatics to the level seen in the steroid sensitive asthmatics. Interestingly, in this thesis, IL-10 production achieved with the combination of vitamin D and dexamethasone from PBMC culture of SR STRA remained less than that seen in cultures of cells from SS STRA. Further stratification of IL-10 secretion according to all 4 individual responses (symptom, lung function, FeNO\textsubscript{50} and sputum eosinophils) after intramuscular triamcinolone revealed similar trends. It was interesting to note that the IL-10 response was most marked in non-asthmatic controls and SS STRA. The differences between the adult and paediatric studies could be secondary to other factors (e.g. genetic) for defective IL-10, or perhaps dose, duration or route of vitamin D exposure (in vivo versus in vitro) may be important. The difference may also be related to different adult and paediatric phenotypes. CD8 depleted PBMC cell cultures employed here do have predominant CD4+ T cells but also other cells, including some antigen presenting cells, gamma-delta T cells, B cells, monocytes and dendritic cells, which may have been important in the results. The variations in the genes regulating steps in the vitamin D axis pathway could also explain differences between paediatric and adult studies. However, the number of children studied in this thesis was too small to look for genetic associations. Goleva et al\textsuperscript{418} showed that serum vitamin D status in asthmatic children correlated inversely with both ICS dose and in vitro steroid responsiveness but no significant association was seen for
adult asthmatics. This perhaps suggests that the effects were stronger in childhood asthmatics. This study was the first to compare an age-specific relationship between serum vitamin D levels and cellular sensitivity to in vitro steroid responsiveness in 53 children and 50 adult asthmatics. Although severity of asthma was not mentioned, 25% of subjects were not prescribed ICS and the remaining were on moderate dose of ICS (median daily dose of 320 microgram), suggesting they were mild-moderate asthmatics. The difference between the results reported in this thesis and the adult (Xystrakis et al\textsuperscript{163}) study and Goleva et al\textsuperscript{418} study suggest adult data should not be directly extrapolated to children and findings from oral vitamin D supplementation trials in adult asthmatic patients might not necessarily be applicable to children with asthma.

From this work it was difficult to elucidate whether IL-17A caused disease or had a protective role. Nanzer et al\textsuperscript{164} and Chambers and Hawrylowicz (unpublished data) have shown that steroids enhance IL-17A synthesis in CD4+ T cell culture from adult asthmatics. Also, in a retrospective analysis of the data, Nanzer et al\textsuperscript{164} observed a positive association between inhaled corticosteroid dosages and the amounts of blood PBMC IL-17A released in vitro (Pearson correlation \( r = 0.5, P <0.05 \)), supporting the possibility that at least some of the IL-17A produced by PBMCs in adult asthmatic patients results from corticosteroid exposure. In the work of this thesis, IL-17A secretion was significantly enhanced by dexamethasone treatment in vitro in STRA peripheral immune cell cultures. This is interesting given the successful use of steroids to control a large proportion of steroid-responsive asthmatic disease and it could be postulated that IL17A is protective in children with STRA. The exact role of IL-17A in asthma is also yet to be fully defined, with data from murine models indicating that IL-17A from gamma-delta T cells is protective in the resolution of acute airway inflammation.\textsuperscript{67} Importantly, the CD8-depleted PBMC cell culture employed in this thesis contains gamma-delta T cells, and they cannot be ruled out as a source of steroid-induced IL-17A. The work of this thesis suggests further investigation of the relationship between IL-17A and vitamin D in children with STRA is warranted.
In this thesis, serum vitamin D was positively associated with BAL IL-10 levels. This is the first in vivo study to demonstrate this association. Both corticosteroids and vitamin D have been proposed as therapeutic candidates to drive IL-10 expression in asthma. However it was not possible to identify the source of IL-10 detected in the BAL or perform functional work to clarify how reduced IL-10 levels in the BAL may contribute to asthma severity or steroid resistance. No other cytokines (IL-6, IL-17A, Th2 cytokines) measured in the BAL significantly correlated with serum vitamin D levels. Signature Th2-associated cytokines in BAL, for example IL-5 and IL-13 were largely undetectable. This is consistent with observations made in similarly characterised paediatric asthmatics, where Th2 cytokines were not prevalent and could only be detected in a small subgroup of individuals.

VDBP is a multifunctional transport protein and is bound via chondroitin sulfate proteoglycans with the surfaces of a large number of cells including neutrophils, fibroblasts, B cells, T cells and smooth muscle cells. VDBP has direct effects and also influences vitamin D availability. The influence of VDBP on vitamin D availability is complex and whether variation in the amount of circulating VDBP affects metabolism and availability of vitamin D is still not established. Direct effects of VDBP are known to influence immune functions including macrophage activation and neutrophil chemotaxis. Very little is known about the role of VDBP in asthma and atopy but it was reported to be significantly elevated in a mouse model of allergic asthma.

The work of this thesis has shown for the first time that local (BAL), but not systemic (serum), levels of VDBP are significantly higher in children with STRA and that BAL VDBP (but not serum VDBP) levels inversely correlate with asthma severity, as assessed by ACT, spirometry, and inhaled corticosteroid usage. The implications of these results with regard to vitamin D deficiency in STRA are unclear. It is known that polymorphisms in the VDBP gene are determinants of vitamin D status and importantly VDBP can regulate the bioavailability of vitamin D to cells. Polymorphisms of VDBP genes determine the activity of vitamin D on monocytes in vitro. Recently Lee et al have shown that proteomic analysis of BAL fluid from 67 mild to moderate adult asthmatics identified increased (more than fivefold)
concentrations of a VDBP when compared to BAL fluid from 22 adult controls. This is in agreement with the work of this thesis. Looking at the individual cells, the BAL VDBP levels of adults with asthma was very weakly positively correlated with BAL neutrophils ($r^2=0.2$) but not with BAL eosinophils or macrophages. However, mixed BAL inflammation (BAL eosinophils>3% and BAL neutrophils >10%) was associated with the highest VDBP levels and pauci-cellular (BAL eosinophils<3% and BAL neutrophils <10%) had lowest VDBP concentration. However, no correlation between airway inflammation (sputum/EB/BAL neutrophils or sputum/EB/BAL eosinophils) or BAL Th1, Th2, Th17 cytokines and BAL VDBP was noted in this study. As discussed earlier, this may be related to relatively small numbers and it is also possible that the substantial anti-inflammatory treatment prescribed for these children may have masked a relationship between VDBP and airway inflammation. Importantly, in a murine model, Lee et al. also showed that VDBP mRNA and protein levels were elevated in ovalbumin challenged mice and intranasal administration of anti-VDBP antibody dose-dependently reduced the ovalbumin induced enhancement of airway hyperresponsiveness. The neutralization of VDBP with anti-VDBP antibody led to significant improvements in airway (BAL) inflammatory cell recruitment, including eosinophils, neutrophils and lymphocytes, and decreased peribronchial infiltration of inflammatory cells and numbers of goblet cells, in a dose dependent manner. Anti-VDBP antibody also significantly reduced levels of BAL Th1 cytokine (IFNγ) and Th2 cytokines (IL-5, IL-5, IL-13). As blockage of VDBP with anti-VDBP antibody attenuated airway inflammation (both eosinophilic and neutrophilic), it could be postulated that VDBP is one of the global factors involved in asthma rather than restricted to neutrophil activation. This hypothesis was further supported by the finding inhibition of Th2 and Th1 cytokines by the anti-VDBP antibody. It was interesting to note global effects of VDBP blockade on airway inflammation and airway hyperresponsiveness but the underlying mechanisms are poorly understood. It could also be postulated that the changes in quantity or function of the VDBP could be accompanied by changes in the relative proportions of free and bound 25-hydroxyvitamin D, with the free proportion being the potential rate-limiting factor for $1\alpha,25$VitD3 production and thus influencing the availability of
vitamin D to local tissues (for example airway). As discussed before, vitamin D has immunomodulatory properties and can modulate allergic inflammation and the modulation of vitamin D by VDBP in the airways is a possible explanation for some of the effects of VDBP in asthma. The authors also postulated an interaction of VDBP with the complement pathway in the airways. VDBP is known to act with neutrophils to increase their chemotaxis to C5a peptide (discussed in chapter 6). Thus, airway neutrophilic inflammation induced by C5a may be dependent on the presence of VDBP in the airways. The modulation of vitamin D by VDBP in the airways is a possible explanation for the effects of VDBP in asthma. In summary, Lee et al’s work confirms the contribution of VDBP to allergic airway inflammation and remodelling and provides a new hypothesis which should lead to a study of the effects of blocking VDBP (perhaps with local pulmonary delivery only) as a therapeutic strategy. However, further studies are needed to fully understand the relationship of vitamin D and VDBP both locally in the lungs and systemically, in children with asthma.

