Exploring the relationship between obstructive sleep apnoea, asthma and atopic disease in children.

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Declaration of originality

I confirm that I carried out all the data collection in this thesis with the exception of the data relating to the severe therapy resistant asthmatics (STRA), which was collated by the asthma team, Royal Brompton Hospital.

I interpreted the STRA sleep studies along with the sleep team at Royal Brompton Hospital. The Ambulatory sleep studies were interpreted by myself and reviewed by the sleep team.

I confirm that all experimental work in this thesis was carried out by me except for the processing of the plasma samples for total and specific IgE analysis, which was carried out by the biochemistry laboratory team at Royal Brompton Hospital.

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Abstract

OSA in children is now recognised as a common health problem with an estimated prevalence of up to 5.7% and an associated with delayed growth and development, cardiovascular consequences and metabolic disorders. The role that $CD4^+$ T-cells play in the pathology has been demonstrated with higher peripheral Th17 and lower Treg cell counts in adults and children with confirmed OSA suggesting a T-cell driven inflammatory basis to the underlying pathology. Links have therefore been proposed between OSA and other inflammatory disorders involving T-cells such as asthma and atopic disease. An OSA incidence of 63% has been reported in 'poorly controlled' asthmatics with improvement in asthma control following adenotonsillectomy. The current published literature however lacks sleep study confirmation of OSA plus well-defined evidence of asthma and atopy to really explore the relationship.

I hypothesised that the incidence of OSA in children with Severe Therapy Resistant Asthma would be higher than reported as the background incidence in childhood and that there would be a higher prevalence of atopy in children with sleep study proven OSA undergoing adenotonsillectomy compared to those without OSA. I also hypothesised that the tonsils tissue in child with OSA would contain more Th17 cells, Less Treg cells and have a Th2 and ILC2 phenotype compare to the tonsils of children without OSA.

All children defined as severe therapy resistant asthmatics (STRA) from September 2013 – August 2016 underwent sleep studies to assess for evidence of sleep-disordered breathing. Data was collected on their demographics as well as lung health, medication burden and levels of atopic disease.

Separately, children with proven OSA undergoing adenotonsillectomy as a treatment option were recruited and their level of atopy assessed using clinical questionnaire and evidence of peripheral blood sensitisation. Children undergoing surgery for recurrent tonsillitis were recruited as a disease control and underwent the same process. The extracted tonsil tissue was analysed to explore cellular phenotype between those with confirmed OSA and those without. The T-cells were one area of interest along with innate lymphoid cells (ILCs), a recently described novel class of leukocytes assigned critical roles in asthma pathology.

Amongst the STRA group 21 children underwent a sleep study with 3 (14%) demonstrating evidence of OSA. No difference could be identified based on their demographics, medical therapy, respiratory health or level of atopy.

For the children undergoing tonsillectomy, 24 with OSA and 21 disease control subjects were recruited. The rate of peripheral sensitisation was higher in the Non-OSA group (OSA group 5 (20%) vs Non-OSA group 10 (48%), P=0.11). From the extracted tissue, a lower proportion of T-regulatory cells (Tregs) ($CD4^+CD25^+Foxp3^+$) was observed in the tonsils of children with OSA compared to controls as both cell counts per gram of tissue and percentage of total cells. A similar result was seen on the peripheral blood of these subjects.

Regarding the innate lymphoid cells (ILCs) no difference was observed between OSA and Non-OSA subjects. A higher proportion however was observed in tonsil tissue amongst those with proven atopy compared to the non-atopic subjects.

The incidence of OSA within the STRA subjects was not found to be higher than the background incidence in children and less severe than previously published.

For the subjects recruited from those listed for tonsillectomy, no difference in atopic status was observed from questionnaire data or laboratory analysis suggesting this was not impacting the development of OSA however this was a small study population affecting any conclusion drawn.

The tonsil and peripheral blood of children with OSA had significantly fewer Tregs compared to controls. This finding has not been reported previously in tonsil tissue with a control group analysis and supports similar findings from adenoidal tissue and peripheral blood implicating Tregs in the underlying pathology. The higher proportion of tonsil ILCs in subjects with atopic disease is also a significant finding and supports the notion of the innate immune systems role in atopic disease.

In conclusion, I observed no difference in the incidence of OSA amongst STRA children compared to the incidence in childhood or atopy amongst children with confirmed OSA compare to those without OSA. I did however identify lower Treg cells in the tonsil and peripheral blood of children with OSA compared to disease control subjects and an association between ILCs and atopy.

Publications arising from this work

Peer review publication

Sleep disordered breathing in children

Grime C, Tan, HL

Indian journal of paediatrics 2015: 8 (10); 945-955

Abstract publication

Innate lymphoid cells are proportionally higher in children with atopy.

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European Respiratory Society Congress, 2015

Children with Obstructive Sleep Apnoea have lower T-regulatory cells in the upper airway and peripherally compared to controls.

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Table of content

Abstract	**Page**	**3**
Publications		**5**
Acknowledgements		**6**
Figures index		**14**
Tables index		**20**
Abbreviations		**22**
Chapter 1: Introduction		**25**
1.1. Obstructive sleep apnoea		**25**
1.1.1 Definitions and prevalence of OSA		25
1.1.2. Signs and symptoms of OSA		26
1.1.3 Aetiology of OSA		27
1.1.4. OSA as an inflammatory disorder involving T-lymphocytes		30
1.1.4.a. T-regulatory cells		31
1.1.4.b. Th17 cells		32
1.1.4.c. Th17 and Tregs in context of OSA		34
1.1.5 Consequences and associated effects of OSA		34
1.1.6. Clinical evaluation and diagnosis of OSA		37
1.1.7. Treatment of OSA		41
1.2. Asthma, allergy and atopy		**46**
1.2.1 Definition and prevalence of asthma and atopy		46
1.2.2. Pathophysiology and basic mechanisms of allergic disease and asthma		47
1.2.2.a. Innate immune system		47
1.2.2.b. Adaptive immune system		49
1.2.2.c. Immunoglobulin E		51
1.2.2.d. Pathology of the allergic disease		51
1.2.4. Clinical evaluation and diagnosis of asthma and allergic disease		55

1.2.5. Treatment of allergic disease 56

1.3. The Association between OSA, Asthma and Atopic disease. 58

1.4. Hypotheses 65

1.5. Aims 65

1.6. Objectives 66

Chapter 2: Material and methods 67

2.1 Severe therapy resistant asthmatics undergoing polysomnography and cardiorespiratory polygraphy. 67

2.1.1. Severe therapy resistant asthma. 67

2.1.2. Assessment of sleep disordered breathing. 69

2.2. The prevalence of atopy amongst children undergoing tonsillectomy for the treatment of OSA. 71

2.2.1. Recruitment 71

2.2.2. Questionnaire data collection. 72

2.2.3. Blood sample collection. 72

2.3. The cellular phenotyping of collected tissues. 73

2.3.1. Tonsil tissue processing 73

2.3.2. Peripheral blood processing. 74

2.3.3. Flow cytometry. 75

2.4 Statistics 79

2.5. Ethics 80

Chapter 3. OSA in Severe Therapy Resistant Asthma 82

3.1 Introduction 82

3.2 Research question 82

3.4 Methods 82

3.5 Results 83

3.5.1. Evidence of sleep disordered breathing 85

3.5.2. Medical therapy received 90

3.5.3. Respiratory Health 91

3.5.4. Level of Atopic disease 93

3.6 Discussion 94

3.6.1. Principle findings 94

3.6.2. Strengths and weaknesses of study 95

3.6.3. Findings in relation to other studies 96

3.7 Conclusion 97

Chapter 4. Atopy in children with OSA 99

4.1. Introduction 99

4.2. Research Questions 99

4.3. Methods 100

4.3.1. Home cardiorespiratory polygraphy 101

4.3.2. Home cardiorespiratory polygraphy preliminary study 102

4.4. Results 105

4.4.1. Sleep-disordered breathing within recruited subjects 106

4.4.2. Atopy within recruited subjects 110

4.6. Discussion 113

4.6.1. Principle findings 113

4.6.2. Strengths and weaknesses of study 114

4.6.3. Findings in relation to other studies 114

4.7. Conclusions 116

Chapter 5.Lymphoid cell populations in tonsils and peripheral blood from children with obstructive sleep apnoea 117

5.1. Introduction 117

5.2. Research question 118

5.4. Methods 118

5.4.1. Preliminary work 118

5.4.1.a. Antibody choice and titration 118

5.4.1.b. Gating strategies 124

5.4.2. Subjects. 129

5.5. Results **132**

5.5.1 Total Cells 132

5.5.2 T-cells in tonsils and peripheral blood from children with OSA compared to controls 134

5.5.2.a Tonsil tissue T-cell results- OSA vs Non-OSA subjects. 134

5.5.2.b Peripheral blood T-cell results- OSA vs Non-OSA subjects. 137

5.5.3. ILCs in tonsils and peripheral blood from children with OSA compared to controls 141

5.5.3.a. ILCs in tonsil tissue results- OSA vs Non-OSA subjects. 141

5.5.3.b. ILCs in peripheral Blood Results- OSA vs Non-OSA subjects . 144

5.6 Discussion **146**

5.6.1. Principle findings 146

5.6.2. Strengths and weaknesses of study 147

5.6.3. Findings in relation to other studies 149

5e. Conclusions **151**

Chapter 6. Lymphoid cell phenotype of tonsils and peripheral blood from atopic and non-atopic children **152**

6.1 Introduction **152**

6.2. Research question **152**

6.3. Methods **153**

6.3.1 Recruitment 153

6.5 Results **157**

6.5.1. T-cells **157**

6.5.1.a. Lymphoid cell composition of tonsil tissue and peripheral blood from atopic and non-atopic (complete aeroallergen panel) children 157

6.5.1.b. Lymphoid cell composition of tonsil tissue and peripheral blood from atopic and non-atopic (all results) children 161

6.5.1.c. Lymphoid cell composition of tonsil tissue and peripheral blood from children with raised serum IgE and normal serum IgE 166

6.5.1.d. T-cell counts vs total IgE 170

6.5.2 Innate Lymphoid Cells **170**

6.5.2.a. ILCs in tonsil tissue and PBMCs comparing atopic and non-atopic (complete aeroallergen panel) children. 170

6.5.2.b. ILCs in tonsil tissue and PBMCs comparing atopic and non-atopic (all results) children 175

6.5.2.c. ILCs in tonsil tissue and PBMCs comparing children with raised serum IgE and normal serum IgE 179

6.5.2.d. Tonsil lineage negative cells vs total IgE. 185

6.6 Discussion 185

6.6.1. Principle findings 185

6.6.2. Strengths and weaknesses of study 186

6.6.3. Findings in relation to other studies 188

6.7. Conclusion 191

Chapter 7: Final Discussion 192

7.1. Principle findings in relation to other studies 192

7.1.1. The incidence of OSA amongst children with severe therapy resistant asthma (STRA) is comparable with the suggested incidence in the general paediatric population. 192

7.1.2. Children undergoing tonsillectomy for proven OSA do not have a higher incidence of atopy than a disease control group. 194

7.1.3. Children with OSA have a lower proportion of T-regulatory cells in tonsils and peripheral blood compared to control subjects. 195

7.1.4. The tonsils of atopic children have a higher proportion of innate lymphoid cells particularly ILC group 2 cells compared to non-atopic. 195

7.2. Strengths 196

7.3. Limitations 197

7.4. Future work 198

7.4.1. OSA amongst Asthmatic children 198

7.4.2. Atopy amongst children with OSA 199

7.4.3. Anti-inflammatory immune function and OSA 199

7.4.4. The role of ILC2s in allergic disease pathology 200

7.5 Conclusion 201

References **202**

Appendix **218**

Figures Index

Figure 1.1. A demonstration of the critical closing pressure in a Starlings Resistor. Page 28

Figure 1.2. Three-dimensional display of the upper airway from a control subject (A) and a subject with OSA (B). Images from 'Upper airway size analysis by magnetic resonance imaging of children with obstructive sleep apnea syndrome', Am J Respir Crit Care Med 2003 30

Figure 1.3. Sleep study examples. 40

Figure 1.4. Diagnostic and treatment algorithm proposed by the ERS task. 'Obstructive sleep disordered breathing in 2-to 18-year-old children: diagnosis and management, European Repsiratory Journal 2016. 42

Figure 1.5. Comparison of T-cells and ILCs demonstrating cytokine production profile and transcription factors for both. The brave New World of Innate Lymphoid Cells, Nature Immunology 2015. 48

Figure 1.6. Allergen exposure leading to an adaptive immune response differentiating a naïve T-cell. The pathophisology, diagnosis and treatment of allergic rhinitis, Allergy, Asthma and Immunology Research 2010. 50

Figure 2.1. A representation of the workings of a flow cytometer. 75

Figure 2.2. The Fluorescence minus one for IL17 78

Figure 2.3. Flow cytometry plot of a sample containing antibodies for IL17. 78

Figure 2.4. An example of a gating strategy used scientifically when flow cytometry plots are analysed using the flowjo software. 79

Figure 3.1. Events scored for the STRA subjects sleep studies. 87

Figure 3.2. Oxygen saturations comparing those with evidence of OSA on sleep study and those without evidence. 87

Figure 3.3 Transcutaneous carbon dioxide measured in kPa comparing those with evidence of OSA 88

Figure 3.4. Comparison of total AHI, REM related AHI and Non-REM related AHI for STRA children who had full PSG. 89

Figure 3.5. Comparison of OSA and Non-OSA STRA patient who had full PSG. Total REM time. 89

Figure 3.6. Comparison of OSA and Non-OSA STRA patient who had full PSG. Arousal index. 90

Figure 3.7. Total daily inhaled corticosteroid dose as budesonide equivalent doses comparing OSA and Non-OSA subjects. 90

Figure 3.8. Percentage predicted forced vital capacity (FVC) and Forced expiratory value In one second (FEV1) comparing OSA and Non-OSA subjects. 91

Figure 3.9. Individual subject scores for the Paediatric asthma Quality of Life Questionnaire (PAQLQ) comparing OSA and Non-OSA subjects. 92

Figure 3.1.1. Individual subject scores for the Asthma Control test (ACT) comparing OSA and Non-OSA subjects. 92

Figure 3.1.2. Total IgE and the sum of the specific IgE from the standard aeroallergen for the OSA and Non-OSA subjects as international units. 93

Figure 3.1.3. Total IgE as Z-scores for age for the OSA and Non-OSA subjects as international units. 93

Figure 3.1.4. Peripheral eosinophil counts from blood sample & BAL eosinophil from those with evidence of OSA on sleep study and those without. 94

Figure 4.1. Recruitment flow diagram. 100

Figure 4 2. Demonstration of the *Somnotouch* home sleep study device being worn. 102

Figure 4.3. Total AHI, central AHI and obstructive AHI comparing in-lab CR-Poly results and home CR-poly results. 104

Figure 4.4. Bland-Altman of AHI & Obstructive AHI. 104

Figure 4.5. Extracted tonsil tissue weight comparing OSA to control. 108

Figure 4.6. The scores generated from the Modified Epworth Sleepiness Questionnaire comparing OSA to controls. 108

Figure 4.7. Apnoea-hypopnoea index comparison for subjects with and without OSA. 109

Figure 4.8. Obstructive Apnoea-hypopnoea index comparison for subjects with and without OSA. 109

Figure 4.9. Results from the aeroallergen panel of all subjects recruited. 111

Figure 4.1.1. Sum of the specific IgE test results for subjects with and without OSA. 112

Figure 4.1.2. Total IgE for subjects with and without OSA. 112

Figure 4.1.3. Total IgE as Z-scores for subjects with and without OSA. 113

Figure 5.1. Flow cytometry gating strategy for identifying T-cells, NK-cells and B-cells in human tonsil tissue and PBMCs. 124

Figure 5.2. Flow cytometry strategy for identifying Th subsets. 125

Figure 5.3. Flow cytometry gating strategy to identify lineage negative cells. 126

Figure 5.4. Improved antibody staining and gating strategy for lineage negative cell population. 126

Figure 5.5. Lineage negative cells that display the extracellular marker $CD127^{+}$ and the ILC2 marker CRTH2. 128

Figure 5.6. Lineage negative cells that stain positive for the transcription factor Gata3 and secrete IL-4 and IL-5. 128