7.6 Future work

Need for double blind randomized placebo-controlled clinical trial of vitamin D supplementation in children with STRA

This work shows very interesting associations between the vitamin D axis and the pathophysiology of asthma and strongly supports the need for a double blind, randomized, placebo-controlled clinical trial of vitamin D supplementation in children with STRA. This work also suggests the findings from vitamin D supplementation in adult asthmatic patients might not necessarily be generalized to children with STRA. Recently an IOM Committee conducted a comprehensive review of the evidence for both skeletal and extraskeletal outcomes of vitamin D supplementation. The Committee concluded that available scientific evidence supports a key role for vitamin D in skeletal health, however, for other outcomes the evidence was inconsistent and inconclusive as to causality, and insufficient to inform nutritional requirements. It was stated that clinical trial evidence for these
extraskeletal outcomes was limited and generally uninformative. The panel concluded that more research and reassessment of laboratory ranges for serum vitamin D should be done to avoid problems of both under and overtreatment.

The primary end point of the clinical trial should be whether vitamin D supplementation improves asthma control and reduces exacerbations in children with STRA. As discussed earlier in this chapter, vitamin D deficiency is associated with more viral respiratory infections in patients with asthma, and viruses often trigger exacerbations in STRA, and may even reduce steroid sensitivity. This work has shown an association between low serum vitamin D levels and acute exacerbations in children with STRA, however whether this is due to an altered response to infections is yet to be elucidated. Bronchial epithelial cells cultures from STRA children have profoundly impaired interferon-β and interferon-λ mRNA and protein in response to rhinovirus as compared to the healthy controls suggesting innate anti-viral responses to rhinovirus are impaired in the STRA. It would be interesting to investigate the effect of adding vitamin D to bronchial epithelial cell cultures from children with STRA and determine anti-viral responses. Interventional studies are also needed to study the interaction between viruses, vitamin D and asthma exacerbations in children with STRA during an acute exacerbation using either blood or sputum to assess the primary role of vitamin D in modulating the severity and/or duration of illness.

The clinical trial would likely have to be a multi-centre UK study. This thesis and previous work also reinforces the need for a very detailed evaluation of potentially reversible factors (e.g allergen exposure, compliance) to ensure as far as possible that the children have truly severe therapy resistant disease. Compliance needs to be evaluated by prescription pick up and either directly observed therapy or inhalers with microchips which show exactly when doses were taken would have been used. Paediatric respiratory nurses also need to check for the availability and utilisation of medications in the home and school. Also, allergen exposure and passive smoke exposure needs to be assessed by a scientific sampling. This detailed
assessment is similar to the recently proposed international approach.\textsuperscript{423-425}

From the work of this thesis, it was difficult to propose a unifying hypothesis to account for the effects of vitamin D; it would be pertinent to study immunopathology following vitamin D supplementation. However it is important to recognize that simple deficiency of serum vitamin D does not necessarily result in loss of activity of genes activated by the active form of vitamin D.\textsuperscript{426} For example, as discussed in chapter 1 section 1.4.12 the antimicrobial peptide cathelicidin is present in skin, monocytes and lung and is strongly induced by increased vitamin D in cell culture. However, low vitamin D levels are associated with increased parathyroid hormone levels (discussed in chapter 1 section 1.4.2) and recently, parathyroid hormone has also been found to induce cathelicidin, thus counteracting the potential for decreases in levels of this antimicrobial peptide and maintaining appropriate immune defence against infection.\textsuperscript{426, 427} This hypothesis of the existence of systems to compensate for local vitamin D insufficiency in airways and immune function supports the important role of this molecule in immunity and sheds light on the difficulty in consistently detecting clinical phenotypes associated with specific systemic serum vitamin D values.\textsuperscript{426, 427} Paul et al\textsuperscript{409} recently reviewed the potential protective effects of vitamin D on asthma morbidity, and these are summarized in Figure 7.2.
The question of how much vitamin D is needed to maintain pulmonary health should also be investigated further in clinical trials in children. It should be noted that there may be different levels appropriate for different organ systems, and it is not right to extrapolate between systems. It will be helpful when technology is available to readily assess vitamin D and other metabolites of the vitamin D axis locally in BAL, sputum and EB. Vitamin D deficiency is defined by most experts as a 25(OH)D3 level of less than 50 nmol/L (20ng per milliliter) which is based entirely on the levels required to maintain bone health.\textsuperscript{185, 190-194} The current IOM guidelines from 2011 recommended a lower limit of 50 nmol/L (20 ng per milliliter) and an upper limit of 125 nmol/L (50 ng/ml).\textsuperscript{203} This upper limit was not based on concern for outright vitamin D toxicity which only occurs at much higher levels.\textsuperscript{428} Rather, the recommendation is based on the U-shape of some studies\textsuperscript{429-431} where both vitamin D levels at the low end and high end exhibited elevated risks of health outcomes (neonatal vitamin D status and risk of schizophrenia\textsuperscript{429}, serum vitamin D levels and risk of pancreatic cancer\textsuperscript{430} and serum total IgE levels\textsuperscript{202}). The capacity to compensate for lower vitamin D levels results in a nonlinear relationship between intake and biological
effects, however, explaining these findings is challenging and needs further investigations in a large prospective controlled study in children with asthma. Again, this study would have to be a multi-centre study, across the UK, and likely international.

The doses of supplementation should ideally not be chosen randomly but pragmatically stratified based on individual vitamin D status or genotype and response to supplementation should also be monitored. The optimal dose of vitamin D to maintain pulmonary health in children with STRA perhaps needs to be tested first by more mechanistic studies in peripheral and local (BAL & bronchial) cell cultures (in vitro and in vivo). This will ensure negative results are not related to inadequate supplementation.

**Further mechanistic work**
The interactions of vitamin D with cells of immune system are summarized in Figure 7.3
**Figure 7.3 Overview of vitamin D and its interactions with cells of immune system.** Reproduced from ‘Vitamin D in Allergic disease’

**Measuring vitamin D levels in BAL and sputum**

The synthesis of active form of vitamin D, 1α,25VitD3 is dependent on serum 25(OH)D3 levels, the primary circulating form of vitamin D. Until recently, it was thought that the conversion to 1α,25VitD3 occurred only in the kidneys, but increasing evidence indicates that the cells of most organs express the vitamin D receptor and have the capacity to synthesize vitamin D locally. The enzyme (25-hydroxyvitamin D-1alpha-hydroxylase) has also been recently found in epithelial cells of the lung, immune cells such as lung macrophages, monocytes, dendritic cells, and T lymphocytes. As a result, there are several pathways that can influence an individual’s ability to produce and respond to adequate local quantities of the active form of vitamin D which is probably the true determinant of physiological function. However, very little is known regarding the levels and clinical significance of vitamin D within lung tissues or along mucosal surfaces. It would be important to
determine the clinical significance of local activation and vitamin D levels in children and the relationship to circulating levels. Importantly, in this thesis local (BAL) VDBP not systemic (serum) VDBP was associated with asthma severity in children with STRA, reinforcing the importance of assessing the vitamin D axis locally. Recently, Liu et al. have developed techniques to measure vitamin D levels in BAL. They noted an association of vitamin D with airway (BAL) inflammation following allergen challenge in adults. Also, Wood et al have recently developed techniques to measure VDBP in sputum of adults with COPD. It would be valuable to measure vitamin D (both 25(OH)D3 and 1.25(OH)D3, although technical challenges still remain) levels locally in the BAL and sputum and assess relationships with airway inflammation, remodelling and steroid responsiveness.

**Importance of raised BAL VDBP in STRA**

The cross-sectional design of this study cannot attribute causality, but strongly suggests that further study of VDBP as a potential biological modulator and functional work to clarify how VDBP may contribute to asthma severity should be a key part of the future investigation of supplementation of vitamin D in lung disease. It could be that VDBP is a marker of disease severity and/or steroid resistance, and this possibility needs to be investigated. It is plausible that in asthma excessive airway VDBP drives alveolar macrophages into a more inflammatory state, diverting them from their tolerogenic role. This could be investigated by using an efferocytosis assay, which assesses macrophage activation by measuring the proportion of macrophages that engulf apoptotic neutrophils. As discussed earlier, VDBP had dual potential functions (regulating vitamin D availability and own direct immunological effects). The influence of VDBP on vitamin D availability is complex and is still poorly understood and further studies are needed to assess the effects of variation in concentrations of airway (sputum) VDBP.

Focusing exclusively on total serum or other fluid vitamin D levels is too simplistic, and overlooks the role of VDBP in determining free vitamin D levels, a consequence of the basic biochemical properties of VDBP. It would be valuable to assess the impact of VDBP on free and total vitamin D levels. It
could be postulated that the high VDBP levels might lead to a reduction in free vitamin D levels leading to the increased risk. Under these kinds of circumstances, total vitamin D levels would not provide a complete picture of the vitamin D status of the individual.

**IL-17A and vitamin D in children with STRA**

A further pathway that should be explored in children with STRA is the Th17 axis and its relationship with vitamin D. There was a trend (non-significant) for IL-17A to be elevated in BAL of some children with STRA, and IL-17A secretion in STRA PBMC was significantly enhanced by dexamethasone treatment *in vitro* and there is recent evidence that this may drive steroid resistant disease. Nanzer and colleagues have shown that adults with severe asthma exhibit increased levels of Th17 cytokines, which are not inhibited by steroids, and vitamin D inhibited Th17 cytokine production identifying novel properties of vitamin D in adult asthmatic patients.

A study specifically investigating the role of IL-17A and vitamin D in paediatric STRA and their impact on steroid responsiveness needs to be undertaken in a larger cohort of childhood STRA. The cellular source of IL17A needs to be identified by flow cytometry in BAL and immunohistochemistry in EB and its secretion from epithelial cell cultures would help to elicit whether it is only released from inflammatory cells or whether structural cells also contribute to its production in STRA. This is particularly important because a number of anti-IL17 antibody strategies are being trialed in rheumatoid arthritis and adult asthma. However, before this approach is extended into paediatric asthma, it is important to determine whether IL-17A is driving asthma, or in fact may be protective. It may well be that the cellular source of IL-17A is important in determining its effects.

**7.7 Summary**

In summary, the work of this thesis highlights the potential pathophysiological importance of vitamin D in children with STRA. The original hypotheses of this thesis were partly proven, in that children with STRA have significantly lower serum vitamin D levels and these were associated with worse lung function,
poorer asthma control and more steroid use. Importantly, low serum vitamin D levels were associated with increased ASM mass. Defective IL-10 protein expression in BAL and peripheral immune cell cultures was noted in children with asthma. The circulating levels of serum vitamin D directly correlated with IL-10 levels and CD4+FoxP3+ cells in BAL. Moreover, the inclusion of vitamin D in PBMC culture enhanced IL-10 responses to dexamethasone.

This work also highlights the importance of evaluating not just systemic (serum) vitamin D but also the pulmonary vitamin D axis. Other findings are hypothesis generating, and need to be validated by larger and more extensive studies. These include the role of IL-17A and vitamin D in children with STRA, and the role of pulmonary VDBP as a therapeutic target.

This thesis has prepared the ground for a double blind, randomized, controlled clinical trial of vitamin D supplementation in children with STRA in collaboration with other centres to allow recruitment of sufficient numbers of children. Realistically, this will be the best test of whether the vitamin D axis is relevant to the pathophysiology of STRA, or whether the associations described here and elsewhere are in fact of no clinical significance.
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Appendix 1. Difficult Asthma Proforma

Stage 1

**DIFFICULT ASTHMA PROTOCOL**

<table>
<thead>
<tr>
<th>Name</th>
<th>Hospital Number</th>
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**BACKGROUND**

- Referring Consultant (circle): AB / CH / IBL / JD / MRO / NW / SS / other
- DOB
- Age (decimal years)
- Date of referral
- Sex (circle): male / female

**Birth History**

- Gestational age: ___ weeks
- Mode of delivery: vaginal / instrumental / elective LSCS / emergency LSCS
- Birth weight: ___ kg
- IUGR: yes [ ] no [ ]
- CLD of prematurity: yes [ ] no [ ]
- (Or dependent at 28 days or 36 weeks CA)
- Ventilated after birth: yes [ ] no [ ]
- Oxygen after birth: yes [ ] no [ ]
- Infant feeding: breast / bottle / mixed
- Duration of breast feeding: ___ weeks
- Age weaned (introduction of solids): ___ months
- Exclusion diet: yes [ ] no [ ]
- details