Figure 5.7. Lineage negative cells produce IL-4, IL-13, IL-5, IL-17 and IFNγ when stimulated in preparation for FACS analysis. 129

Figure 5.8. Recruitment diagram demonstrating group numbers when analysing for differences based on OSA diagnosis in the tonsil cell samples. 130

Figure 5.9. Flow diagram showing potential number of tonsil samples analysed for T helper cells at each stage of the FACS panel development. 130

Figure 5.1.2. Flow diagram showing potential number of tonsil samples analysed for ILCs at each stage of the FACS panel development. 131

Figure 5.1.3. Recruitment diagram demonstrating group numbers for analysis of cells from peripheral blood when analysing for differences based on OSA diagnosis. 131

Figure 5.1.4. Flow diagram showing potential number of peripheral blood samples analysed for T helper cells at each stage of the FACS panel development. 132

Figure 5.1.5. Flow diagram showing potential number of peripheral blood samples analysed for ILCs at each stage of the FACS panel development. 132

Figure 5.1.6. Differential cell counts per gram of tonsil tissue for B-cells, T-cells, NK-cells and lineage negative cells. 133

Figure 5.1.7. Differential cell counts per ml of blood for B-cells, T-cells, NK-cells and lineage negative cells. 133

Figure 5.1.8. Similar total cell counts obtained from a pair of pharyngeal tonsils comparing OSA subjects to non-OSA. 134

Figure 5.1.9. T-cells, B-cells and NK cell counts per gram of tonsil tissue comparing OSA subjects to Non-OSA subjects. 135

Figure 5.2.1. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing OSA subjects to Non-OSA subjects. 135

Figure 5.2.2. T-cell counts per gram of tissue comparing OSA subjects to Non-OSA subjects. 133

Figure 5.2.3. T-cells as a percentage of total cell counts comparing OSA subjects to Non-OSA subjects. 136

Figure 5.2.4. Tonsil Th subtype results comparing OSA against Control. 137

Figure 5.2.5. T-cells, B-cells and NK cell counts per ml of blood comparing OSA subjects to Non-OSA subjects. 138

Figure 5.2.6. T-cells, B-cells and NK cell median percentages of total cells comparing OSA subjects to Non-OSA subjects. 138

Figure 5.2.7. T-cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. 139

Figure 5.2.8. T-cells median percentages of total T-cells ($CD3^+$) comparing OSA subjects to Non-OSA subjects. 139

Figure 5.2.9. PBMC Th subsets results comparing OSA to Controls. 140

Figure 5.3.1. Total tonsil lineage negative cells comparing OSA to Controls. 141

Figure 5.3.2. Tonsil cytokine producing ILCS comparing OSA to controls. 142

Figure 5.3.3. Tonsil cytokine producing CD127+ ILCs comparing OSA to controls. 143

Figure 5.3.4. ILC2s comparing OSA to Control. 144

Figure 5.3.5. Total PBMC lineage negative cells comparing OSA to controls. 145

Figure 5.3.6. PBMC cytokine producing ILCs comparing OSA to controls. 146

Figure 5.3.6. Immunopathology of Treg depletion, dysfunction or resistance resulting in autoimmunity. from Nature reviews: Immunology, Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. 150

Figure 6.1. A recruitment diagram, demonstrating group proportions when analysing for differences based on atopic status considering those with and without a complete set of specific IgE tests. 153

Figure 6.2. A recruitment diagram, demonstrating group proportions when analysing for differences based on the presence or absence of a raised total IgE results. 154

Figure 6.3. A flow diagram showing potential number of samples analysed for T helper cells at each stage of the FACS panel development. 155

Figure 6.4. Flow diagram showing potential number of samples analysed for innate lymphoid cells at each stage of the FACS panel development. 155

Figure 6.5. A recruitment diagram demonstrating group proportions of peripheral blood samples available to analyse when dividing samples based on atopic status. 156

Figure 6.6 A recruitment diagram demonstrating group proportions of peripheral blood samples available to analyse when dividing samples based on presence or absence of a raised total IgE. 156

Figure 6.7. A flow diagram showing potential number of samples analysed for T helper cells at each stage of the FACS panel development. 157

Figure 6.8. A flow diagram showing potential number of samples analysed for ILCs at each stage of the FACS panel development. 157

Figure 6.9. A comparison of the T-cells, B-cells and NK-cells in Tonsil tissue and PBMCs between atopic (red) and non-atopic(complete aeroallergen panel) (blue) subjects. 158

Figure 6.1.1. A comparison of T-cells in tonsil tissue and PBMCs between atopic (red) and non-atopic (complete aeroallergen panel) (blue) subjects. 159

Figure 6.1.2. A comparison of Th cells defined by their cytokine production in tonsil tissue and PBMCs between atopic (red) and non-atopic (complete aeroallergen panel) (blue) subjects. 160

Figure 6.1.3. A comparison of the T-cells, B-cells and NK-cells in tonsil tissue and between atopic (red) and non-atopic(all results) (green) subjects. 162

Figure 6.1.4. A comparison of T-cells in tonsil tissue and PBMCs between atopic (red) and non-atopic (all results) (green) subjects. 163

Figure 6.1.5. A comparison of Th cells defined by their cytokine production in tonsil tissue and PBMCs between atopic (red) and non-atopic (all results) (blue) subjects. 165

Figure 6.1.6. Comparison of Th2 cells ($CD4^{+}Ll4^{+}$) as cell counts per gram of tonsil tissue between atopic and Non-atopic (all results) subjects. 166

Figure 6.1.7. A comparison of Tonsil and PBMCs T-cells, B-cells and NK-cells between subjects with raised IgE (Red) to those with normal IgE (Purple). 167

Figure 6.1.8. A comparison of Tonsil and PBMCs T-cells between subjects with raised IgE (Red) to those with normal IgE (Purple). 168

Figure 6.1.9. A comparison of Tonsil and PBMCs Th cells defined by cytokine secretons and Tregs between subjects with raised IgE (Red) to those with normal IgE (Purple). 169

Figure 6.2.1. A comparison of tonsil lineage negative cells identified by published extracellular markers as cell counts per gram of tissue between atopic (red) with non-atopic (complete aeroallergen panel) subjects (Blue). 171

Figure 6.2.2. A comparison of peripheral blood (PBMCs) lineage negative cells identified by published extracellular markers between atopic (Red) and non-atopic (complete aeroallergen panel)(Blue) subjects. 172

Figure 6.2.3. A comparison of tonsil and PBMCs lineage negative cytokine producing cells comparing atopic (Red) subjects with non-atopic (complete aeroallergen panel) (Blue). 173

Figure 6.2.4. A comparison of tonsil lin^{neg} $CD127^{+}$ and cytokine producing cells between atopic (Red) and non-atopic (complete aeroallergen panel) (Blue). 174

Figure 6.2.5. A comparison of tonsil ILC2s ($Lin^{-}Gata3^{+}$) between atopic (Red) and non-atopic (complete aeroallergen panel) (Blue) subjects. 175

Figure 6.2.6. A comparison of tonsil lineage negative cells identified by published extracellular markers between atopic (red) with non-atopic (all results) (Green). 176

Figure 6.2.7. A comparison of peripheral blood (PBMCs) lineage negative cells identified by published extracellular markers between atopic (Red) and non-atopic (sufficient IgE)(Green) subjects. 177

Figure 6.2.8. A comparison of tonsil and PBMCs lineage negative cytokine producing cells comparing atopic (Red) subjects with non-atopic (sufficient IgE) (Green). 178

Figure 6.2.9. A comparison of tonsil lin^{neg} $CD127^{+}$ and cytokine producing cells between atopic (Red) and non-atopic (sufficient IgE) (Green). 179

Figure 6.3.1. A comparison of tonsil ILC2s ($Lin^{-}Gata3^{+}$) between atopic (Red) and non-atopic (sufficient IgE) (Blue) subjects. 180

Figure 6.3.2. A comparison of tonsil lineage negative cells identified by published extracellular markers between subjects with raised total IgE (Red) and normal total IgE (Purple). 181

Figure 6.3.3. A comparison of PBMCs lineage negative cells identified by published extracellular markers between subjects with raised total IgE (Red) and subjects with normal total IgE (Purple). 182

Figure 6.3.4. A comparison of tonsil and PBMCs lin^{neg} cytokine producing cells between subjects with raised total IgE (Red) and subjects with normal total IgE (Purple). 183

Figure 6.3.5. A comparison of tonsil $lin^{neg}CD127^{+}$ and cytokine producing cells between samples from subjects with raised total IgE (Red) against those with normal total IgE (Purple). 184

Figure 6.3.6. A comparison of tonsil ILC2s ($Lin^{-}Gata3^{+}$) between samples from subjects with raised total IgE (Red) against those with normal total IgE (Purple). 185

Figure 7.1. Collaborative work combining data on total ILCs in four different compartment in children (BAL, Sputum, PBMCs and tonsils) demonstrating a higher proportion in atopic individuals defined by the presence of positive specific serum IgE tests to an aeroallergen panel. 201

Tables Index

Table 1.1. Signs and symptoms associated with childhood OSA divided into nocturnal and daytime features. Page 26

Table 1.2. Common disorders predisposed to OSA in children either due to anatomical narrowing of the upper airway or compression of a collapsible upper airway. 29

Table 1.3. Summary of the published literature on the subject of immunological features noted in children with OSA including whether the sleep disordered breathing was diagnosed with sleep study, whether a disease-free control group was used for comparison and what tissue was used for the evaluation. 33

Table 1.4. Summary table of published work studying the association between children with evidence of sleep disordered breathing and atopic disease excluding asthma. 62

Table 1.5. Summary table of published work studying the association between children with a history of habitual snoring and asthma. 63

Table 1.6. Summary table of published work studying the association between children with evidence of sleep disordered breathing and asthma. 64

Table 3.1. Demographics of the 21 children who underwent sleep studies during their assessment as part of the problematic asthma investigations. Mean values with standard deviations. 83

Table 3.2. Quantifiable results obtained from the cardio-respiratory sleep studies undertaken by the STRA children comparing those with OSA to those without. 84

Table 3.3. Demographics of the 3 children with evidence of OSA and 18 children without evidence of OSA on cardiorespiratory polygraphy. 85

Table 3.4. Comparison of answers to a series of questions focusing on signs and symptoms of sleep disordered breathing between those with sleep study proven evidence of OSA and those without. 86

Table 4.1. Comparison of data available from in-laboratory cardiorespiratory polygraphy study and home cardiorespiratory polygraphy study. 101

Table 4.2. Demographics for subjects recruited for sub-study comparing home and in-laboratory cardiorespiratory polygraphy studies. 103

Table 4.3. Demographics of recruited subjects. Values demonstrated are median values with interquartile ranges. 106

Table 4.4. Answers to direct questioning for features suggestive of sleep disturbance. 107

Table 4.5. Answers to direct questioning for features suggestive of atopic disease. The values are proportion of subjects answering yes to questioning with percentages. 110

Table 4.6. Answers to direct questioning for features suggestive of a family history of atopic disease. 110

Table 4.7. Infant birth details and feeding patterns comparing subjects with OSA and controls. 111

Table 5.1. Flow cytometry lineage panel 1. 120

Table 5.2. Flow cytometry lineage panel 2. 121

Table 5.3. Flow cytometry T-cell panel 1. 122

Table 5.4. Flow cytometry T-cell panel 2. 123

Table 5.5. CD4+ T-cell subsets and the definitions used to identify them through flow cytometry. 124

Table 5.6. Lineage negative ILC groups and the extracellular and cytokine markers used to identify them. 127

Abbreviations

AD	Allergic dermatitis
AHI	Apnoea-hypopnoea index
AR	Allergic rhinitis
AT	Adenotonsillectomy
BAL	Bronchoalveolar lavage
BiPAP	Biphasic positive airway pressure
BNP	Brain naturetic peptide
CAS 15	Clinical Assessment Score 15
CASQ	Cleveland Adolescent Sleepiness questionnaire
CHAT	Childhood adenotonsillectomy trial
ChelWest	Chelsea and Westminster Hospital
CPAP	Continuous positive airway pressure
CRP	C-reactive protein
EEG	Electroencephlogram
EMG	Electromyogram
EOG	Electro-oculogram
FcεrR1	High IgE affinity receptor
Foxp3	Fork head box p3 transcription factor
FRC	Functional residual capacity
RP	Cardiorespiratory polygraphy
HRP	Home respiratory polygraphy
IgE	Immunoglobulin E
IGF-1	Insulin growth factor 1
IL-1	Interleukin 1
IL-4	Interleukin 4
IL5	Interleukin 5
IL-6	Interleukin 6

IL-8	Interleukin 8
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-23	Interleukin 23
ILCs	Innate lymphoid cells
ILC1	Group 1 innate lymphoid cells
ILC3	Group 3 innate lymphoid cells
ILC2	Group 2 innate lymphoid cells
INFγ	Interferon gamma
IPEX	Immundysregualtion polyendocrinopathy enteropathy
LRT-1	Cysteinyl leukotriene receptors 1
LTR-2	Cysteinyl leukotriene receptors 2
MALT	Mucosal-associated lymphoid tissue
mESS	Modified epworth sleepiness score
NADPH	Nicotinamide adenine dinucleotide phosphate
OAHI	Obstructive apnoea-hypopnoea index
OSA	Obstructive sleep apnoea
Pcrit	Critical closing pressure
PSG	Polysomnogrpahy
RcεR11	low IgE affinity receptor
RBHT	Royal Brompton and Hare field NHS foundation trust
REM	Rapid eye movement sleep
STRA	Sever therapy resistant asthma
Tregs	T-regulatory cells
TRT	Total recording time
Th	Helper T-cells
Th1	Type 1 Helper T-cells

Th2	Type 2 Helper T-cells
Th17	Type 17 Helper T-cells
TNF-A	Tumor necrosis factor alpha
TST	Total sleep time
TUCAS	Tucson Children's Assessment of Sleep Apnea

Chapter 1. Introduction

1.1. Obstructive sleep apnoea

1.1.1. Definitions and prevalence of OSA

The latest edition of the International Classification of Sleep Disorders (ICSD-3) classifies sleep disordered breathing into 4 main categories: obstructive sleep apnoea (OSA) disorders, central sleep apnoea syndromes, sleep-related hypoventilation disorders, and sleep-related hypoxemia disorder[1]. Obstructive sleep apnoea disorders encompasses a range of phenomena beginning with the more benign Primary Snoring with no effect on gas exchange and ending with Obstructive Sleep Apnoea (OSA) at the severe end of the spectrum [2]. OSA is the phenomenon of repeated, episodic reduction or cessation of airflow during sleep with preserved or increased respiratory effort resulting in hypoxia, hypercarbia, pronounced intra-thoracic pressure changes and sleep fragmentation.

The reported prevalence of OSA in children depends greatly on the populations studied and criteria used for investigation and diagnosis. Many published studies use small study populations, lacking standardised criteria for diagnosis or only target sleep studies on high-risk populations. That noted, the prevalence has been reported to range from 0% to 5.7% [3-13]. Three studies of note employing large study populations and full polysomnography (PSG) to report OSA (O'Brien et al. 2003[10], Bixler et al. 2009[5] & Li et al. 2010[8]) and estimate the prevalence in the general paediatric population more precisely, ranging from 1.2% to 5.7%. A wider ranging prevalence has been reported by studies utilizing ambulatory home sleep studies of 0.8% to 24%[14-19] however this is heavily influenced by two outlier studies (Casteronovo et al. 2003[14] & Goodwin et al. 2005[15]) for which the diagnostic criteria of OSA was not as precise as others. Home sleep studies are not considered to be the gold standard for diagnosis.

A gender difference in prevalence is unclear with three studies demonstrating no difference between the sexes [11, 12, 18] while two showed a higher rate amongst males[8, 15]. An association with childhood obesity is considered well established [4, 8, 12, 13] however three studies did not demonstrate this relationship [7, 11, 15]. One study did show an

association with premature birth [17]. Several authors have studied adenotonsillar hypertrophy based on direct visual scoring of tonsil size but surprisingly only two studies reported a relationship [8, 13].