**Clinical Features**

- Age of first reported symptoms: ___ months / years
- Courses of steroids in past year: ___ Date of last systemic steroids: ___
- Admissions in past year: ___ Date of last admission: ___
- Admissions to PICU ever: yes [ ] no [ ]
- Intubated for asthma ever: yes [ ] no [ ]
- Viral induced symptoms: yes [ ] no [ ]
- Viral illness in past 2 weeks: yes [ ] no [ ] possibly [ ]
- Persistent productive cough: yes [ ] no [ ]
Exercise induced symptoms: yes □ no □
Reduced exercise tolerance: yes □ no □ doesn’t exercise □
Main trigger (circle max 3): viral illness / exercise / anxiety / pets / pollen / dust / cigarette smoke / other allergy / other __________
Asthma pattern (circle 1): persistent symptoms / acute exacerbations only / both
Main symptoms (circle any): wheeze / cough / SOB / other ________________
Nocturnal symptoms: yes □ no □
Sudden deterioration: yes □ no □
Known food allergy: yes □ no □ details □
Known other allergy: yes □ no □ details □
History of eczema: yes □ no □
History of hayfever: yes □ no □
History of rhinitis: yes □ no □
Details of allergy symptoms:

Family and Social History
Family history of asthma: mother / father / siblings / none
Family history of eczema: mother / father / siblings / none
Family history of hayfever: mother / father / siblings / none
Pet (type and number): 1. ________________ 2. ________________ 3. ________________ 4. ________________
Parental smoking (circle): mother / father / both / other household member / none
Location of smoking: inside / outside
Smoking during pregnancy: mother / father / both / neither
Active smoking: yes □ no □
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<th>Question</th>
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<td>Main trigger (circle max 3):</td>
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<td>Viral illness / exercise / anxiety / pets / pollen / dust / cigarette smoke / other allergy / other</td>
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<td>Asthma pattern (circle 1):</td>
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<td>Persistent symptoms / acute exacerbations only / both</td>
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<td>Main symptoms (circle any):</td>
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<td>Wheeze / cough / SOB / other</td>
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<td>Nocturnal symptoms:</td>
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<tr>
<td>Sudden deterioration (brittle asthma)</td>
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<tr>
<td>Known food allergy:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known other allergy:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of eczema:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of hayfever:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of rhinitis:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Details of allergy symptoms:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Family and Social History**

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family history of asthma:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Family history of eczema:</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Family history of hayfever:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pet (type and number):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2.</td>
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<td>3.</td>
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<td></td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Parental smoking (circle):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location of smoking:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking during pregnancy:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active smoking:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Exercise induced symptoms: yes ☐ no ☐
Reduced exercise tolerance: yes ☐ no ☐ doesn’t exercise ☐
Main trigger (circle max 3): viral illness / exercise / anxiety / pets / pollen / dust / cigarette smoke / other allergy / other _______________________
Asthma pattern (circle 1): persistent symptoms / acute exacerbations only / both
Main symptoms (circle any): wheeze / cough / SOB / other _______________________
Nocturnal symptoms: yes ☐ no ☐
Sudden deterioration: yes ☐ no ☐
Known food allergy: yes ☐ no ☐ details _______________________
Known other allergy: yes ☐ no ☐ details _______________________
History of eczema: yes ☐ no ☐
History of hayfever: yes ☐ no ☐
History of rhinitis: yes ☐ no ☐

Details of allergy symptoms:

Family and Social History
Family history of asthma: mother / father / siblings / none
Family history of eczema: mother / father / siblings / none
Family history of hayfever: mother / father / siblings / none
Pet (type and number): 1. ______________________ 2. ______________________ 3. ______________________ 4. ______________________
Parental smoking (circle): mother / father / both / other household member / none
Location of smoking: inside / outside
Smoking during pregnancy: mother / father / both / neither
Active smoking: yes ☐ no ☐
DIFFICULT ASTHMA PROTOCOL

Name ___________________________ Hospital Number __________________

Induced Sputum
Baseline FEV1 (post salbutamol) __________ 20% fall in FEV1 __________

<table>
<thead>
<tr>
<th>Time</th>
<th>Duration</th>
<th>Completed</th>
<th>FEV1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
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<td></td>
</tr>
<tr>
<td>10 mins post induction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample produced: __________ Comments: __________

Cell differential:

<table>
<thead>
<tr>
<th></th>
<th>Eosinophils</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Epithelial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Psychosocial Questionnaire
Completed: yes [ ] no [ ] [ ] Comments: __________
Psychology referral made: yes [ ] no [ ] [ ] Comments: __________

Prescription
GP and/or pharmacist contacted: yes [ ] no [ ] [ ] Comments: __________
% uptake: <50 [ ] 50-80 [ ] >80 [ ] Comments: __________

School
School contacted: yes [ ] no [ ] [ ] Comments: __________
School attendance (%): __________
# STAGE 1 ASTHMA CONTROL TEST

1. In the past *4 weeks*, how much of the time did your *asthma* keep you from getting as much done at work, school or home?

<table>
<thead>
<tr>
<th>All of the time</th>
<th>Most of the time</th>
<th>Some of the time</th>
<th>A little of the time</th>
<th>None of the time</th>
</tr>
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<td></td>
</tr>
</tbody>
</table>

2. During the past *4 weeks*, how often have you had shortness of breath?

<table>
<thead>
<tr>
<th>More than once a day</th>
<th>Once a day</th>
<th>3 to 6 times a week</th>
<th>Once or twice a week</th>
<th>Not at all</th>
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</tr>
</tbody>
</table>

3. During the past *4 weeks*, how often did your *asthma* symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?

<table>
<thead>
<tr>
<th>4 or more nights a week</th>
<th>2 or 3 nights a week</th>
<th>Once a week</th>
<th>Once or twice in the past 4 weeks</th>
<th>Not at all</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. During the past *4 weeks*, how often did you have to use your rescue (blue) inhaler or nebuliser medication?

<table>
<thead>
<tr>
<th>3 or more times a day</th>
<th>1 or 2 times a day</th>
<th>2 or 3 times per week</th>
<th>Once a week or less</th>
<th>Not at all</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. How would you rate your *asthma* control over the past *4 weeks*?

<table>
<thead>
<tr>
<th>Not controlled at all</th>
<th>Poorly controlled</th>
<th>Somewhat controlled</th>
<th>Well controlled</th>
<th>Completely controlled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total score: 

Completed by (circle): child / parent / both
<table>
<thead>
<tr>
<th>Name</th>
<th>Dose</th>
<th>Route</th>
<th>Frequency</th>
</tr>
</thead>
</table>

**Medicines previously tried**

**STAGE 1**

**STAGE 2**

**STAGE 3**

Other comments re: medication
### Stage 2

**DIFFICULT ASTHMA PROTOCOL**

Name: ___________________________ Hospital Number: ___________________________

**STAGE 2**

- Height: __________ Centile: __________ Weight: __________ Centile: __________
- Viral infection in past 2 weeks: yes / no / possibly
- High dose systemic CS in past 2 weeks: yes / no

**Exhaled Nitric Oxide**

<table>
<thead>
<tr>
<th>Flow</th>
<th>Attempt</th>
<th>$F_2$NO (ppb)</th>
<th>Measured Flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>50ml/s</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100ml/s</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
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<td></td>
<td>3</td>
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<tr>
<td></td>
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<td></td>
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<tr>
<td>200ml/s</td>
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<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td></td>
<td></td>
</tr>
<tr>
<td>260ml/s</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Ambient NO**

- $J_{NO}$
- $C_{Nit}$

**Lung Function and Bronchodilator Reversibility**

<table>
<thead>
<tr>
<th></th>
<th>FVC</th>
<th>FEV1</th>
<th>FEV1/FVC</th>
<th>PEF</th>
<th>FEF 25-75</th>
<th>FEF 25</th>
<th>FEF 50</th>
<th>FEF 75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre salbutamol</td>
<td>measured</td>
<td>% predicted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post salbutamol</td>
<td>measured</td>
<td>% predicted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% reversibility</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 1 -
**STAGE 2**

**Induced Sputum**

Baseline FEV1 (post salbutamol): [ ]

20% fall in FEV1: [ ]

<table>
<thead>
<tr>
<th>Time</th>
<th>Duration</th>
<th>Completed</th>
<th>FEV1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
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</tr>
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<td></td>
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</tr>
</tbody>
</table>

Sample produced: [ ]

Cell differential:

<table>
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<th>Eosinophils</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Epithelial</th>
</tr>
</thead>
</table>

Absolute count: [ ]

% of total: [ ]

**Bronchoscopy**

Macroscopic appearance (circle): normal / structural abnormality / increased mucus / inflammation / inflammation and mucus / other

Comments: [ ]

**Biopsy**

Number of biopsies taken: [ ]

Quality (circle): evaluable / non-evaluable

RBM score (0-3): [ ]

Eosinophil score (0-6): [ ]

Neutrophil score (0-6): [ ]

Smooth muscle (circle): present / absent

Comments: [ ]
DIFFICULT ASTHMA PROTOCOL

Name ___________________________ Hospital Number ___________________

STAGE 2

BAL
BAL eosinophils (%): □ □
BAL neutrophils (%): □ □

Fat laden macrophages (circle): none / scanty / moderate / large

Virology: positive □ negative □
Microbiology: positive □ negative □
Organism (circle): haemophilus / pneumococcus / pseudomonas / staph aureus / brucellosis catarrhalis / other

pH Study
% time pH<4: □ □
corrected % time pH<4 (dual probe): □ □

Anti-reflux medication started: yes □ no □

Full blood count
Hb □ □ WCC □ □ Eosinophils □ □ % eosinophils □ □

Immunoglobulins

<table>
<thead>
<tr>
<th>Total IgG</th>
<th>IgG1</th>
<th>IgG2</th>
<th>IgG3</th>
<th>IgG4</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal/low/high</td>
<td>normal/low/high</td>
<td>normal/low/high</td>
<td>normal/low/high</td>
<td>normal/low/high</td>
<td>normal/low/high</td>
<td>normal/low/high</td>
</tr>
</tbody>
</table>

RASTs

<table>
<thead>
<tr>
<th>Allergen</th>
<th>IU</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grasses</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Cat</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Dog</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>HDM</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Aspergillus</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Trees</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Peanut</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Egg</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Alternaria Alternata</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Cladosporium</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td>positive / negative</td>
</tr>
<tr>
<td>Cockroach</td>
<td></td>
<td>positive / negative</td>
</tr>
</tbody>
</table>

Total IgE: □ □
STAGE 2

Serum steroid levels (if on prednisolone or triamcinolone)
Prednisolone □
Cortisol □

Theophylline level (if on theophylline or aminophylline)
Theophylline □

Vaccine Response

<table>
<thead>
<tr>
<th>Result</th>
<th>Pertussis</th>
<th>Tetanus</th>
<th>HiB</th>
<th>Diphtheria</th>
<th>Pneumococcus</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/abnormal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Triamcinolone
Administered: yes □ no □
Dose given: 80mg □ 40mg □ other (specify) □

Other comments:
**DIFFICULT ASTHMA PROTOCOL**

Name: ___________________________ Hospital Number: ___________________________

**STAGE 2**

**ASTHMA CONTROL TEST**

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</table>

Total score: [ ]

Completed by (circle): child / parent / both

- 5 -
## Stage 3

### Exhaled Nitric Oxide

<table>
<thead>
<tr>
<th>Flow</th>
<th>Attempt</th>
<th>$F_NO$ (ppb)</th>
<th>Measured Flow</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>100ml/s</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
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<tr>
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<td>3</td>
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<td>3</td>
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<td></td>
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<tr>
<td></td>
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</tbody>
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**Ambient NO**  

**$J_{NO}$**  

**$C_{av}$**

### Lung Function and Bronchodilator Reversibility

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<th>PEF</th>
<th>FEF 25-75</th>
<th>FEF 25</th>
<th>FEF 50</th>
<th>FEF 75</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pre</strong></td>
<td>measured</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% predicted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Post</strong></td>
<td>measured</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>% predicted</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**% reversibility**

-1-
## Induced Sputum

**STAGE 3**

Baseline FEV1 (post salbutamol)  

<table>
<thead>
<tr>
<th>Time</th>
<th>Duration</th>
<th>Completed</th>
<th>FEV1</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mins post induction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sample produced  
Comments

**Cell differential:**

<table>
<thead>
<tr>
<th>Absolute count</th>
<th>Eosinophils</th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>Lymphocytes</th>
<th>Epithelial</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**High Resolution CT Scan** (if indicated)

Date: __________

Brief description: __________

Summary (circle):  
normal / airway dilatation / air trapping / bronchial wall thickening / bronchiectasis / small airways disease / large airway disease / structural abnormality

**Asthma Control in past 4 weeks**

Courses of oral steroids  
Hospital Admissions

GP attendances  
Diary completed

A&E attendances  
Viral infections

Viral infection in past 2 weeks: yes / no / possibly

Description of exacerbations
# Difficult Asthma Protocol

**Stage 3 Asthma Control Test**

1. In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or home?

<table>
<thead>
<tr>
<th>All of the time</th>
<th>Most of the time</th>
<th>Some of the time</th>
<th>A little of the time</th>
<th>None of the time</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

2. During the past 4 weeks, how often have you had shortness of breath?

<table>
<thead>
<tr>
<th>More than once a day</th>
<th>Once a day</th>
<th>3 to 6 times a week</th>
<th>Once or twice a week</th>
<th>Not at all</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

3. During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?

<table>
<thead>
<tr>
<th>4 or more nights a week</th>
<th>2 or 3 nights a week</th>
<th>Once a week</th>
<th>Once or twice in the past 4 weeks</th>
<th>Not at all</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

4. During the past 4 weeks, how often did you have to use your rescue (blue) inhaler or nebuliser medication?

<table>
<thead>
<tr>
<th>3 or more times a day</th>
<th>1 or 2 times a day</th>
<th>2 or 3 times per week</th>
<th>Once a week or less</th>
<th>Not at all</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

5. How would you rate your asthma control over the past 4 weeks?

<table>
<thead>
<tr>
<th>Not controlled at all</th>
<th>Poorly controlled</th>
<th>Somewhat controlled</th>
<th>Well controlled</th>
<th>Completely controlled</th>
</tr>
</thead>
<tbody>
<tr>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