1.1.2. Signs & Symptoms of OSA

A publication in the British Medical Journal in September 1889 referenced 'backward and stupid' children who suffered deafness and headaches as a result of enlargement of lymphoid tissues and benefited from naso-pharyngeal 'scarification'. The author, William Hill, was unknowingly describing the features of severe OSA and suggesting an appropriate treatment. Signs and symptoms of OSA include snoring, witnessed pauses in breathing, increased work of breathing, paradoxical chest and abdominal movement, sweating, restless sleep and mouth breathing. Some children may sleep with their necks in extension to maintain their airway. In contrast to adult OSA, daytime somnolence is uncommon. Daytime symptoms are often nonspecific and can include hyperactivity, difficulty in concentrating, moodiness and sleepiness (Table 1.1).

Nocturnal symptoms	Daytime symptoms (non-specific)
Habitual snoring	Hyperactivity
Gasping	Difficulty concentrating
Increased work of breathing (accessory muscles use)	Behavioural difficulties
Paradoxical breathing	Excessive daytime sleepiness
History of apnoeas	Moodiness
Sleeping with head in hyperextended position	Learning difficulties.
Nocturnal enuresis	
Cyanosis	
Morning headaches	

Table 1.1. Signs and symptoms associated with childhood OSA divided into nocturnal and daytime features.

1.1.3. Aetiology of OSA

The aetiology of childhood OSA is multifactorial but can be explained by three main themes: intrinsic upper airway narrowing; increased upper airway collapsibility; and exogenous tissue deposition for example, fat in the obese.

Anatomical causes of a narrowed upper airway include micrognathia, macroglossia and mid-face hypoplasia. There are therefore numerous syndromes involving craniofacial abnormalities that would predispose an individual to OSA (e.g., Treacher Collins syndrome, Crouzon's syndrome, Aperts syndrome, Pierre Robin sequence) as well as Beckwith Wiedemann syndrome, achondroplasia and trisomy 21 that result in the features named above. African and Caribbean ethnicity is also associated with a narrowed upper airway [20] along with sickle cell disease due to a persistent tonsillar hyperplasia [21].

The collapsibility of the upper airway can be explained by considering it as a Starling resistor- essentially a tube with a collapsible segment (Figure 1.1). Under conditions of flow limitation, the maximum inspiratory airflow is determined by the pressure change upstream (nasal) to the collapsible segment and is relatively independent of the downstream (tracheal) pressure generated by the diaphragm. Collapse occurs when the pressure outside the collapsible segment is greater than within the segment- the critical closing pressure (Pcrit). Marcus et al[22] determined the Pcrit in children who were asleep by measuring the maximal inspiratory airflow against the level of negative nasal pressure applied via a nasal mask and found that children with OSA had demonstrably more collapsible upper airways during sleep.

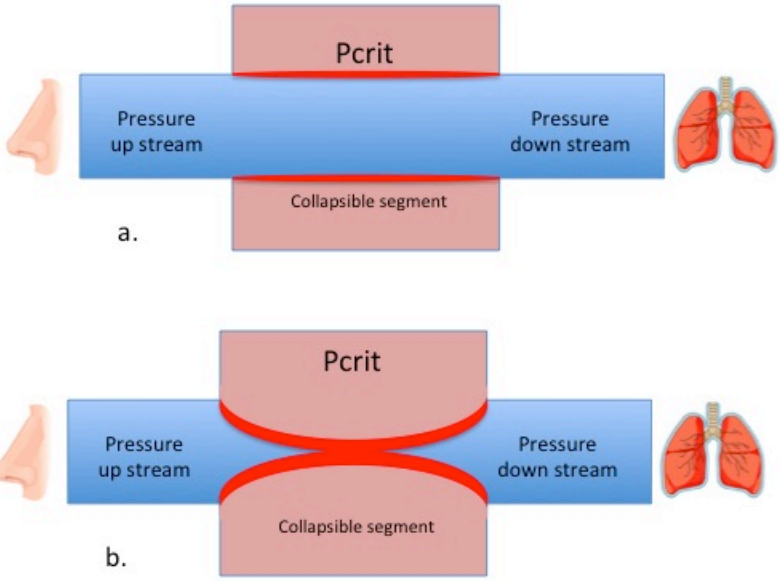


Figure 1.1. A demonstration of the critical closing pressure in a Starlings Resistor. a. The pressure upstream at the nose and downstream generated by the diaphragm are both greater than Pcrit. b. Here the Pcrit is greater than up and downstream pressures resulting in the collapsible segment to close over. In OSA an increase in the upstream pressure through the nose is needed to overcome Pcrit and open the airway allowing inhalation.

The presence of upper airway inflammation such as asthma or allergic rhinitis and altered neurological reflexes involving muscles of the upper airway as in neuromuscular disorders are common factors leading to increased upper airway collapsibility (Table 1.2.).

The deposition of exogenous tissue occurs in conditions such as mucopolysaccharidoses but is most commonly seen with the rising obesity epidemic in childhood. Fat infiltrates occur within compartments of the upper airway structures and neck contributing to narrowing and collapsibility. In addition, obesity results in generalised sleep disordered breathing secondary to a reduction in the functional residual capacity of the lungs due to abdominal visceral fat impinging on the chest cavity, limiting diaphragmatic descent particularly when supine. Chest wall adipose tissue can also decrease lung compliance leading to hypoventilation, atelectasis and ventilation perfusion mismatch [23].

A reciprocal interaction between obesity and OSA has also been postulated. Leptin, a key hormonal regulator of appetite and metabolism that is predominantly secreted by adipocytes, promotes satiety and reduced food intake. Conversely, ghrelin is an appetite stimulating hormone secreted by the gut. OSA can induce leptin resistance and increase

ghrelin levels, which can potentiate obesogenic behaviour, in particular, the intake of high calorie comfort foods [24].

The number of referrals to sleep units qualifying for a sleep study on the basis of a raised BMI is growing considerably resulting in recommendations to reclassify the underlying causes of OSA into groups with or without the present of obesity. Redline et al. demonstrated that obese children are at greater risk of OSA with a 12% increased risk with every 1Kg/m^2 they gain in BMI above the mean for height and gender[25].

Conditions associated with OSA in children	
Narrow upper airway	**Collapsible upper airway**
Adenotonsillar hypertrophy	Cerebral palsy
Obesity	Neuromuscular disorders (e.g., Duchenne's Muscular Dystrophy)
Micrognathia (e.g, Pierre Robin Sequence)	Myelomeningocele
Craniofacial syndromes (e.g., Crouzon's syndrome, Apert's syndrome, Treacher-Collin's Syndrome)	Allergic rhinitis
Achondroplasia	Asthma
Macroglossia (e.g., Beckwith Wiedemann syndrome)	
Mucopolysaccharidoses	
Sickle cell disease	
Afrocaribbean ethnicity	
Trisomy 21	

Table 1.2. Common disorders predisposed to OSA in children either due to anatomical narrowing of the upper airway or compression of a collapsible upper airway.

Overriding all these disorders is the presence of adenotonsillar hypertrophy. It is the most common anatomical cause of upper airway narrowing in children [26], contributes to all of the causes mentioned above and still remains the main focus for treatment. As the upper airway structures increase in size with age, the tonsils grow disproportionately faster resulting in a narrowing of the upper airway space before regression occurs in late childhood [27]. Activation of the upper airway musculature and increased central ventilator

drive compensate for this airway space reduction[28]. OSA is therefore the result of this interaction failing to compensate either due to a severe anatomic restriction or neuromuscular failure.

The size of the pharyngeal tonsils, more commonly referred to as adenoids, and the palatine tonsils has been shown using magnetic resonance imaging (MRI) to be enlarged in children with OSA compared to matched controls with a resulting decrease in upper airway volumes[29], findings were confirmed utilising MRI and direct endoscopy of the upper airway [30, 31].

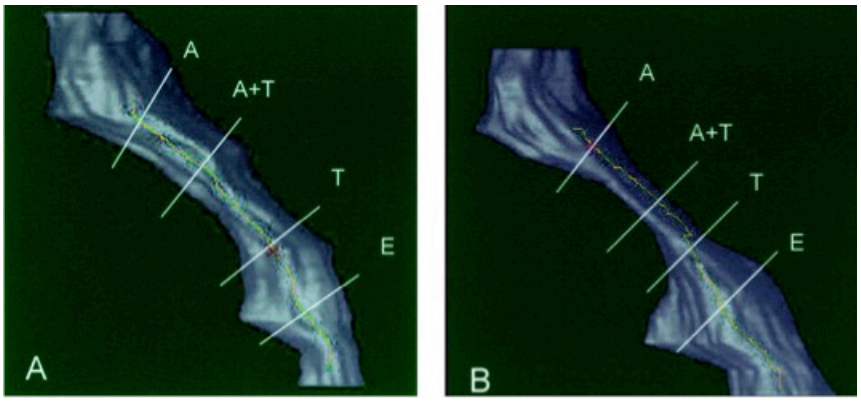


Figure 1.2. Three-dimensional display of the upper airway from a control subject (A) and a subject with OSA (B) with the grey area representing the space between structures. The line marked A represents the level of the adenoids, A+T the level of the overlap between adenoids and tonsils, T the level of the tonsils and E the level of the epiglottis. In the subject with OSA, the space between structures (grey area) if significantly reduced at the adenoids and tonsil level. Images from 'Upper airway size analysis by magnetic resonance imaging of children with obstructive sleep apnea syndrome', Am J Respir Crit Care Med 2003 [29]

The reason for increased cellular proliferation resulting in an increased volume of these lymphoid tissues is unclear. The proliferative rates of $CD4^+$ and $CD8^+$ T-cells isolated from tonsillar tissue has been shown to be higher in children with OSA compared to disease control subjects (recurrent tonsillitis). Furthermore, increased pro-inflammatory cytokine expression was demonstrated from the same extracted tissue implicating a T-cell driven inflammatory process to the underlying pathology[32]. This is discussed further in the next section.

1.1.4. OSA as an inflammatory disorder involving T lymphocytes

OSA is now accepted as a disease characterised by chronic, low-grade systemic inflammation. C-reactive protein (CRP) is a serum marker of inflammation, synthesised by

the liver and regulated by cytokines [33]. Unlike cytokines, CRP levels are quite stable in the same individual across a 24 hour period and reflect the level of inflammatory response [34]. A raised CRP associated with OSA was first demonstrated in adults with proven OSA compared to controls [35]. This finding has been confirmed in the plasma of children [36] with a significant reduction post adenotonsillectomy [37, 38].

The role immune cells play in OSA pathology has also been explored (Table 1.3.). Elevation of the pro-inflammatory cytokine Interleukin 6 (IL-6) and reduced levels of the anti-inflammatory cytokine IL-10 have been reported (albeit inconsistently) in the peripheral blood of children and adults. Gozal et al [39] demonstrated this difference in a group of 20 non-obese children with moderate to severe OSA compared to a control group of the same size. Higher levels of IL-6 and lower levels of IL-10 were associated with worsening OSA severity. Tam et al [40] however did not find any difference in a similar sized group of children with moderate OSA but did show higher levels of plasma interferon Gamma (IFN-γ) and IL-8 in the OSA subjects. Adult studies have both demonstrated [41] and failed to show a reduction in IL-10 in OSA subjects [42]. Some of the same authors who demonstrated a reduction in CRP post adenotonsillectomy have also published reduction in plasma TNF-α and IL-6 and an increase in IL-10 post surgical intervention [39, 43].

Two groups of $CD4^+$T helper cells in particular have been implicated in OSA pathology. Regulatory T-cells (Treg) modulate the immune system by maintaining tolerance of self-antigens and T helper 17 Cells (Th17) have the capacity to secrete IL17 and have been shown to produce inflammation and tissue damage in autoimmune disease[44].

1.1.4.a. T-regulatory cells (Treg)

Naturally occurring Tregs arise from the thymus, are defined as $CD4^+$ and possess the IL2 receptor CD25 and the fork-head box P3 transcription factor (Foxp3). They modulate multiple immune cells including Th1 cells, Th2 cells and Th17 cells, basophils, eosinophils and mast cells. This can either be by directly interacting with cells or secretion of anti-inflammatory cytokines. Tregs play a key role in the control of all immune responses including responses to self-antigens, tumors, microbes and grafts as well as inflammation [45-47]. A deficiency in Tregs is implicated in the pathology of Immunodysregulation, polyendocrinopathy, enteropathy (IPEX). Tregs have also been implicated in the pathology

of several autoimmune and inflammatory diseases: type 1 diabetes, multiple sclerosis, systemic lupus erythematous (SLE), rheumatoid arthritis, IBD, psoriasis and asthma [48].

1.1.4.b. Th17 cells

Th17 cells differentiate from naïve T-cells under the influence of transforming growth factor β (TGF-β), IL-23 and tumour necrosis factor α (TNF-α). They secrete several cytokines including IL-17A which can enhance neutrophil chemotaxis and has a role in the immunological response to bacterial and fungal infections. IL-17A has also been implicated in chronic inflammation and autoimmunity. It was first shown to induce IL-6 from fibroblasts in cultured synoviocytes from patients with rheumatoid arthritis [49]. Th17 cells have since been implicated in the pathology of psoriasis [50], multiple sclerosis [51], inflammatory bowel disease [52] and have been shown to be raised in the circulating blood of adult patients with allergic rhinitis and allergic asthma [53].

	No. Recruited	Sleep study	Control Subjects	Sample source	Finding
Tam 2006[40]	113	Yes	Yes	Plasma	Significantly elevated IFN-γ and trend towards elevated IL-8 in OSA.
Tauman 2007[54]	111	Yes	Yes	Plasma	Significantly elevated log plasma IL-6 levels in moderate- severe OSA.
Gozal 2008 [39]	40	Yes	Yes	Plasma	Higher circulating IL-6 and lower IL-10 in OSA.
Serpero 2009 [55]	57	Yes	Yes	Tonsils	Basal proliferative rate and TNF-α, IL-6 production increased in OSA.
Kim 2009 [32]	45	Yes	Yes	Tonsil	Tonsil cells in culture, basal proliferative rate, TNF-α, IL-6 and IL-1α higher in OSA.
Gozal 2010 [43]	298	Yes	Yes	Plasma	Significantly elevated TNF-α levels in moderate-severe OSA.
Sade 2011[44]	20	No	No	Adenoids	Negative correlation between Th17/Treg ratio and sleep questionnaire clinical scores.
Israel 2013[56]	50	Yes	Yes	Tonsil & adenoids	NF-kB p65 and p50 subunits and IL-1α over expressed children with OSA.
Tan 2013a[57]	56	Yes	Yes	Plasma	Significantly lower plasma Treg in moderate- severe OSA.
Tan 2013b[58]	50	Yes	Yes	Plasma	Significant negative correlation between Treg suppression and sleep pressure score.
Anderson 2014[59]	24	Yes	No	Tonsil	Significantly higher CD4+ cells and higher culture supernatant IL17A, IL-1β and IL-10 in moderate – severe OSA.
Gilesles-Hillel 2014 [60]	204	Yes	Yes	Plasma	Significantly elevated IL-6 levels in children with OSA independent of obesity.
Ni 2015 [61]	156	Yes	Yes	Plasma & adenoids	Higher peripheral blood and adenoidal Th17 cells and lower Treg cells in OSA subjects with positive correlation between cell ratio and adenoidal size.

Table 1.3. Summary of the published literature on immunological features noted in children with OSA including whether the sleep disordered breathing was diagnosed with sleep study, whether a disease-free control group was used for comparison and what tissue was used for the evaluation

1.1.4.c. Th17 and Treg in the context of OSA

In the extracted adenoidal tissue of children, Sada et al [44] found a negative linear correlation for Th17/Treg ratio and a clinical symptom score for sleep disordered breathing showing that children with higher Th17 and lower Treg cells in their adenoids had a more significant questionnaire score suggestive of sleep disordered breathing. Ni et al [61] found the same ratio in adenoids and peripheral blood correlated with adenoidal hypertrophy and upper airway obstruction. These findings are also present in adults newly diagnosed with OSA based on polysomnography. Peripheral blood samples demonstrated a higher proportion of Th17 cells and reduction in Tregs with a higher ratio of both in severe OSA and lower ratio in milder disease. [62]

Treg cells have been further implicated in pathology with evidence that the promoter region of the Foxp3 gene exhibits a severity dependant increase in methylation in paediatric OSA [63]. The addition of a methyl group may change the primary DNA sequence, altering gene transcription and ultimately reduced or impaired Tregs in children with OSA.