**Total score:** □

Completed by (circle): child / parent / both
DIFFICULT ASTHMA PROTOCOL

Name ____________________________ Hospital Number ____________________________

Medication Strategies Recommended

- Optimise steroids
- Reduce steroids
- High dose LABA
- Azathioprine
- Cyclosporin
- Methotrexate

S/c terbutaline
Azithromycin
Omalizumab
Optimise anti-reflux therapy
Other

Summary of findings

- Allergen presence in sensitised subject
  * +ve SPT / RAST in asth pts; ++/+++ SPT / RAST confirmed and lack of avoidance measures; +++ SPT / RAST to mould and dust or damp house

  Non atopic (all SPTs and RASTs –ve)
  Significant comorbidity (details below)

- Major causal psychosocial issues

  Significant comorbidity (details below)
  Not asthma (details below)

- Suspected VCD / disordered breathing

  No cause for poor symptom control found

- Passive / active smoking

  Ongoing poor adherence (circle):
  yes / no / possibly / not known

Other comments (including discussion at DA meeting)

— 5 —
Appendix 2. Sputum Processing Form

<table>
<thead>
<tr>
<th>Specimen Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tube weight</th>
<th>Total weight (tube + sample)</th>
<th>Sample weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Whole sputum (g)</th>
<th>Selected sputum – tube 1 (g)</th>
<th>Volume of DTT added (ml)</th>
<th>Volume of PBS added (ml) [total of 1 in 9 dilution]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 x B</td>
<td></td>
<td>4 x B</td>
<td></td>
</tr>
</tbody>
</table>

**Counts: Improved Neubauer Haemocytometer**

Resuspend cell pellet in 1ml of PBS, then dilute 10µl into a known volume of Trypan Blue e.g. 10µl cells + 40µl Trypan Blue for a 1 in 5 dilution (i.e. dilution factor = 5) or 1 in 10 Trypan Blue (dilution factor = 2)

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>a</th>
<th>Viable</th>
<th>Dead leukocytes</th>
<th>Squamous Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower left 4x4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle 5x5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper right 4x4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>b</td>
<td>c</td>
<td>d</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leukocyte count (x 10⁴/ml)</th>
<th>a x e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte viability (%)</td>
<td>(b / e) x100</td>
</tr>
<tr>
<td>Squamous cells (%)</td>
<td>(d / (d + e)) x 100</td>
</tr>
</tbody>
</table>

**Cytospin preparation**

Dilution required to prepare 2x10⁵ leukocytes/ml

100µl of cells at 2x10⁵ leukocytes/ml in PBS per cytospin block

<table>
<thead>
<tr>
<th>Cell Differential</th>
</tr>
</thead>
<tbody>
<tr>
<td>counts for 400 leukocytes</td>
</tr>
<tr>
<td>% of 400 leukocytes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Absolute</th>
<th>Neu</th>
<th>Mac</th>
<th>Lym</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>% of Total</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
</tbody>
</table>

**Specimen Identification Number**

_____µl sputum supernatants store at -80°C

Number

<table>
<thead>
<tr>
<th>Slides unfixed/-70°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slides MeOH/Diff-Quik/mount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
</tr>
</tbody>
</table>

273
Appendix 3  Parent information (asthma)

PARENT/GUARDIAN INFORMATION SHEET

Vitamin D level and asthma control in children

Thank you very much for taking the time to read this leaflet.

We would be very grateful if you would consider allowing your child to take part in a research study. Before you decide whether your child should take part, it is important for you and your child to understand why the research is being done and what it will involve. We would therefore be grateful if you would take the time to read the following information and discuss it with your child, friends, relatives and your child’s GP if you wish. Also, please feel free to ask the doctor doing this research or your child’s consultant here at the Brompton Hospital if there is anything that is not clear, or if you would like more information.

Why is the study being done?
This study will look at ways to improve asthma control in children with severe disease that is unresponsive to steroid treatment.

Inhaled steroids are the main treatment that we use for asthma. However, they do not always work well in children with severe asthma. These children often have a poor quality of life because of inadequate asthma control. Making steroids work better in these children would represent a major medical advance. We do not know why steroids work so badly in some children with severe asthma. We know that adults with severe asthma produce lower levels of a natural chemical messenger, interleukin-10, in response to steroids. Also, when vitamin D (sometimes called sunshine vitamin) is added with steroids to blood cells from severe asthmatics they respond better to steroids. However, this work has not yet been done in children.

In this study, we hope to find out how much of the chemical interleukin 10 is produced in response to steroids in children with severe asthma and compare that to children without asthma. We also want to find out whether addition of vitamin D to blood cells from children with severe
asthma increases the production of the helpful Interleukin -10 messenger. If increased production of Interleukin-10 is achieved in the presence of vitamin D, then this may result in severe asthmatic children becoming more responsive to steroid treatment. If successful, then the experiments proposed in this study may lead to a new treatment (vitamin D supplementation) for children with severe, steroid resistant asthma.

**Why has my child been asked to take part?**
We would like to invite your child to participate in this study because he/she has severe asthma and is looked after by the doctors at Royal Brompton Hospital which is where this study is being done.

**What does the study involve?**
If you are happy for your child to take part in this study, we will do a blood test and take up to 5 teaspoons of blood from your child. We will try to take the blood whilst they are undergoing a general anesthetic (for their operation) or at the same time as they are having any planned blood tests (clinically indicated) so as to avoid any additional distress to your child. If your child is not anaesthetized when we do the blood test we will use an anaesthetic (numbing) cream on their skin (like EMLA or AMETOP) to minimize any pain or discomfort.

This blood will be taken to the laboratory to analyze levels of various chemical messengers and molecules like interleukin-10 and vitamin D levels. We will also perform tests in the laboratory to find out whether the active form of vitamin D can magnify the helpful (interleukin-10) messenger response when steroids are given.

We will also ask you some questions about your child’s diet in order to get an idea of their daily Vitamin D intake. As vitamin D status in children is affected by developmental status, we will also ask your child to indicate which developmental stage they are at using a short picture questionnaire.

**Are there any risks or side effects involved in taking part?**
The main risks with blood tests are pain, discomfort or bruising at the site where the needle goes in. These complications are usually minor and go away shortly after the tests are done. As the amount of additional blood needed is small, it will not adversely affect your child. Your child’s treatment will be unaffected regardless of whether he/she takes part in the study.
Who is organizing and funding the research?
This study is organized by the doctors from Royal Brompton Hospital and Imperial College, London in collaboration with Kings College, London. Equipment needed for the study is funded by a research grant from The British Medical Association. No doctor conducting the research will be given any remuneration for including your child in this study.

What does my child gain from being in the study?
There is no immediate benefit to your child from taking part in this study.

What happens when the research study stops?
Your child’s treatment will be continued during and after this study and will not be affected by your child’s participation in this study.

What if something goes wrong?
Imperial College holds insurance policies which apply to this study. If your child experiences harm or injury as a result of taking part in this study, you and your child will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation.