1.1.5. Consequences and associated effects of OSA

OSA results in a pattern of short intermittent high-frequency hypoxia with cycles of oxygen desaturation lasting 15-60 seconds followed by re-oxygenation occurring throughout a night's sleep. This is in contrast to sustained or low frequency intermittent hypoxaemia that lasts from a few minutes to hours that can be seen during rapid ascent and descent from altitude or in chronic lung disease during sleep. This latter pattern of intermittent hypoxia can promote both adaptive responses such as increased erythropoesis and maladaptive responses such as pulmonary hypertension. The high frequency intermittent hypoxia seen in OSA however generally leads to maladaptive responses only. The hypoxaemia and subsequent re-oxygenation results in an ischaemia-reperfusion type injury contributing to production of reactive oxygen species and oxidative stress[64] along with the inflammatory processes discussed previously. The impact of this can be seen in many different systems throughout the body in particular the cardiovascular and neurocognitive.

The cardiovascular consequences of OSA are well established in adults and now in children. One of the earliest reports of the disorder was by Guilleminault in 1976 who published a series of eight children with severe OSA resulting in *cor pulmonale*, the most serious

consequence of the disease [26]. Fortunately, this is now rarely seen. Children with OSA tend to exhibit more subtle evidence of cardiovascular dysfunction such as dysregulation of blood pressure, a phenomenon shown to improve following treatment in the form of adenotonsillectomy [65, 66]. Possible explanations for this include sympathetic neural activity resulting from the hypoxia and partial sleep loss resulting in neurogenic hypertension[67], dysfunction in the Renin- Aldosterone system [68] and lower baroreflex sensitivity[69].

Endothelial dysfunction has also been reported with consistent post-occlusive hyperaemic responses in children with OSA compared with controls. The majority once again demonstrated resolution following surgery except for those with a strong family history of cardiovascular disease[70, 71]. An association between adenotonsillar hypertrophy and raised brain naturetic peptide (BNP) released by cardiac myocytes in response to cardiac wall distension has also been reported[72]. The left ventricular strain and reduced contractility seen in OSA may be due to the effects of BNP. Greater overnight changes in BNP levels have been demonstrated in children with moderate to severe OSA (moderate relating to the number of apnoea and hypopnoea occurring per hour, the apnoea-hypopnoea index (AHI) of 5-10 events per hour and severe >10 events per hour) compared with mild OSA (AHI 1-5 events per hour) or controls likely related to the more frequent and pronounced negative intra-thoracic pressure swings seen in more severe OSA[73].

Other forms of treatment for OSA have also shown improvements in cardiovascular health. In adults, continuous positive pressure ventilation (CPAP) has been shown to reduce serum cardiovascular risk factors such as highly sensitive c-reactive protein (hs-CRP), homocysteine and mean total cholesterol levels following introduction with less significant reductions associated with poor compliance[74]. Raised CRP has also been reported in children with sleep disordered breathing, correlating with severity[36].

The impact on neurocognitive development is now also well established in children. Sleep is a fundamental requirement for growth and development with memory consolidation occurring during rapid eye movement sleep[75] and growth hormone release during slow wave sleep[76]. The mechanism for which OSA impacts neurocognition is likely to relate to the high frequency intermitted hypoxia fragmenting and impacting on memory laydown and growth hormone secretion. Animal models exposed to intermittent hypoxia demonstrate impaired cognition lasting for several days after recovery and increased

apoptosis in the hippocampal and cortical regions[77]. Long-term intermittent hypoxia can also enhance the production of angiotensin II and up- regulate Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which promotes oxidative tissue injury in the hippocampal catecholamine neurons[78].

One of the earliest studies to highlight a detrimental effect on academic performance and OSA was a prospective study looking at 297 first-grade children whose school performance was in the lowest 10th percentile of their class. Screening for OSA revealed that 18% had the condition. Compellingly, children who underwent treatment showed significant academic improvement in the subsequent year while those for whom their OSA remained untreated did not[79]. However this study lacked randomisation.

More recently, The Tucson Children's Assessment of Sleep Apnea (TuCASA) study identified a negative correlation between the AHI and immediate recall, Full Scale IQ, Performance IQ and maths achievements while nocturnal hypoxemia adversely affected nonverbal skills[80]. Even mild intermitted hypoxia has repeatedly been associated with adverse cognitive outcomes[81, 82]. There is also recent intriguing preliminary functional MRI data suggesting OSA influences cognitive and empathic processing[83].

In light of the daytime symptoms of hyperactivity and difficulty in concentrating exhibited by children, links with Attention deficit hyperactivity disorder (ADHD) and OSA have been proposed with improvements observed following adenotonsillectomy[84]. It is vital therefore to screen all children for whom a diagnosis of ADHD is being considered for evidence of sleep-disordered breathing. The hyperactive features demonstrated could be the result of OSA disturbed sleep.

However, not all children with OSA exhibit neurocognitive deficits. Genetic and environmental modifiers are likely playing a role in phenotypic expression. Genetic variants in NADPH oxidase 2, Insulin growth factor 1 (IGF-1) and apolipoprotein E are some of the candidate genes thus far identified as influencing neurocognitive consequences in children with OSA[85, 86]. Differences in plasma CRP levels, previously mentioned to be higher in children with OSA, appear to also differentiate children with the same severity of OSA who do or do not exhibit cognitive deficit[87].

Similar to adult published work, links between childhood OSA and metabolic dysregulation have been demonstrated albeit less robustly. Clear indications of a causal mechanism

between metabolic disorders and OSA in children are lacking. Mouse models of intermittent hypoxia demonstrate marked changes in insulin resistance, impaired b-cell function and increased oxidative stress that only partially improved after cessation of the hypoxia[88]. Experimental models of sleep restriction have also shown alterations in neuropeptides that regulate appetite[89].

Adolescents with OSA, the majority of whom were overweight, were reported to have a 6.5 times increased odds of metabolic syndrome compared with those without OSA[90]. In younger children, OSA has been shown to be associated with reduced insulin sensitivity only when obesity was concurrently present, and improvement in homeostasis model assessment (HOMA- a model for quantifying insulin resistant and beta-cell function) was reported following effective OSA treatment[91, 92]. Children with adenoidal or adenotonsillar hypertrophy have also been shown to have lower high-density lipoproteins[93].

A higher prevalence of nocturnal enuresis has been reported in children with OSA. A systemic review including 14 studies found a third of children with sleep disordered breathing were diagnosed with nocturnal enuresis [94]. It has been postulated that this may be due to the dampening effects of OSA on arousal response to changes in bladder pressure, or may be related to elevated BNP levels which impact on the renin-angiotensin pathway, vasopressin and excretion of sodium and water[95]. These studies however all used a clinical diagnosis of sleep-disordered breathing.

Based on the work described above, OSA is no longer considered to be an inconsequential night time phenomenon. Untreated, the disease can result in an array of consequences. Efficient and accurate assessment and diagnosis is therefore vital.

1.1.6. Clinical evaluation and diagnosis of OSA

The assessment of a child with suspected OSA should start with a thorough history including daytime and night time symptoms as well as the presence of associated morbidities such as neuro-behavioural deficits, sleepiness, failure to thrive and systemic hypertension. A physical examination should pay close attention to the size of the palatine tonsils in relation to the size of the throat, allergic rhinitis or any other condition likely to increase nasal airflow resistance and the relative size and position of the mandible.

Unfortunately, the accuracy of OSA diagnosis based on history and physical examination alone has been shown to be poor even when performed by sleep physicians [96]. The latest American Academy of Pediatrics (AAP) guidelines quote a positive predictive value of 65% for history and 46% for clinical examination. Unsurprisingly, they therefore recommend that all children with symptoms of OSA should be referred for further investigations. In settings where access to sleep studies is limited, formalised clinical assessment and examination may be of benefit. The Clinical Assessment Score 15 (CAS-15) correctly diagnosed 72% of children with polysomnograpy identified sleep disordered breathing and can be carried out in an office environment [97].

There are several modalities used to investigate SDB. Interventions such as the paediatric sleep questionnaire have their uses for research purposes when assessing differences between groups, but are not reliable for many individual cases [98, 99]. Other devices such as the Tucson Children's Assessment of Sleep Apnea Study (TuCASA) questionnaire or the Cleveland Adolescent Sleepiness questionnaire (CASQ) has been validated for clinical use outside of research[100, 101] however it is unlikely that any questionnaire-based diagnosis will supersede the data produced from sleep studies.

The current gold standard for diagnosis of OSA is an overnight in-lab polysomnography (PSG) study. PSG provides an objective quantification of disturbance in respiratory parameters and sleep patterns, allowing the classification of patients into different disease severities, thereby enabling clinicians to tailor clinical management accordingly (Figure. 1.3.a). The ICSD-3 PSG criterion for diagnosis requires either (1) one or more obstructive events (obstructive or mixed apnoea or obstructive hypopneas) per hour of sleep or (2) obstructive hypoventilation, manifested by PaCO2 >50mmHg for >25% of sleep time, coupled with snoring, paradoxical thoracoabdominal movements, or flattening of the nasal airway pressure waveform implying flow limitation[1].

PSG however may not be widely available in all countries. It is labour intensive, requiring in-hospital monitoring of the patient by skilled staff overnight and subsequent scoring and analysis, which is expensive both in terms of time and resources. In countries were PSGs are not available, alternative and less intensive studies are performed. The basic study would be a limited channel nocturnal oximetry study (Fig. 1.3.b.) measuring oxygen saturations, heart rate with the option of additional transcutaneous carbon dioxide (CO_2) monitoring. These studies have been shown to have a high specificity but low sensitivity in the diagnosis of

paediatric OSA [102]. A negative result indicates the need for further investigations however this level of study has an important role as a screening tool or in resource poor settings as an aid to diagnosis used in conjunction with a formalised clinical assessment and examination.

A more detailed but less labour intensive study is the Respiratory Polygraph study (RP). This has cardiorespiratory measurements but lacks electroencephalography (EEG), electromyography (EMG) and electrooculography (EOG) leads used to stage sleep and is performed quite commonly in Europe (Fig. 1.3.c.). The limitation of the RP is the possible underscoring of hypopnoea resulting in arousal that would require sleep stage assessment to identify. This can be overcome to some extent by the use of a video recording of the child during sleep to effectively define sleep onset and end as well as noting arousals from sleep[103]. The Apnoea-Hypopnoea Index (AHI) generated from a RP has been shown to correlate well with the value generated from PSG [104] however one study did show that on Bland-Altman analysis the AHI may be underestimated[105].

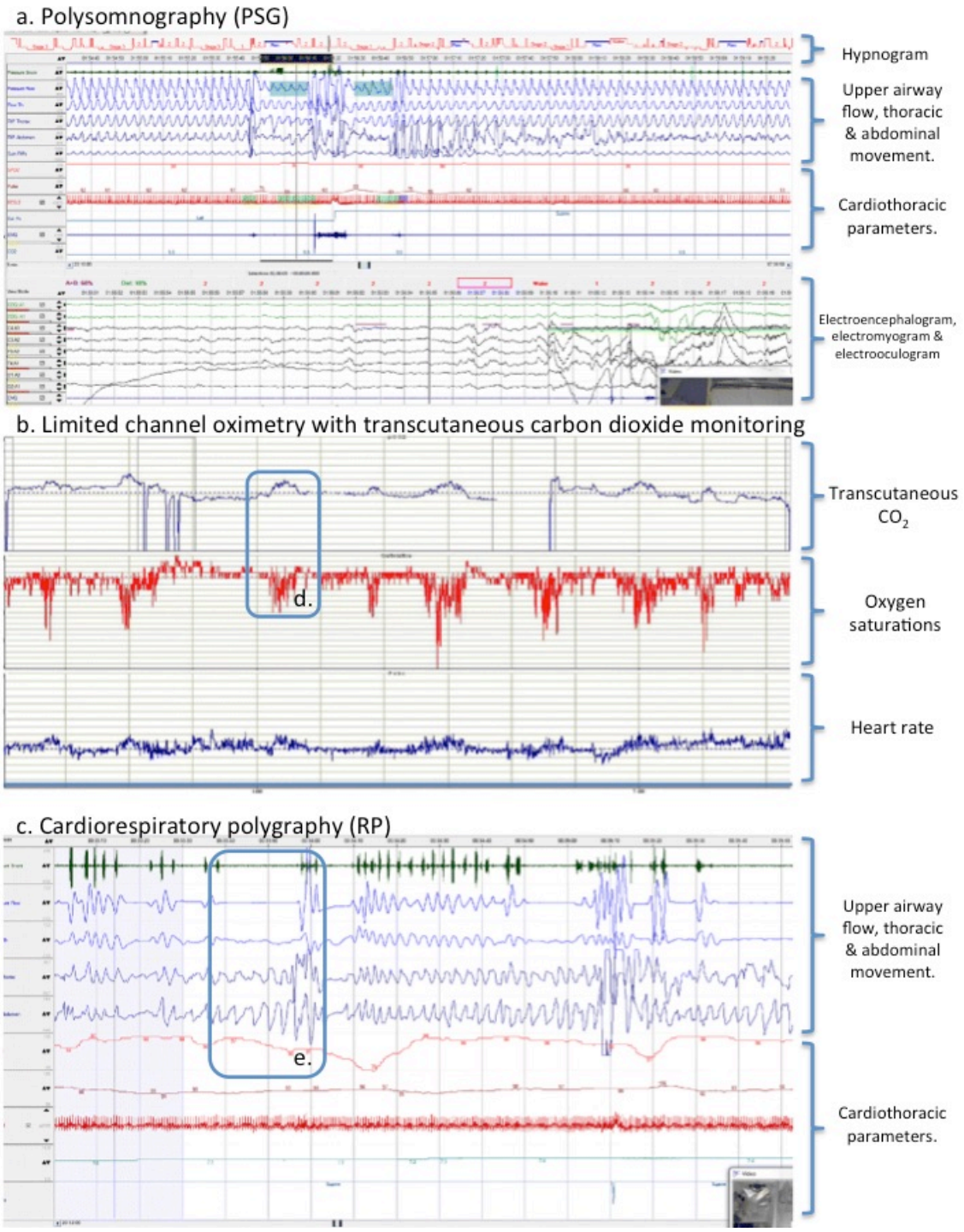


Figure 1.3. a. Example of obstructive hypopnoea seen in PSG. **b.** Example of oximetry and transcutaneous CO_2 study of child with OSA. Note the clusters of desaturations associated with rise in CO2 (**d.**) (occurring during rapid eye movement (REM) sleep). **c.** Example of obstructive apnoea seen in respiratory polygraphy (Note the paradoxical breathing seen during the apnoea- **e**.).

An additional form of sleep study to consider would be the Ambulatory home sleep study or Home respiratory polygraphy (HRP) studies. This investigative system is particularly useful in rural settings where access to specialist sleep units is limited or in-lab resources are not available. The home sleep study provides the potential level of data as the RP with the limitation of no directly observed monitoring to replace disconnected leads to correct lost signals throughout the night. While HRPs have been shown to correlate well with in-lab PSGs [104], underscoring has been reported due to the use of total recording time (TRT) as the denominator for calculating the events per hour score compared to a more accurate total sleep time (TST) generated from direct observation of the start and end of a night's sleep[106]. Whatever the available resources or expertise, whilst these investigations may not have the sensitivity and specificity of PSGs, as long as the clinicians analysing the data and making clinical judgements based on the outcomes are aware of the limitations, the information provided is still useful.

It is important to mention that despite improvements in access to sleep studies and considering the limitations of clinical assessment discussed above, the majority of surgical interventions in the UK and abroad still take place without PSG or RP confirmation of OSA.

1.1.7. Treatment of OSA

The cut offs for treatment based on sleep study data in childhood vary internationally. Most sleep centres consider an obstructive AHI (OAHI) of ≤ 1 to be normal, 1<OAHI ≤5 to be mild OSA, 5<OAHI≤10 to be moderate OSA and OAHI >10 to be severe [107]. Most clinicians would instigate treatment either surgical or a form of ventilation support for moderate to severe OSA but there remains considerable variability in practice in the management of mild OSA. The type of treatment must take into consideration the patient and the effect the disease is having on their quality of life. Clinical algorithms proposed by the European Respiratory Society Task force [108] take into account PSG results and severity of symptoms, risk factors and the presence of any OSA-related morbidity to provide a clinically relevant approach to diagnosis and prioritization of treatment (Figure 1.4.).



Figure 1.4. Diagnostic and treatment algorithm proposed by the ERS task force. 'Obstructive sleep disordered breathing in 2-to 18-year-old children: diagnosis and management, European Respiratory Journal 2016 [108].

Adenotonsillectomy (AT) remains the first line treatment for children with moderate to severe OSA and is to be considered as a treatment option in milder disease. It involves the surgical removal of the pharyngeal tonsils (or adenoids) and the complete removal of the palatine tonsils along with the capsule of the tonsil in most cases and leaves underlying

muscle disrupted. This is usually performed utilizing a range of ablation or electro-cautery dissection techniques. The removal significantly improves signs and symptoms immediately and at follow-up after 40 days but only results in complete disease resolution in a 27.2% of cases [109]. The reported percentage of complete resolution of disease in moderate to severe cases was between 13% (when AHI ≥5/h TST was used as cut off) to 73% in obese children using the lower cut off of AHI ≥1/h TST. Risk factors for persistence of disease include obesity, asthma, neuromuscular disease, high Mallampati score (measure of palatine tonsil size), African-American ethnicity, craniofacial anomalies, chromosomal abnormalities and a high perioperative AHI (>20/h TST).

The Childhood Adenotonsillectomy trial (CHAT) was the first therapeutic randomised controlled trial to compare adenotonsillectomy with watchful waiting, for the management of OSA. The primary outcome measure was attention and executive – function score on the Developmental Neurological Assessment[110]. No difference was observed comparing surgery with watchful waiting. The trial was however limited to only those with mild OSA and no significant oxygen desaturations due to ethical considerations and children under 7 years were excluded. The follow up period was also limited to 7 months. Tonsillectomy did result in significant improvement in the trails secondary outcomes such as symptoms of OSA and parent-rated generic and OSA-specific quality of life measures [111].

Adenotonsillectomy is a surgical procedure and not without risk. The CHAT study also provided trial data on the post-operative complications including pulmonary oedema, hypoxemia, bronchospasm, dehydration, haemorrhage and fever encountered by a total of 8 children out of a total of 464. No clear demographic difference or PSG features could be identified to predict complications prior to surgery [112]. Alternative surgical methods have therefore been proposed, one such being a sub-total tonsillectomy or tonsillotomy. The aim here is to remove the lymphoid tissue and crypt epithelium, whilst preserving the faucial arches and mucosal plicae. A recent systematic review has reported comparable efficacy to tonsillectomy in the treatment of OSA with less post-surgical pain and analgesia use, less post-operative haemorrhage and higher patient/ parent satisfaction scores[113]. However, there is a risk of regrowth of which patients and parents must be made aware. This occurred in 13% (5/39) of tonsillotomy cases in a recent randomised control trial comparing the two techniques [114].

In cases of OSA that are the result of craniofacial abnormalities, skeletal advancement procedures may be beneficial. Mandibular distraction osteogenesis involves the lengthening of the mandible bringing the muscular insertion of the tongue forward and thus increasing the anterior-posterior dimensions of the airway. This technique has been found useful for children with micrognathia or retrognathia enabling relief from symptoms, prevention and decannulation of tracheostomy[115]. Children with maxillofacial abnormalities and dental malocclusion may benefit from rapid maxillary expansion which involves the use of an orthodontic procedure to gradually widen the maxillary bone at the mid-palatal suture increasing the volume of the nasal cavity and decreasing nasal resistance. This procedure has been shown to relieve symptoms, reduce AHI score and normalise sleep architecture[116].

For children with residual disease after surgery or for those without adenotonsillar hypertrophy causing an upper airway obstruction, positive airway pressure therapy (PAP) in the form of continuous positive airway pressure (CPAP) or bi-level positive airway pressure (BiPAP) may be indicated to splint open the upper airways during sleep[26]. The goal is to maintain airway patency throughout the respiratory cycle improving functional residual capacity (FRC) and decrease the work of breathing. A post-operative AHI of 5 or above would be an indication for ventilator support [117]. It is often required as first line treatment in children with obesity, craniofacial abnormalities or neuromuscular disorders.

In children with significantly raised CO_2 overnight, those with higher-pressure requirement during sleep or those with neuromuscular disease where the OSA is the result of muscle weakness and not necessarily obstruction, BiPAP is indicated. This mode of ventilation provides a higher inspiratory pressure coinciding with a subject's inspiration, which terminates at the end of inspiration providing the continued pressure support of CPAP during expiration maintaining airway patency. Studies comparing the two primary methods have not demonstrated a difference in adherence or outcome for children[118]. In practice BiPAP is often used when higher pressures are required.

PAP can be difficult to instigate and maintain. A multi-disciplinary approach is needed to desensitise children to the device and establish it as a part of their daily routine minimising potential side effects. Ensuring a comfortable fit of the headgear, minimising erythema and preventing tissue damage while proving an appropriate seal, is essential. Children tend to prefer nasal mask compared to full face (which includes nasal and oral cover) however

significant nasal obstruction or persistent mouth breathing may dictate the need for an oro-nasal interface. Long-term follow up is required to monitor and upsize devices as the child grows, avoiding flattening of the mid-face or maxillary retrusion from the pressure effect.

High flow oxygen via nasal cannula (HFNC) has been proposed as a possible 'gentler, kinder' alternative to PAP. Most HFNC experience derives from its use in acute respiratory distress or neonatology. There have however been several small studies investigating its role in OSA, which have demonstrated a reduction in AHI and improvement in oxygenation [119, 120] however larger comparison trials against PAP are needed.

Medical therapies have gained acceptance for milder disease in an attempt to avoid surgery. Cysteinyl leukotrienes are major mediators of inflammation and serve as neutrophil chemo attractants and activators. The Cysteinyl leukotriene receptors 1 and 2 (LT1-R and LT2-R) are both present on nasal mucosa. The leukotrienes B4 and cysteinyl leukotrienes C4, D4 and E4 bind to these receptors resulting in airway constriction, increased vascular permeability, oedema, and an influx of eosinophils and neutrophils. The palatine tonsils of children with proven OSA compared with those of children with recurrent tonsillitis and no evidence of OSA show higher protein expression of the LT1-R and LT2-R suggesting the leukotrienes may be contributing to the lymphoid proliferation found in OSA patients[121-124].

A role of leukotriene antagonists has emerged as a treatment of mild OSA in a similar fashion to their established role in the treatment of asthma, with improved AHI following treatment. In a group of 23 children diagnosed with mild OSA (AHI>1 and <5) given a 16 week course of *Montelukast*, there was not only a reduction in the AHI on repeat sleep study but also a reduction in adenoidal size based on lateral X-ray compared to a control group[125]. Other sleep study parameters showed a non-significant reduction compared to the control group.

As inflammation appears to contribute to OSA, a role for glucocorticoids has been proposed as another alternative to surgical interventions. In an in vitro model, a mixed cell culture obtained from extracted tonsil tissue responded to corticosteroid treatment with reduced proliferative rates, increased apoptosis and reduced inflammatory mediator production, namely TNF-α, IL-8 and IL-6[125]. Clinical applications for corticosteroids administered directly to the nasal mucosa have shown improvement in symptoms and polysomnography

results in mild OSA with no rebound symptoms at 8 weeks after treatment was discontinued [126-128]. The use of combination of oral leukotriene antagonist and intranasal corticosteroids post adenotonsillectomy has also been shown to be effective for residual post-operative OSA [129].

1.2. Asthma, Allergy and Atopy

1.2.1. Definitions and Prevalence of asthma, allergy and atopy

The term allergic diseases comprise a group of chronic illnesses that are genetically heterogeneous. The four most common that affect children are allergic asthma, allergic rhinitis, atopic dermatitis and food allergies. Atopy describes a genetic tendency to develop allergic disease and is associated with a heightened immune response to common allergens especially inhaled or food allergens [130, 131]. Atopy is generally defined as one or more positive reactions to an aeroallergen skin prick test or serum specific Immunoglobulin E (IgE).

The most common allergic disease in childhood is asthma, which is a heterogeneous collection of disorders with reversible bronchial obstruction and evidence of chronic airway inflammation as the common feature. This airway hyper-responsiveness may resolve spontaneously as the child ages or require medication for life [132]. Most cases are mild and can be cared for in a primary care setting however a spectrum of disease exists including severe therapy resistant asthma at the extreme.

The prevalence of asthma has increased worldwide along with the prevalence of atopy [133-136]. Currently respiratory allergic disease (asthma and allergic rhinitis) affects 700 million subjects worldwide with 6 million children affected by asthma in the United States [137, 138] but rates vary widely between countries. In the UK, childhood asthma saw a 2-3 fold increase in prevalence over the second half of the 20th century with a possible plateau or even down turn in the early 21st Century[139]. Currently there are 1.1 million children being treated for asthma in UK with a child being admitted to hospital every 20 minutes due to an exacerbation (www.asthma.org.uk).

1.2.2. Pathophysiology and basic mechanisms of allergic disease and asthma

The pathophysiology of allergic disease involves the interplay between the adaptive and innate immune system.

1.2.2.a. Innate immune system

The pulmonary epithelium responds to environmental insults by secreting inflammatory mediators and antimicrobial peptides[140]. In the case of microbial infection, pathogen-associated molecular patterns (PAMPS) provide exogenous signals to alert the immune system to the presence of pathogens [141]. Cells release damage-associated molecular patterns (DAMPS) as endogenous danger signals to alert the immune system to unscheduled cell death, microbial invasion or as a stress response[142]. These molecules bind to specific receptors such as Toll-like receptors (TLR) and induce autophagy[143]. Damage and receptor binding results in the release of the innate mediators IL-25, and IL-33 and the cytokine protein Thymic Stromal Lypoprotein (TSLP), which activate lineage negative innate lymphoid cells (ILCs).

ILCs are a relatively recently described novel class of leukocytes that lack the classical lineage markers assigned to T-cells, B-cells and other known immune cells and are induced by innate epithelial cytokines. They have been classified according to their cytokine secretion patterns which appear to mirror the classical T-helper cells groups (Innate Lymphoid Cell Group 1, 2 and 3). They provide early signals to help eliminate pathogens but they also secrete cytokines that help in generating the adaptive immune response [144] (Figure 1.6.).

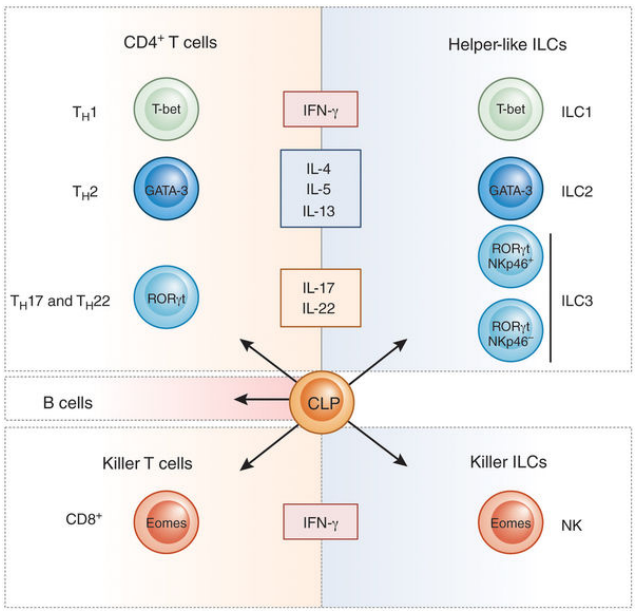


Figure 1.5. Comparison of T-cells and ILCs demonstrating cytokine production profile and transcription factors for both. The brave New World of Innate Lymphoid Cells, Nature Immunology 2015 [145].

In an effort to unify the field, Spitz and Artis proposed a nomenclature for these new cells reflecting their cytokine profile and transcription factors[146]. Innate lymphoid cells group 1 (ILC1) express the transcription factor T-bet and produce IFN-γ. Innate lymphoid cells group 3 (ILC3) have the retinoic acid orphan receptor γt (RORγt) and produce IL-22 and IL17.

Group 2 innate lymphoid cells (ILC2) produce Th2 cytokines (IL-4, IL-5, IL-9 & IL -13) and have been found at mucosal surfaces such as tonsils and gut mucosa, and also circulating in peripheral blood[144]. Several different groups have now established the role ILC2 cells play in allergic disease in murine models [147-149]. In a T-cell eliminated mouse asthma model, ILC2 are induced by IL-33 and in turn induce more IL-33 production establishing positive feedback [150]. The same group reinforced the role of ILC2 and IL-33 when higher levels of IL-33 were found in adult asthmatic bronchoalveolar lavage (BAL) compared to other diseases (interestingly including other allergic diseases).

In humans, ILCs have now been established as existing at mucosal surfaces including the airways [151]. ILCs have also been found in induced sputum samples from adult asthmatics [152] and children with severe therapy resistant asthma[153]. ILC2 cells specifically have

been identified within nasal polyps of patients suffering chronic allergic rhinosinusitis [154] and have been assigned critical roles in atopic dermatitis and asthma pathology [155, 156].

1.2.2.b. Adaptive immune system

The adaptive immune response is orchestrated by lymphocytes in two ways, an antibody-mediated and cell-mediated response. The antibody-mediated response is the result of B-cell differentiation and resulting production of immunoglobulins. Immunoglobulin E (IgE) for example can then circulate in the blood stream, permeating tissues and bodily fluids to ultimately bind to host cells such as mast cells or basophils forming a receptor cross-link. When IgE encounters an antigen the receptors cross link causing degranulation of the host cell and release of type 2 cytokines.

The cell-mediated functions of the adaptive immune system are the result of T-cells with their ability to act directly against foreign antigen. Cytotoxic T-cells expressing the CD8 glycoprotein are able to bind to antigen bound to Major Histocompatibility Complex I (MHC1) molecules on the surface of cancer and virus-infected cells. Memory T-cells provide the immune system with a long lasting 'memory' of an antigen encounter and the capacity to rapidly expand into effector T-cells on next exposure.

The Helper T-cells (Th) have the ability to assist with immunological processes such as maturation of B-cells or memory T-cells and activation of $CD8^+$ T-cells and macrophages. They express the glycoprotein CD4 and on activation by an antigen-presenting cell, can secrete a range of cytokines depending on their subtype differentiation. There are at least three subtypes of differentiated Th cells from naïve T-cells, Th1, Th2 and Th17 cells, the latter have been discussed in regards to the pathology of OSA.

The transcription factor T-bet is the major factor for differentiating Th1 cells from naïve T-cells that are then able to produce IFN-γ. Their major function is in defence against bacteria and protozoa, activating macrophages. Over activation against auto-antigens can result in type 4 delayed hypersensitivity disorders.

Gata3 is a transcription factor present on all naïve T-cells but when up regulated, leads to differentiation into Th2 cells capable of producing IL-4, IL-5, IL-13 and in lesser amounts IL-9. Th2 cells have a role in immune defences against extracellular parasites such as helminths however their main effector cells are eosinophils, basophils, mast cells and B-

cells. Over activity can lead to type 1-IgE mediated hypersensitivity disorders such as allergic dermatitis and food allergies.

Allergic disease involves the presentation of an allergen to antigen presenting cells such as dendritic cells. The immature dendritic cells are present at mucosal surfaces and extend projections between epithelial cells to sample the environment. They phagocytose allergens and pathogens, process them and present them on their cell surface using MHC molecules. They up regulate CCR7, a chemotactic receptor that induces them to travel to the lymph node where they activate T cells and B cells [157]. This results in T-cell differentiation in the presence of IL-4, into an allergen specific T helper (Th2) cell, which travels back to the epithelium and on exposure to the allergen, releases IL-5, IL-13 and IL-4 generating a positive feedback loop [158].

The presentation of the antigen via dendritic cells and MHC molecules induces B-cells in the lymph node to secrete IgE into the circulation to trigger an allergic response on contact with the allergen again in the future. The reaction in allergic disease is the result of mast cell degranulation and the release of histamine and leukotrienes [159]. The degranulated mast cells secrete tryptase that acts upon the factor II receptor like 1(F2RL1) on the surface of endothelial and epithelial cells, fibroblasts and smooth muscle cells. Activation of F2RL1 results in tumour necrosis factor α (TNFα), IL-1 and IL-4 secretion, which in-turn leads to the recruitment of basophils and eosinophils (Figure 1.5.).