If your child is harmed due to someone's negligence, then you may have grounds for legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Chief Investigator (Dr Atul Gupta telephone number 02073528121, extn 2257). The normal National Health Service complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact Imperial College Clinical Research Governance Office.

Will my child’s taking part in this study be kept confidential?
All information which is collected about your child during the course of the research will be kept strictly confidential. Any information about your child which leaves the hospital will have your name and address removed so that your child will not be identified.

Your child’s GP will be notified of their participation in the trial but we will seek your agreement prior to doing this.

What will happen to the results of the research study?
The results of the research will be analysed by the research team and will be presented at research meetings. The results will be published in medical journals but without identification of any participants in the report. All information collected will be confidential, and no information will appear in any medical publication that will allow your child to be identified.

If you would like to find out about the publication, please let us know and we will send you a copy once the results are published. Please remember that it can take at least a year after the end of the study to have the results published.

Who has reviewed the study?
The study has been given a favourable opinion by the Brompton, Harefield and NHLI Research Ethics Committee.

Does my child have to take part?
It is up to you and your child to decide whether or not to take part. If you and your child do decide to take part you will be given this information sheet to keep and you will be asked to sign a consent form. If your child decide to take part you are still free to withdraw your child from the study at any time without giving a reason. The care and the management your child will receive will be exactly the same and will not be affected by your participation in this study.

Who can I contact for further Information?
If you need further information about this study, you can contact Dr Atul Gupta on 02073528121, extn 2257.

Thank you for reading this document. You and your child are encouraged to ask any questions you may have. Please remember that you may withdraw from this study at any time and without giving any reason. Please understand that your child need not take part in this trial if you do not wish to. If your child does take part, they may withdraw at any time, and does not need to give any reason for doing so.

Thank you very much for your kindness in considering this project.
Appendix 4 Parent information (control)

Royal Brompton & Harefield NHS Trust

PARENT/GUARDIAN INFORMATION SHEET

VITAMIN D LEVEL AND ASTHMA CONTROL IN CHILDREN

Thank you very much for taking the time to read this leaflet.

We would be very grateful if you would consider allowing your child to take part in a research study. Before you and your child decide whether to take part, it is important for you and your child to understand why the research is being done and what it will involve. We would therefore be grateful if you would take the time to read the following information and discuss it with your child, friends, relatives and your child’s GP if you wish. Also, please feel free to ask the doctor doing this research or your child’s consultant here at the Brompton Hospital if there is anything that is not clear, or if you would like more information.

Why has my child been asked to take part?
As a parent of a non-asthmatic child, we would like to invite you to consider allowing your child to participate in a research project which is trying to find ways to improve asthma control in children with severe disease. In order for us to be able to fully understand and accurately interpret our results, it is essential that we compare the measurements we obtain from asthmatics to those made in non-asthmatic children. We would therefore be most grateful if you and your child would consider taking part in this project.

Why is the study being done?
This study will look at ways to improve asthma control in children with severe disease that is unresponsive to steroid treatment.

Inhaled steroids are the main treatment that we use for asthma. However, they do not always work well in children with severe asthma. These children often have a poor quality of life because of inadequate asthma control. Making steroids work better in these children would represent a major medical advance. We do not know why steroids work so badly in some children with severe asthma. We know that adults with severe
asthma produce lower levels of a natural chemical messenger, interleukin-
10, in response to steroids. Also, when vitamin D (sometimes called
sunshine vitamin) is added with steroids to blood cells from severe
asthmatics they respond better to steroids. However, this work has not
yet been done in children.

In this study, we hope to find out how much of the chemical interleukin 10
is produced in response to steroids in children with severe asthma and
compare that to children without asthma. We also want to find out
whether addition of vitamin D to blood cells from children with severe
asthma increases the production of the helpful Interleukin -10 messenger.
If increased production of Interleukin-10 is achieved in the presence of
vitamin D, then this may result in severe asthmatic children becoming
more responsive to steroid treatment.

**What does the study involve?**

If you are happy for your child to take part in this study, we will do a
blood test and take up to 5 teaspoons of blood from your child. We will try
to take the blood whilst they are undergoing a general anesthetic (for
their operation) or at the same time as they are having any planned blood
tests (clinically indicated) so as to avoid any additional distress to your
child. If your child is not anaesthetized when we do the blood test we will
use an anaesthetic (numbing) cream on their skin (like EMLA or AMETOP)
to minimize any pain or discomfort.

This blood will be taken to the laboratory to analyze levels of various
chemical messengers and molecules like interleukin-10 and vitamin D
levels. We will also perform tests in the laboratory to find out whether the
active form of vitamin D can magnify the helpful (interleukin-10)
messenger response when steroids are given.

We will also ask you some questions about your child’s diet in order to get
an idea of their daily Vitamin D intake. We will also ask you some
questions about your child’s diet in order to get an idea of their daily
Vitamin D intake. As vitamin D status in children is affected by
developmental status, we will also ask your child to indicate which
developmental stage they are at using a short picture questionnaire.

We will also do a blowing test (basic lung function test ‘spirometry’) to
measures how much air your child breathes in and out and how fast
he/she blows it out. During the test, we will ask your child to take a deep
breath in and then blow as hard as he/she can into a tube connected to a
small machine. Spirometry (blowing test) is done to look for diseases and
conditions that affect how much air you can breathe in and to look for
diseases that affect how fast you can breathe air out. We will explain blowing test results to you and your child.

**Are there any risks or side effects involved in taking part?**
The main risks with blood tests are pain, discomfort or bruising at the site where the needle goes in. These complications are usually minor and go away shortly after the tests are done. As the amount of additional blood needed is small, it will not adversely affect your child. Your child’s treatment will be unaffected regardless of whether he/she takes part in the study.

**Who is organizing and funding the research?**
This study is organized by the doctors from Royal Brompton Hospital and Imperial College, London in collaboration with Kings College, London. Equipment needed for the study is funded by research grant from British Medical Association. No doctor conducting the research will be given any remuneration for including your child in this study.

**What does my child gain from being in the study?**
There is no immediate benefit to your child from taking part in this study.

**What happens when the research study stops?**
Your child’s treatment will be continued during and after this study and will not be affected by your child’s participation in this study.

**What if something goes wrong?**
Imperial College holds insurance policies which apply to this study. If your child experience harm or injury as a result of taking part in this study, you and your child will be eligible to claim compensation without having to prove that Imperial College is at fault. This does not affect your legal rights to seek compensation. If your child is harmed due to someone’s negligence, then you may have grounds for a legal action. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been treated during the course of this study then you should immediately inform the Chief Investigator (Dr Atul Gupta telephone number 02073528121, extn 2257). The normal National Health Service complaint complaints mechanisms are also available to you. If you are still not satisfied with the response, you may contact the Imperial College Clinical Research Governance Office.

**Will my child’s taking part in this study be kept confidential?**
All information which is collected about your child during the course of the research will be kept strictly confidential. Any information about your
child which leaves the hospital will have your name and address removed so that your child will not be identified.

Your child’s GP will be notified of their participation in the trial but we will seek your agreement prior to doing this.