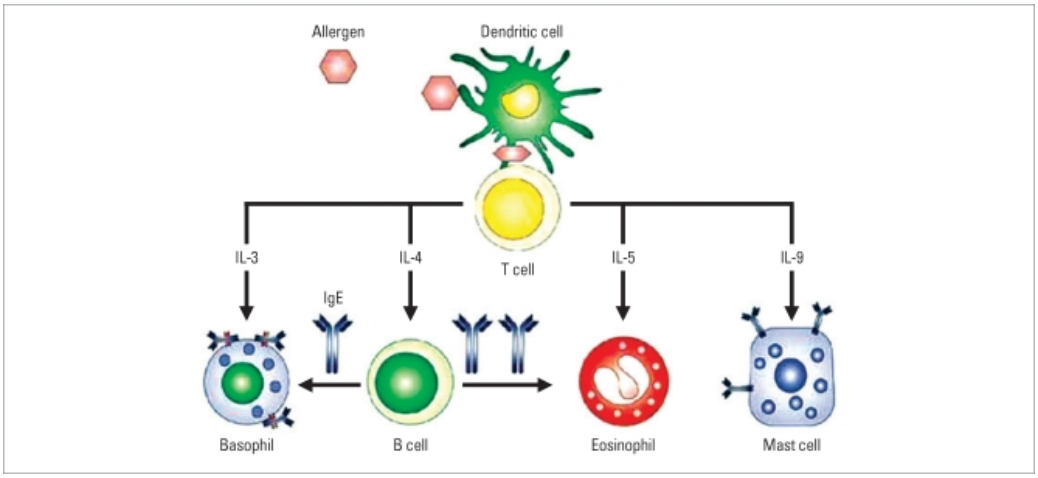


Figure 1.6. Allergen exposure leading to an adaptive immune response differentiating a naïve T-cell. The pathophisology, diagnosis and treatment of allergic rhinitis, Allergy, Asthma and Immunology Research 2010 [160].

The pathogenesis of asthma is considered to be driven by Th2 cells and adaptive immunity as with the other allergic diseases. This has been supported by the fact that sensitisation to aeroallergens was strongly associated with asthma[161] and reinforced by the presence of Th2 cells expressing the signature cytokines interleukin 13, 4 and 5 infiltrating the airway [162-164]. IL-13 induced airway hyper-responsiveness goblet cell metaplasia and mucous production. IL-5 is a key eosinophil mediator. This concept however has been modified of late to include the emerging role the innate immune system plays in health and disease.

1.2.2.c. Immunoglobulin E

In all allergic pathologies, IgE is a key component in disease propagation as well as laying the paving stones for future reactions. There are two IgE receptors; High affinity receptor (FcεrR1) and low affinity receptor (RcεR11 or CD23) [165]. The high affinity receptor is expressed on mast cells and basophils. The binding of IgE to these cells sensitizes them to the allergen. Future exposure leads to cross-linking of membrane bound IgE in mast cells and basophils inducing degranulation and the release of histamine, tryptase, cysteine-leukotrienes and platelet-activating factors [166, 167]. The result is oedema and bronchoconstriction of the airway.

The low affinity receptor is found on T-cells, Langerhans cells, macrophages, monocytes, eosinophils and platelets. The binding of the receptor with IgE mediates IgE regulation, activation of monocytes and differentiation of B-cells to secrete more IgE [168]. Secretion of IL-4 and IL-13 by the Th2 cells after allergen presentation leads to up-regulation of the low-affinity receptor and induces the IgE proliferation by B-cells [169].

1.2.2.d. Pathology of allergic disease

Longitudinal cohort studies of children from birth into early adolescence have been used to assess patterns or clusters of subjects with similarities in disease presentation temporality and predominance of one or more allergic disease. The $COPSAC_{2000}$ birth cohort of children born to mothers with a history of asthma identified four such clusters of subjects: asthma-dominated; eczema dominated; sensitisation dominated; healthy subjects [170]. Results from an association of atopy phenotypes study three clusters of children were identified: later sensitisation to indoor allergens; early onset of multiple allergen sensitisation; early sensitisation to outdoor allergens [171].

The signs and symptoms of disease will relate to the location of the allergen interaction and can be site specific or systemic. Allergic rhinitis and food allergies are examples of type I hypersensitivity disorders with a rapid onset of symptoms when exposed to allergen. Type 2 hypersensitivity underlies many cases of asthma where continuous exposure to allergens triggers local inflammation leading to hypersensitivity of the airways, bronchoconstriction and obstruction. Allergic dermatitis is an example of a type IV hypersensitivity disorder with a slower or delayed onset mediated by cell interaction rather than antibody driven.

Allergic rhinitis

The allergic reactions in allergic rhinitis (AR) occur in two phases. The early phase reaction is the result of mast cells releasing histamine, prostaglandins and leukotrienes and develops approximately 30 minutes after allergen exposure. The late phase occurs approximately 6 hours after exposure and is the result of eosinophil chemotaxis brought about by the early phase reaction. The migration of eosinophils, mast cells and T-cells to the nasal mucosa causes tissue destruction resulting in the upper airway obstruction seen in AR.

What follows next is neurogenic inflammation as a result of exposed nerve endings due to tissue destruction by the eosinophils. Non-specific stimulation of the sensory nerve fibres results in the release of neuropeptides inducing smooth muscle contraction, mucous secretion from goblet cells and plasma exudation from capillaries all adding to the airway obstruction [160]. Chronic rhinosinusitis is the description of this disease when symptoms have persisted for at least 12 weeks. In this context, ILC2s ($Lin^{neg}CD127^{+}CRTH2^{+}$) have been implicated in the pathology with increased numbers in nasal polyps from patients with chronic rhinosinusitis compared to tissue from controls subjects without allergic disease [154, 172].

Food allergy

The gastrointestinal tract is the largest immunological organ in the body and is constantly exposed to endogenous antigens. A single cellular layer separates the antigenic load from the collection of immunological cells in the lamina propria including lymphocytes, antigen presenting cells and stromal cells comprising the mucosal-associated lymphoid tissue, MALT. Within the MALT, a population of dendritic cells are exposed to dietary antigens and determine the fate of this reaction ie. immunity vs tolerance. Oral tolerance is the term used when T-cell mediated suppression prevents an allergic reaction. However in 4-6% of

children, this mechanism fails and an immune reaction is generated in response to a specific antigen.

Once the dendritic cells are primed with the antigen, the allergy specific Th2 cells differentiate and stimulate B-cells to produce IgE. The IgE binds to high affinity receptors on the surface of mast cells in skin, gut, respiratory tract and the cardiovascular system. With re-exposure to the antigen, now viewed as an allergen, allergic symptoms can occur within minutes of exposure [173]. Currently, ILCs have not been implicated in the pathology of food allergy.

Allergic Dermatitis

There are two main components to the pathophysiology of allergic dermatitis (AD): epidermal structure and function abnormalities; inappropriate cutaneous inflammation due to immune responses to antigens encountered in the skin. Abnormalities of the stratum corneum (the major epidermal barrier structure) associated with AD include decreased hydration and increased water loss, altered lipid composition, raised skin pH and reduced skin microbial diversity.

The second hallmark feature of AD is inflammatory cell inflammation. Infiltrating differentiated T-cells express various skin-homing adhesion molecules that enable their recruitment into cutaneous sites. As well as recirculating through skin, antigen-primed T-cells persist as resident memory cells in local skin pools and exert rapid recall responses.

Non-lesional skin progresses to AD 'flares' characterised by epidermal thickening, enhances expression of immune-stimulatory chemokines and further infiltration of Th2 cells and epidermal dendritic cells. Th17 cells along with chemokines and cytokines such as thymic stromal lymphopoietin (TSLP) derived from fibroblasts and keratinocytes drive tissue remodelling and fibrosis. TSLP also promotes maturation and proliferation of epidermal dendritic cells, Th2 polarisation and induces B-cell proliferation and differentiation. Treated tissue with resolution of disease will continue to show immunological and structural abnormalities that may contribute to disease re-occurrence at that site [174].

ILC2s have also been implicated in AD with evidence of expanded populations within lesions [175, 176] with increased transcripts for IL-25 and IL-33 also detected in the lesions [175-177]. The lectin inhibitory receptor KLRG1 may have a regulatory role in the control of

ILC2 activation during atopic dermatitis, because the KLRG1 ligand epithelial cadherin (E-cadherin) diminishes ILC2 cytokine production, and E-cadherin is down regulated in atopic dermatitis lesions [175].

Allergic asthma

Similar to the gastro-intestinal tract, the lungs are continuously exposed to pathogens, pollutants and antigens and have to differentiate between pathological and innocuous allergens. The bronchial epithelium acts as an immunologically active barrier to these agents. In asthma, the integrity of the tight junction of the epithelial cells is altered allowing antigens to penetrate [178]. Airway dendritic cells are exposed to antigens and able to deliver them to neighbouring lymph nodes allowing T-cell priming and B-cell differentiation to occur.

The infiltration of immune cells generating an inflammatory environment leads to oedema of the airway cell walls. T-cells are the main effector cell producing IL-13 that leads to airway hyper-responsiveness and mucous production and provide an environment for sustained inflammation. The other effector cells in asthma are the mast cells and eosinophils. Leukotrienes released from mast cells results in smooth muscle contraction, bronchospasm and increased mucous secretion. Eosinophil major basic protein (MBP) increases acetylcholine release and desquamation of the epithelium [179]. As well as epithelial loss, children with allergic asthma develop an increased number of airway blood vessels and a thickened reticular basement membrane [180].

As with the other allergic diseases, a role of ILCs has also been established in asthma. Elevated expression of IL25 and IL33 and TSLP has been shown in human asthmatic lung tissue[181-183] and ILC2s are increased in the peripheral blood of people with asthma relative to those without the condition [184]. Several genes associated with ILC2s are enriched in people with asthma, including the gene that encodes IL1RL1 (a component of the IL-33 receptor), RORα and IL-13, suggesting a role for ILC2s in disease pathogenesis [185].

1.2.4. Clinical evaluation and diagnosis of allergic disease and asthma

The evaluation of allergic disease and diagnosis remains firmly based on clinical symptoms and findings. There are no tools for measuring the severity of a food allergy reaction for example. The mere fact it occurs is sufficient for diagnosis and future management, namely avoidance of the allergen and introduction of emergency rescue medication should it be needed. There are also no laboratory and histological findings for these diseases specifically. Diagnosis relies on laboratory evidence of exposure and immune priming in preparation for future allergen contact. This can be performed using skin prick tests with measurement of the localised reaction or measuring serum levels of total and specific IgE to the allergen in question.

For asthmatic individuals, symptoms usually develop during pre-school years. Even those diagnosed in early adult life commonly have higher rates of bronchial hyper-responsiveness and episodic wheeze in childhood[186, 187]. Most childhood asthmatics or 'pre-school wheezers' demonstrate intermittent airway obstruction in response to illness, provocation from an allergen of some form or from physical exercise. As with the rest of the allergic diseases and recognising its varied nature, several childhood asthma phenotypes have been proposed based on frequency of wheezing, age of symptom onset or level of IgE sensitisation to allergens [131, 188].

The first step in the diagnosis of asthma is a thorough clinical history exploring common asthma-like symptoms such as night time symptoms or triggers. The term wheeze should be used with caution as it can be incorrectly used by parents when describing symptoms [189]. Response to short-acting bronchodilators is then a key feature in diagnosis. This can be measured immediately by observing a 12% or more improvement in peak flow after administration, over a longer period of assessment with serial peak flow measurement or by clinical improvement with exercise as the outcome measure. The sensitivity of these last two measurements are obviously therefore poor [190].

A lack of definitive tests can result in criticism of published research in the field. Using asthma as an example, a lack of standardised diagnostic tests can lead to incorrect labelling of disease [191]. An Australian study of one hundred children with chronic cough showed that after a period of extensive investigations, the incidence of asthma diagnosis dropped

from a pre-assessment 50% to 5% by the end of the study period [192]. For many studies, the fact that a child has previously been prescribed a bronchodilator or is therefore registered with an insurance agency as being asthmatic is sufficient for recruitment to a trial or study population.

In the UK, the NICE guidelines state a single objective measure should not be used for childhood asthma. Measures used to aid diagnosis include spirometry, bronchodilator reversibility, exhaled nitric oxide, peak flow measurement variability, exercise testing and bronchial challenge testing. Children who demonstrate more than 12% reversibility with bronchodilation should perform fractional exhaled nitric oxide (FeNO) which, if greater than 35ppb is positive. If the above tests are positive, a diagnosis of asthma can be made.

Correct administration and adherence to treatment is also important to establish particularly when recruiting asthmatics based on disease severity. Studies have shown very poor techniques from children and care providers when administering metered-dose inhalers with a spacer device or dry power inhalers [193, 194]. Adherence to medication can be assessed by checking how frequently repeat prescriptions are collected. Studies have shown that this can be as low as 50% in childhood asthma [195, 196].

Problematic severe asthma is a term used for children who are still symptomatic despite escalation of treatment to high dose inhaled steroids. These children undergo a staged approach to confirm the diagnosis, check modifiable factors such as technique and adherence and exclude any other pathology that may be impacting upon or be the sole reason for the persistent symptoms [197]. Those with modifiable features are termed Difficult-to-treat asthmatics while those with none identified are referred to as severe therapy resistant asthmatics (STRA). Pathways such as this result in very well defined groups however these terms are not universally used in the same manner therefore careful attention needs to be paid to definitions in published work.

1.2.5. Treatment of allergic disease

Aside from the identification and management of comorbidities, the treatment for children with asthma and allergic disease involves two main strategies. The first is environmental control and allergen avoidance. Removal, or minimising exposure to previously identified

allergens to which a child is sensitized (via skin prick test or specific IgE) may be all that is required particularly for food allergy.

The second strategy involves treatments targeting acute symptoms. These include anti-histamine medication or topical emollients restoring moisture to allergic dermatitis. In asthma the response to inhaled bronchodilators is not only needed for diagnosis, it is the first step in treatment with an appropriate for age delivery method.

The mainstay of treatment to prevent symptoms in allergic disease involves corticosteroids either topically, inhaled, orally or intramuscularly. There are two pathways by which corticosteroids exert their effects: genomic and non-genomic. The genomic pathway involves binding with the Glucocorticoid Receptor (GRα) in cytoplasm before migrating to the nucleus where the resulting complex blocks Nuclear factor-κB (NF-κB) thus reducing gene transcription for pro-inflammatory cytokine production. Binding with GR-α also blocks membrane release of arachidonic acid inhibiting the synthesis of prostaglandins and leukotrienes. The non-genomic pathway involves activation of endothelial nitric oxide (NO) and NO synthesis, which results in vasodilatation. Glucocorticoids also accelerate eosinophil apoptosis [198] and inhaled corticosteroids have also been shown to reduce the number of mast cells in the airway [199].

Aside from corticosteroids, more novel and targeted treatments for allergic disease have been developed and are in clinical use. *Omalizumab* binds to circulating IgE preventing it from binding to the high and low affinity receptors on basophils, mast cells and dendritic cells and exerting its effect. A Cochrane review on its use in adult and childhood asthma concluded it was an effective treatment for reducing exacerbations as an adjunct to inhaled steroids but also allowed reduction in steroid doses prescribed [200]. There are also promising findings for the use of *Omalizumab* in other allergic disease such as atopic dermatitis with 43% of patients showing a clinical response in atopic dermatitis disease severity scores, however further large scale studies are required [201].

1.3. The Association between OSA, Asthma and Atopic disease.

Sleep disturbance in asthma is not a new concept with nocturnal symptoms such as coughing being a common feature of the disease. Compared with non-asthmatics, these children tend to wake more often and have poorer quality sleep [202-205]. There has been no reported difference in sleep latency or duration in asthmatic children [202, 203, 205, 206] however asthma in childhood does appear to be associated with sleepiness and tiredness [202, 205]. The diagnosis of asthma in all these cases is usually clinically based and severity based on the patient's own perception of their symptoms, not on prescribed therapy dose or exacerbation frequency. Using these diagnostic criteria based on patient or parent reporting, those with more severe asthma are thought to have more sleep disturbances [207].

One study used more robust diagnostic criteria and severity score including symptom diaries, twice daily peak flow measurement, bronchodilator use and a record of night time symptoms [208]. The incidence of night-time waking was associated with more significant level of atopy based on skin prick test and total IgE. Those with more reactive airways on methacholine challenge, increased responsiveness to bronchodilators and higher bronchodilator use also showed a stronger association with night-time disturbance.