**What will happen to the results of the research study?**
The results of the research will be analysed by the research team and will be presented at research meetings. The results will be published in medical journals but without identification of any participants in the report. All information collected will be confidential, and no information will appear in any medical publication that will allow your child to be identified. If you would like to find out about the publication, please let us know and we will send you a copy once the results are published. Please remember that it can take at least a year after the end of the study to have the results published.

**Who has reviewed the study?**
The study has been given a favourable opinion by the Brompton, Harefield and NHLI Research Ethics Committee.

**Does my child have to take part?**
It is up to you and your child to decide whether or not to take part. If you and your child do decide to take part you will be given this information sheet to keep and you will be asked to sign a consent form. If you and your child decide to take part you are still free to withdraw your child from the study at any time without giving a reason. The care and the management your child will receive will be exactly the same and will not be affected by your participation in this study.

**Who can I contact for further Information?**
If you need further information about this study, you can contact Dr Atul Gupta on 02073528121, extn 2257.

Thank you for reading this document. You and your child are encouraged to ask any questions you may have. Please remember that your child may withdraw from this study at any time and without giving any reason. Please understand that your child need not take part in this trial if you do not wish to. If your child does take part, they may withdraw at any time, and does not need to give any reason for doing so.

**Thank you very much for your kindness in considering this project.**
Appendix 5 Child information (control)

VITAMIN D LEVEL AND ASTHMA CONTROL IN CHILDREN

Young person Information sheet

Thank you very much for taking the time to read this leaflet

We would like to ask you to take part in a research study. Before you decide whether to take part, it is important for you to understand why the study is being done and what it will involve. We would therefore be grateful if you would take the time to read the following information and discuss it with your family, friends and your GP if you wish. Ask us if there is anything that is not clear, or if you would like more information.

What is research?
Research is done to try and find out answers to important medical questions.

Why is the study being done?
This study is being done to try and find ways to improve asthma control in children with severe disease.

Steroids are the main treatment we use to control asthma symptoms. But some children have very severe asthma that does not respond to steroid treatment. These children remain poorly and have troublesome asthma. They also often have a poor quality of life because of inadequate asthma control. Making steroids work better in these children would be very helpful.

It is known that adults with severe asthma produce lower levels of a special chemical called interleukin-10 in response to steroids. Importantly, when vitamin D (sometimes called sunshine vitamin) is added together with steroids to blood cells from these asthmatic adults their asthma responds better to steroids.

In this study, we want to know whether this special chemical (interleukin 10) is also lower in children with severe asthma. We then want to find out whether adding vitamin D to blood cells from children with severe asthma increases the production of this helpful chemical. If this does happen, then giving vitamin D
to children with severe asthma may make their asthma respond better to steroids and make their asthma control better as well.

**Why have I been asked to take part in this study?**
In order for us to fully understand our results it is really important that we compare the findings from asthmatic children to those made in healthy children. We are therefore asking you as a non-asthmatic child to consider taking part in this study.

**What happens if I agree to take part?**
- If you are happy to take part in this study, we will need you to have a blood test. We can put 'magic cream' on your skin before the blood test to help to make it numb so that it does not hurt. As the amount of blood we need is small, it will not affect you or harm you in any way.
- This blood will be taken to the laboratory where the special tests for this study will be performed.
- We will also ask you and your family a few questions to find out how much Vitamin D is in your daily food.
- As vitamin D status is affected by development, we will also ask you to indicate which developmental stage you are at using a short picture questionnaire.
- We will also ask you to do a blowing test.

**Do I have to take part?**
- It is up to you and your parents to decide whether or not to take part. You do not have to take part in the project, and you do not have to say why either.
- The care and the management you will receive will be exactly the same and will not be affected by your participation in this study.

**Will your taking part in this study be kept confidential?**
All information which is collected during the course of the research will be kept strictly confidential.

**What will happen to the results of this study?**
The results of the research will be presented at research meetings. The results will be published in medical journals. All information collected will be confidential, and no information will appear in any publication that will allow you to be identified.

**Who can I contact for further Information?**
If you need further information about this study, you can contact Dr Atul Gupta on telephone number 02073528121, extn 2257.
Thank you for reading this document. You are encouraged to ask any questions you may have.

*Thank you very much for your kindness in considering this project*
Appendix 6  Child information (asthma)

Royal Brompton & Harefield

VITAMIN D LEVEL AND ASTHMA CONTROL IN CHILDREN
Young person Information sheet

Thank you very much for taking the time to read this leaflet

We would like to ask you to take part in a research study. Before you decide whether to take part, it is important for you to understand why the study is being done and what it will involve. We would therefore be grateful if you would take the time to read the following information and discuss it with your family, friends and your GP if you wish. Ask us if there is anything that is not clear, or if you would like more information.

What is research?
Research is done to try and find out answers to important medical questions.

Why is the study being done?
This study is being done to try and find ways to improve asthma control in children with severe disease.

Steroids are the main treatment we use to control asthma symptoms. But some children have very severe asthma that does not respond to steroid treatment. These children remain poorly and have troublesome asthma. They also often have a poor quality of life because of inadequate asthma control. Making steroids work better in these children would be very helpful.

It is known that adults with severe asthma produce lower levels of a special chemical called interleukin-10 in response to steroids. Importantly, when vitamin D (sometimes called sunshine vitamin) is added together with steroids to blood cells from these asthmatic adults their asthma responds better to steroids.

In this study, we want to know whether this special chemical (interleukin 10) is also lower in children with severe asthma. We then want to find out whether adding vitamin D to blood cells from children with severe asthma increases the production of this helpful chemical. If this does happen, then giving vitamin D to children with severe asthma may make their asthma respond better to steroids and make their asthma control better as well.
Why have I been asked to take part in this study?
We would like you to take part in this study as you have severe asthma and you are being looked after by the doctors at the Royal Brompton Hospital which is where this study is being done.

What happens if I agree to take part?
- If you are happy to take part in this study, we will need you to have a blood test. We can put 'magic cream' on your skin before the blood test to help to make it numb so that it does not hurt. As the amount of blood we will take is small, it will not affect you or harm you in any way.
- This blood will be taken to the laboratory where the special tests for this study will be performed.
- We will also ask you and your family a few questions to find out how much Vitamin D is in your daily food.
- As vitamin D status is affected by development, we will also ask you to indicate which developmental stage you are at using a short picture questionnaire.

Do I have to take part?
- It is up to you and your parents to decide whether or not to take part. You do not have to take part in the project, and you do not have to say why either.
- The care and the management you will receive will be exactly the same and will not be affected by your participation in this study.

Will your taking part in this study be kept confidential?
All information which is collected during the course of the research will be kept strictly confidential.

What will happen to the results of this study?
The results of the research will be presented at research meetings. The results will be published in medical journals. All information collected will be confidential, and no information will appear in any publication that will allow you to be identified.
If the results of this study show that vitamin D can help children with severe asthma respond better to steroids, then we hope to look at vitamin D as a possible additional treatment in severe asthma.

Who can I contact for further Information?
If you need further information about this study, you can contact Dr Atul Gupta on telephone number 02073528121, extn 2257.
Thank you for reading this document. You are encouraged to ask any questions you may have.

Thank you very much for your kindness in considering this project