Opposing these findings is a life-long cohort study of over two thousand new-borns in Australia which showed persistent nocturnal waking from sleep (defined as more than twice in a night) during early life was associated with non-atopic asthma at aged fourteen years with an adjusted odds-ratio of 2.18 (95% CL 1.15-4.13) but not atopic asthma [209]. This second study used physician diagnosis, deficits in lung function and methacholine challenge results as the diagnosis of asthma but only skin prick testing to define atopy with no clarification of the allergens used. Direct comparison is difficult when different diagnostic criteria are used between studies.

Children with OSA also demonstrated elevated exhaled nitric oxide, a non-invasive measure of lung inflammation, that is also raised in conditions such as asthma. This contributes to the hypothesis that OSA drives airway inflammation. The postulated theory is that snoring results in vibrations of airway soft tissues that over time can result in tissue damage and

inflammation [210]. Tonsillar hypertrophy has also been associated with a clinical history of wheeze [211] however the description or reporting of wheeze by parents or care providers can be misleading and in fact reflect upper airway pathologies rather than lower airway bronchoconstriction [212].

The 'One Airway Hypothesis' suggests that the upper airway and lower airways influence each other[213] with topical nasal administration of steroids for rhinitis reducing asthma exacerbations[214] and nasal epithelium demonstrating increases in eosinophil numbers and IL-5 expression following direct segmental bronchial provocation with grass pollen allergen in sensitised non-asthmatic individuals [215].

There have been several large-scale population studies in adults with thousands of subjects recruited that demonstrate an association between asthma and sleep disordered breathing. Adults with physician diagnosed asthma and asthma-related symptoms are twice as likely to have witnessed apnoea during sleep [216, 217]. These results were based on questionnaire data with no objective assessment of sleep-disordered breathing. Female asthmatics are 1.8 times more likely to snore [218] however this study failed to take into account obesity and again lacked objective evidence of sleep disordered breathing. Studies utilising ambulatory cardio-respiratory polygraphy and in-laboratory polysomnography, have shown that adults with confirmed OSA are between 1.5 – 3 times more likely to have asthma [219-221]. However, extrapolation of these findings to a paediatric population is difficult owing to the different asthma phenotypes in adulthood. The obese non-eosinophilic phenotype for example has a female and late onset preponderance.

Interestingly one study showed the introduction of CPAP for treatment of confirmed OSA resulted in improved peak flow rates for those individuals with asthma [222] although obesity was a confounding factor. Adults with rhinitis are also more likely to demonstrate witnessed apnoea. In a five and a half year retrospective cohort study, adults with confirmed OSA were more likely to have been diagnosed with allergic dermatitis previously then those without (Adjusted hazard regression of 1.5, 95% CI=1.15–1.95, P=0.0025) [223], showing a relationship between OSA and atopy in general.

For children, the evidence for an association between OSA, asthma and atopy is much less robust with smaller study populations and a heavier reliance on questionnaire diagnosis for OSA, asthma and atopy. Studies that address the association between atopic diseases, but

excluding asthma, rely predominantly on questionnaire identification of snoring, and use this as a marker for sleep disordered breathing (Table 1.4). Six studies showed an association between snoring and parent reported allergic rhinitis or allergic dermatitis [224-229]. One group utilised a specific IgE test to confirm atopy and showed 40% of habitual snorers could be defined as atopic compared to 26.5% of the non-snorers ($p=0.005$) in children as young as twelve months of age [230]. The validity of parent or care provider diagnosis versus physician diagnosis or objective evidence is poor and should be taken into account when interpreting these studies.

Two groups used sleep study evidence for the confirmation of OSA. In 1997, McColly in a study of 47 children used polysomnography and demonstrated an association in those children diagnosed with OSA and positive specific IgE serum samples [231]. However, Carr et al more recently showed no association between evidence of sleep disordered breathing demonstrated using limited channel sleep study and atopy confirmed with specific IgE in 117 children.

Several groups have utilised habitual snoring as a marker for OSA to investigate a relationship with childhood asthma (Table 1.5.). A strong association was demonstrated in six studies with odds-ratios ranging from 2.4 to 9.7 when the diagnosis relied solely on parents reporting their child as being wheezy or having asthma [203, 225, 226, 228, 229, 232]. However, when the definition required a physician diagnosis of asthma, the association was less significant with odds-ratios ranging from 1.28 – 2.03 [211, 233, 234]. No groups utilised any other methods of asthma diagnosis when relating disease to habitual snoring.

In addition to showing a relationship between physician confirmed asthma and habitual snoring, Kaditis in 2010[211] also demonstrated that children with tonsillar hypertrophy were more likely to wheeze regularly based on parental recall. This raises the association between sleep disordered breathing and asthma (Table 1.6.). In a study population of over nine hundred children, Desager [202] reported decreased quality of sleep and increased tiredness in school aged wheezy children. This study used parent recall over the past twelve months to confirm wheeze. The authors did perform lung function looking at exercise-induced changes but found no association with the answers to questionnaires suggestive of disturbed sleep making the association with wheeze and asthma less convincing.

Ross et al [235] used a more robust definition of asthma including physician diagnosis, a review of medication used in the past twelve months and a lifestyle impact questionnaire. The authors reported in just over two hundred children, odds-ratio of 5.02 (95% CI 1.88-13.44) between severe asthma and sleep disordered breathing diagnosis was based on questionnaire data.

Using questionnaires for sleep disordered breathing diagnosis and physician diagnosed asthma, Li in 2015 recruited over twenty thousand children in a large multi-centred questionnaire based study in China and demonstrated an adjusted odd-ratio of 1.92 (95% CI 1.34-2.76) for the presence of two conditions [234]. A target question regarding whether a physician had ever diagnosed asthma was employed rather than the objective measures used previously. However, with such a large study population the results are intriguing.

There are two studies published using sleep study evidence for OSA but only parental reporting of asthma or asthma-related symptoms. Using ambulatory home cardiorespiratory studies, Sulit [236] in 788 children showed an association between OSA and a history of wheeze in a study of obese children. However, Ramagopal in 226 children employed the gold standard for diagnosis, polysomnography and did not find a relationship [237]. The same group did however publish the results from a group of 50 of children with PSG confirmed OSA and showed a lifetime association with asthma, again parent reported and only in those with severe OSA (AHI >10 events per minute) [238]. Three groups used sleep study confirmation (one ambulatory CR-Poly and two full PSG) and physician confirmed asthma, all of which found an association between the two [25, 239, 240]. One of these studies reported higher sleep disordered breathing events during REM-sleep [240].

Of most significance is a publication in 2011 by Kheirandish-Gozal et al. An OSA incidence of 63% was reported in a population of ninety-two children with 'poorly controlled' asthma. While OSA was defined using full polysomnography, this work relied upon patient reporting of asthma symptoms to define severity [241] and not prescribed medication. As well as having a significantly higher proportion of asthmatics with OSA, the severity of OSA was also noteworthy with a mean obstructive AHI of 14.6±8.2. Interestingly a proportion of these children who underwent adenotonsillectomy as treatment for their OSA showed improvement in their asthma symptoms, again perceived by the patients themselves.

	Number of participants	AGE Range	OSA Diagnosis		Atopy diagnosis		Association outcome	
			Sleep study	Questionnaire	Specific IgE/ Skin prick	Questionnaire		
Corbo 1989 [224]	118	6-11	No	Yes- Snore only	No	Yes	Habitual snoring associated with allergic rhinitis.	OR 2.93 (1.7-5)
Teculescu 1992 [225]	190	5-6	No	Yes	No	Yes	Habitual snoring related to reported allergy	RR 2.83
McColly 1997 [231]	39	1-7	Yes- PSG	Yes	Yes	No	56% of allergic subjects had evidence of OSA.	p<0.01
Erus 2004 [226]	2147	5-13	No	Yes	No	Yes	Habitual snoring associated with allergic rhinitis.	OR 2.1 (1.2-4)
Chug 2004 [227]	11,114	4-7	No	Yes- Snore only	No	yes	Atopy associated with habitual snoring.	OR 7.45 (3.49-15.97)
Kalra 2006 [230]	681	12-13 mths	No	Yes	Yes	Yes	Significant proportion of snoring children are atopic.	p=0.005
Carr 2007 [242]	117	1-14	Yes- Limted channel	yes	Yes	No	None	
Kuehni 2008 [228]	6811	1-4	No	Yes- Snore only	No	Yes	Habitual snoring association with allergic dermatitis.	OR 1.67 (1.38-2.02)
Li 2010 [229]	6349	5-14	No	Yes- Snore only	No	Yes	Habitual snoring association with allergic rhinitis.	OR 2.9 (2-4.2)

Table 1.4. Summary table of published work studying the association between children with evidence of sleep disordered breathing and atopic disease excluding asthma.

	Number of participants	Age Range	Habitual Snore Diagnosis based on questionnaire	Asthma diagnosis			Association Outcome	
				Patient reported	Physician diagnosed	Bronchodilator reversibility or Bronchospasm challenge.		
Corbo 1989 [224]	118	6-11	Yes	Yes	No	No	None	
Teculescu 1992 [225]	190	5-6	Yes	Yes	No	No	Habitual snoring related to exercise induced wheeze.	RR 4.5
Lu 2003 [233]	974	2-5	Yes	Yes	Yes	No	Asthma association with habitual snoring.	OR 2.03 (1.34- 3.1)
Erus 2004 [226]	2147	5-13	Yes	Yes	No	No	Habitual snoring associated with asthma.	OR 4.6 (1.7-13.1)
Valery 2004 [232]	1650	0-17	Yes	Yes	No	No	Wheeze in previous 12 months associated with snoring.	OR 5.4 (3.6-8.1)
Kuehni 2008 [228]	6811	1-4	Yes	Yes	No	No	Habitual snoring association with >10 episodes of wheeze.	OR 9.7 (5.8-16.16)
Verhulst 2008 [203]	695	6-12	Yes	Yes	No	No	Wheeze associated with habitual snoring	OR 2.8 (1.6-4.7)
Kaditis 2010 [211]	442	2-16	Yes	Yes	Yes	No	Wheeze associated with habitual snoring	OR 1.88 (1.24-2.86)
Li 2010 [229]	6349	5-14	Yes	Yes	No	No	Asthma association with habitual snoring.	OR 2.4 (1.2-5.2)
Li 2015 [234]	20,672	5-14	Yes	Yes	Yes	No	Asthma association with habitual snoring.	OR 1.28 (1.01-1.62)

Table 1.5. Summary table of published work studying the association between children with a history of habitual snoring and asthma.

	Number of participants	Age Range	OSA Diagnosis		Asthma diagnosis			Association Outcome	
			Sleep study	Questionnaire	Patient reported	Physician diagnosed	Bronchodilator reversibility or Bronchospasm challenge.		
Redline 1999 [25]	399	2-18	Yes- Ambulatory	Yes	Yes	Yes	No	Association with Asthmatics and OSA.	p=0.019
Sulit 2005 [236]	907	8-11	Yes- Ambulatory	Yes	Yes	No	No	Sleep disordered breathing associated with wheeze.	OR 1.89 (1.26-2.85)
Desager 2005 [202]	943	6-14	No	Yes	Yes	No	Yes	None	
Ramagopal 2008 [237]	236	Mean 7.18	Yes- PSG	No	Yes	No	No	None	
Ramagopal 2009 [238]	50 (all OSA)	Mean 9.3	Yes- PSG	No	Yes	No	No	Lifetime experience of asthma associated with more severe OSA.	p=0.033
Kaditis 2010 [211]	442	2-16	No	Yes	Yes	Yes	No	History of wheeze associated with presence of tonsillar hypertrophy.	OR 2.36 (1.46-3.79)
Kerirandish -Gozal 2011 [241]	92	3-10	Yes- PSG	Yes	Yes	Yes	No	Asthma associated with OSA.	OR 40.9 (12.9-140.1)
Ross 2012 [235]	201		No	Yes	Yes	No	No	Sleep disordered breathing associated with severe asthma.	OR 5.02 (1.88-13.44)
Gutierrez 2015 [240]	141	2-12	Yes- PSG	No	Yes	Yes	No	Higher rate of REM related OSA in asthmatics.	RR 1.87
Li 2015 [234]	20,672	5-14	No	Yes	Yes	Yes	No	Asthma association with OSA.	OR 1.92 (1.34-2.76)
Goldstein 2015 [239]	229	2-15	Yes- PSG	Yes	Yes	Yes	No	Asthma associated with positive PSG result for sleep-disordered breathing.	OR 2.94 (1.82 -4.75)

Table 1.6. Summary table of published work studying the association between children with evidence of sleep disordered breathing and asthma.

OSA is a prevalent upper airway inflammatory paediatric disease with an undefined underlying pathology. The relationship between OSA and asthma and atopic disease may shed some light on its aetiology and provide additional treatment strategies that would prevent the need for surgery. Aside from the difficulties of recruitment to paediatric research compared to adults, the current literature in children is lacking robust definitions for the diseases. While several groups have utilised sleep studies to reliably diagnose OSA, criticism of the atopy diagnosis, asthma diagnosis and the methods of defining severe or problematic asthma can be raised. Therefore, a study of well phenotyped asthmatic childre and children with clearly defined evidence of atopy along with sleep study evidence of OSA is needed to investigate this relationship. It is for these reasons that I investigated the following hypotheses and research questions.

1.4. Hypotheses

1. The incidence of OSA in children with Severe Therapy Resistant Asthma is higher than reported as the background incidence in childhood.
2. There is a higher prevalence of atopy in children with sleep study proven OSA undergoing adenotonsillectomy compared to those without OSA.
3. The palatine tonsils from children with sleep study proven OSA have more Th17 cells and less Treg cells than a control group without OSA.
4. The tonsils from atopic subjects undergoing adenotonsillectomy will contain a stronger Th2 cellular phenotype than children without evidence of atopy undergoing the same procedure.
5. Increased type 2 innate lymphoid cells (ILC2s) are present in tonsils from atopic children compared to children without evidence of atopy.

1.5. Aims

1. To recruit children aged 6-16 years with a diagnosis of severe therapy resistant asthma and assess the presence of OSA using cardiorespiratory polygraphy or full polysomnography.
2. To determine atopic status in children undergoing tonsillectomy for OSA or recurrent tonsillitis using questionnaires and serum specific IgE levels to common aero-allergens.

3. To recruit children scheduled for tonsillectomy that have had and assessment of atopic status and collect tonsil tissue for flow cytometry analysis to phenotype T-cell subsets and ILCs.

1.6 Objectives

1. To assess sleep disordered breathing in STRA children by performing a full polysomnography (PSG) sleep study during the child's admission to Royal Brompton Hospital (RBT) for investigations as part of the problematic asthma pathway and prior to their intramuscular corticosteroid injection. To analyse the PSG and correlate results to the data obtained during the child's asthma investigations.
2. To apply for ethical approval to recruit children undergoing tonsillectomy for tissue collection, analysis and storage.
3. To approach parents of children undergoing tonsillectomy for the treatment of OSA diagnosed by sleep study at the RBT and obtain consent to collect data in a questionnaire format, collect a blood sample and collect the extracted tonsil tissue for this research.
4. To approach parents of children undergoing tonsillectomy for the treatment of recurrent tonsillitis as a disease control group. Obtain consent to collect data in a questionnaire format, collect a blood sample and the extracted tonsil tissue for this research.
5. To define atopy using serum total specific IgE to an aeroallergen panel using the laboratory standard cut off as >0.35 international units as evidence of sensitization.
6. To process tonsil tissue into a single cell suspension and use flow cytometry to identify T-cells using the extracellular marker CD3 and Helper T-cell (Th) by the extracellular marker CD4 and define subtypes of Th cells using intra-cellular cytokine staining. Quantify T-regulatory cells defined as $CD4^{+}CD25^{+}Foxp3^{+}$.
7. To use flow cytometry to identify tonsillar innate lymphoid cells.
8. To process any remaining blood samples after objective 5 in the same manner as the tonsil tissue to identify Th cells and ILCs.

The following chapter will outline how I recruited children to my study and the techniques and methods undertaken to investigate the hypotheses listed above.

Chapter 2: Materials and Methods

This chapter will address the clinical characterisation of the subjects recruited to the different components of the study and how the samples obtained were processed and analysed. The methods and processes described in this chapter are not novel and are established protocols. There are several specific and new methods I developed for this work that will be described in the relevant results chapters.

2.1. Children with severe-therapy resistant asthma undergoing polysomnography and cardiorespiratory polygraphy.

In order to test the hypothesis that the incidence of OSA in children with severe therapy resistant asthma is higher than that published in the general paediatric population, I recruited children aged 6-16 years with severe therapy resistant asthma (STRA) and assessed the presence of OSA using cardiorespiratory polygraphy or full polysomnography.

2.1.1. Severe-therapy resistant asthma.

Children are referred to the asthma team at Royal Brompton Hospital for assessment of their asthma when management at a primary or secondary care level has failed to control symptoms or reduce exacerbations. The children enter the Problematic Severe Asthma investigation protocol (Appendix 1) and undergo a staged assessment to confirm the diagnosis and to identify modifiable aspects of their disease. Children are eligible for the protocol if they are receiving high doses of medication or multiple medications (BTS/ Sign guidelines stage 4/5) and have one of the following: persistent chronic symptoms; persistent airflow obstruction (FEV1<70% predicted despite bronchodilator reversibility); recurrent severe exacerbations (>2 per year requiring intravenous therapy); recurrent acute exacerbations requiring oral corticosteroids (>6 per year).

First the diagnosis was confirmed. This was not based on one single measurement and required included: lung function; bronchodilator reversibility; exhaled nitric oxide; peak flow measurement variability; exercise testing; bronchial challenge testing. Once diagnosis was confirmed, modifiable factors were investigated. The modifiable factors included

persistent exposure to allergens to which the child is sensitised and nicotine exposure measured by urinary cotinine levels. Medication adherence was measured with general practitioner prescription collection rates and with electronic monitoring devices. Psychological aspects of health were also explored. If modifiable factors were identified, the children were given a diagnosis of 'Difficult asthma' (DA) along with advice and management steps to address these factors. If no modifiable factors were found or if the children had persistent poor control despite addressing modifiable factors, they had 'severe therapy resistant asthma' (STRA). All investigations or assessments were clinically indicated.

The first stage of the assessment of children with problematic asthma involved:

- A nurse led hospital-based assessment involving a detailed history, review of medication and administration techniques, and discussion about possible allergen exposure.
- Skin prick tests to determine atopic status using common aeroallergens (Cat dander, dog dander, house dust mite, *Aspergillus fumigatus,* mixed tree pollen, mixed grass pollen). Atopic status defined as one or more positive reactions.
- Review of general practitioner prescription collection as a measure of compliance to maintenance inhaled steroids.
- Lung function including bronchodilator reversibility and exhaled nitric oxide.
- A nurse led home visit assessing environmental exposures such as cigarette smoke.

Once these factors had been addressed, the STRA patients progressed to the next stage of the assessment. This involved a two-night hospital admission. On the first night, the child underwent polysomnography in the paediatric sleep and ventilation unit at Royal Brompton hospital. The following day, the child underwent a flexible bronchoscopy under general anesthetic using a previously published protocol [243]. While anaesthetised, the child also received an intramuscular injection of the long acting (28 days) corticosteroid Triamcinolone. On occasions when the child was being admitted for a prolonged period of assessment, the sleep study took place at some point during the admission and not always before the bronchoscopy.

The children also underwent non-invasive assessments that were repeated before and after the intramuscular triamcinolone. Spirometry using a Vitalograph alpha touch spiromteter (Buckingham, England) was performed to obtain forced expiratory manoeuvers. The best of three technically acceptable blows was selected and the forced vital capacity (FVC) and forced expiratory volume after one second (FEV1) were calculated based on Rosenthal reference equations [244]. The device was calibrated daily using a one-liter syringe. Bronchodilator reversibility was measured by performing the spirometry twice, before and fifteen minutes after the administration of salbutamol based on American Thoracic Society guidelines [245].

Fractional exhaled nitric oxide measurement was made at 50mls/sec ($FeNO_{50}$) flow rate using a chemiluminescence analyzer (NIOX; Aerocrine, Solna, Sweden) with an incentive screen to aid in maintaining flow rate. The mean of three technically successful measurements was calculated.

Two questionnaires were used in the assessment. The Asthma control test (ACT) was used to assess the previous four weeks asthma control. Five questions, each question scored from 1-5, one demonstrating poorest control, are included. A score greater than 19 reflects good control[246] (Appendix 2). The other was the Paediatric Asthma Quality of life Questionnaire (PAQLQ)[178, 247]. This includes 23 items in three domains: symptoms; emotional function; activity limitation. Each item is scored using a 7-point scale, one being most affected (Appendix 3).

2.1.2. Assessment of sleep disordered breathing

On arrival in the paediatric sleep and ventilation unit, the parent or caregiver was asked a series of questions referring to nighttime sleep behaviour such as presence of snoring, witnessed apnoea, as part of a non-validated history taking exercise (Appendix 4). No formalized questionnaire was in use at the time of this work. They were also asked to complete the modified Epworth sleepiness scale (mESS) on behalf of their child (Appendix 5). This tool was first introduced in 2001 and further modified in 2009 [248]. It involves asking the parent to score from 0-3 how likely it was that their child would fall asleep in different situations, 0 being not at all and 3 as a high chance. The total sore is then calculated with 11 or above being deemed as excessively sleepy. This cut off has been used in previous publications [249-251] however there is no universally agreed cut off for

children [252]. This is a modified version of the adult Epworth sleepiness scale with alterations to the scenarios making them more applicable to a paediatric population.

The equipment was provided and maintained by *SOMNOmedics GmbH, Germany.* The sleep study involved the following:

- Bilateral electro-oculogram (EOG).
- Eight channels of electroencephalogram (EEG).
- Chin electromyogram (EMG).
- Nasal pressure transducer.
- Oro-nasal thermistor.
- Chest and abdominal movement sensors using respiratory impedance bands.
- Heart rate measurement by Electrocardiogram (ECG).
- Arterial oxygen saturations measured by pulse oximetry.
- Transcutaneous carbon dioxide measurement (TCM40, Radiometer).
- Limb movement sensor.

All signals were digitalized using commercially available polysomnography software (Domino, Somnomedics). The study also included a synchronized video feed. On the occasion that a child did not tolerate the EOG, EEG and EMG leads, the study was changed to a cardiorespiratory polygraphy relying on the video feed to identify arousals or wake periods.

The studies were scored using American Academy of Sleep Medicine criteria[253] by myself and the sleep physiologists at Royal Brompton Hospital (three in total) and reviewed by the sleep consultant. The department twice yearly performs an inter-scorer assessment where all members of the sleep team involved in sleep scoring and staging individually and privately score the same study to ensure common practice within the department. OSA was defined as an apnoea-hypopnea index of over one event per hour and more than 50% of the events were obstructive in nature.

The assessment of the asthmatic children was carried out by the asthma team at RBHT. The sleep behaviour evaluation was carried out by the junior doctor clerking the patient on arrival to the sleep unit. I participated in setting up the sleep study and in the staging and scoring of the studies produced.

2.2. The prevalence of atopy in children undergoing tonsillectomy for the treatment of OSA.

The methods outlines in this section refer to the hypothesis that there is a higher prevalence of atopy in children with sleep study proven OSA undergoing adenotonsillectomy compared to those without OSA. The aim was to determine atopic status in children undergoing tonsillectomy for OSA or recurrent tonsillitis using questionnaires and serum specific IgE levels to common aero-allergens. Ethical approval was obtained from the Health Research Authority, NRES committee London- Riverside (REC Reference 14/LO/0001) for the recruitment and assessment of the children as these investigations were not clinically indicated.

2.2.1. Recruitment

Children assessed for OSA at Royal Brompton hospital underwent tonsillectomy in two surgical centres: Royal Brompton Hospital and Chelsea and Westminster Hospital. Permission to recruit at these centres was obtained from the individual research and development departments. The ENT surgical schedule was reviewed on a weekly basis. Those undergoing tonsillectomy were cross-referenced with the sleep database to identify those who had undergone a sleep study at our centre. The methods for assessment of sleep disordered breathing were the same as above (section 2.1.2).

The parent or caregiver for children due to have adenotonsillectomy were approached on the morning of the scheduled surgery, before the consent process and given an information leaflet (Appendix 6). The children were also given age appropriate information leaflets (Appendix 7). After consent for surgery had been obtained, the parent or caregiver was approached and asked if they would like to participate in the study. If so, consent was taken (Appendix 8).

For each surgical list, those who had been scheduled for a tonsillectomy for indications other than OSA where approached in the same manner. The parents received an information leaflet to explain that they would be recruited as a disease control group (Appendix 6), as did the children (Appendix 7).

2.2.2. Questionnaire data collection.

Once consent had been obtained, the demographics of the children were recorded from medical notes or admission data. Heights and weights of the children were measured by a nurse and confirmed by the recruiting doctor. The parent or caregiver was then asked to complete a series of questions focusing on the child's past medical history and medication history. They were also asked questions focusing on atopy and allergy related illnesses or symptoms. Finally, questions about the child's sleep behaviour were asked (these were derived from the sleep unit admission proforma and the modified Epworth Sleepiness Scale) (Appendix 9).

2.2.3. Blood sample collection.

As part of the general anaesthetic, intravenous access is required and obtained using an age appropriate intravenous cannula. During this process, up to 5mls of venous blood was collected. 2.6mls of blood was injected into two 1.3ml heparinized paediatric blood bottles. The remainder, if available was placed into an EDTA paediatric bottle.

The samples in the heparinized bottles were sent to the laboratory at Royal Brompton Hospital for analysis. Total IgE was measured using the Beckman Access immunoassay and recorded in IU/ml. Specific IgE to cat dander, dog dander, house dust mite, mixed tree pollen, mixed grass pollen and *Aspergillus Fumigatus* was measured using the ImmunoCAP 250 analyser. A positive result was defined as ≥0.35IU/ml. If during the recruitment process, a known allergy was identified, this was added to the specific allergen panel, for example if the child was known to be or suspected to be allergic to peanuts. All samples were processed by the same laboratory following their standard operating procedure.

I undertook all of the recruitment and processing described in this section. I checked and cross-referenced the surgical schedule on a weekly basis. I performed all recruitment and consenting. Either I or the anaesthetist for the surgical list, obtained the blood samples. The biochemistry team at RBHT performed the specific and total IgE analysis in the RBHT biochemistry laboratory.

2.3. Cellular phenotyping of collected tonsils.

The methods described in this section were used to process and analyse inflammatory cells in tonsillar tissue from children with OSA and recurrent tonsillitis

2.3.1. Tonsil tissue processing

Once the tonsils were removed from the child, they were rinsed with saline to remove excess blood and placed in a pre-weighed 50ml falcon tube containing RPMI medium (Roswell Park Memorial Institute Medium, a recognised medium for use in tissue and cell culture). The tissue was weighed and processed using published methods to obtain a single cell suspension from lymphoid tissue [254]:

1. With one tonsil and using a scalpel blade, the tissue was cut up roughly to a pulp, keeping it moist with RPMI. The remaining tonsil was kept in RPMI on ice.
2. The small sections of tissue were then pushed through a 70micron filter with the back of a syringe into a sterile falcon tube. The filter was continuously washed with the RPMI.
3. Approximately one third of the dissected tonsil tissue was filtered before the falcon tube containing the cell suspension was sealed and placed on ice. A new filter and falcon tube was used for the next third of tissue. Any more tissue per tube used resulted in a cell supernatant too concentrated to accurately count (see below).
4. Once a cell suspension had been made from the whole tonsil, all tubes were spun in a centrifuge at 1600rpm for 6 minutes at 4^{0}C. The supernatant was discarded.
5. The cell pellets were re-suspended in 30mls of RPMI.

Total leukocytes were counted in each tube. 10ul of cell suspension was obtained and mixed with 90ul of Trypan blue. Stained cells were counted using a cytometer. The tissue contained very few red blood cells. For this reason, a red cell lysis buffer was not required. Each falcon tube approximately represented the total cell count for one third of a single tonsil. Using the cytometer, the cell count per ml of RPMI for each tube was determined from an average of three counts. The total cell count per tube was therefore the cell count per ml of RPMI multiplied by the volume of RPMI. The total cell count from all three tubes

represented the cells obtained from one tonsil of a predetermined weight and the cell count per gram of tissue could then be derived.

The remaining tonsil was sliced and half was formalin-fixed for 24 hours prior to processing to paraffin embedded blocks. The remainder was fresh frozen as whole tissue segments. This was for future immunohistochemistry staining and whole tissue homogenizing.

2.3.2. Peripheral blood processing.

If peripheral blood was available after samples for total and specific IgE had been obtained (2.2.3.), peripheral blood mononuclear cells were obtained from an EDTA sample with the following method:

1. The whole blood was diluted with 0.9% saline in equal volumes 1:1 dilution.
2. 3mls of lymphoprep (Progen Biotechnik, Germany) were added to a 15ml falcon tube.
3. The diluted whole blood was added carefully so that it sat on top of the lymphoprep layer.
4. This combination was centrifuged at 1800rpm for 20 mins at 21^0C
5. The lymphocyte layer (the cloudy second layer in the tube) was aspirated from the sample.
6. The aspirated lymphocytes in a fresh 15ml falcon tube were diluted with 10mls of RPMI.
7. The tube was then centrifuged at 1600 rpm for 10 minutes at 4^0C and supernatant discarded.
8. The cell pellet was re-suspended in 10mls RPMI and centrifuged again at 1400rpm for 10 minutes at 4^0C before discarding the supernatant.
9. The cell pellet was again re-suspended in an equal volume of RPMI to the original amount of whole blood.

The total white cells were then counted following the same methods as for the tonsil cell suspensions.

2.3.3. Flow cytometry.

Flow cytometry is a method of using a laser to identify and sort cells based on the light scattered by the cells. Cells are passed through the device in a single cell suspension and exposed to an excitation laser beam. The degree of forward scatter to the beam is a measure of the cell size while the degree of side scatter is a measure of granularity.

In addition to size and granularity and using florescent bound antibodies, individual cell surface markers can be identified. Before adding the sample to the flow cytometer, cells are stained with antibodies to specific cell surface markers. The antibodies are bound to a fluorophore which when excited by the correct wavelength, emits a signal detected by the device. The strength of the signal reflects the number of cells in the sample with that specific cell surface marker.

The same principle can be used to identify the intracellular content of cells, particularly the cytokine profile and intra-nuclear markers. By first exciting the cells inducing cytokine production and then fixing and permeabilising the cell membrane, fluorophore bound antibodies can attach to the cytokine or intra-nuclear marker in the same way.

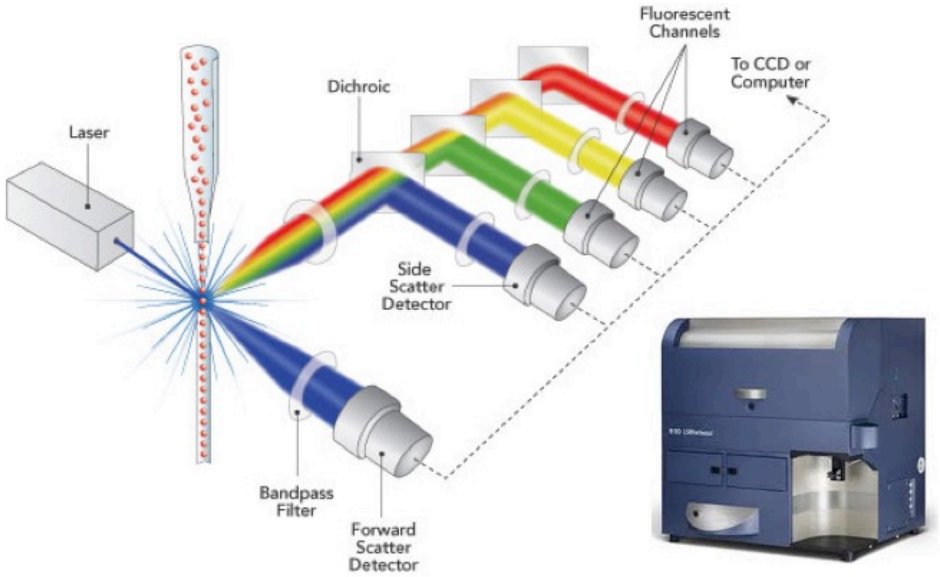


Fig 2.1. A representation of the workings of a flow cytometer. www.semrock.com/flow-cytometry.aspx

Excitation of the cells can be induced using a combination of phorbol 12-myristate 3-acetate (PMA), ionomycin and brefeldin A. PMA is a small molecule which activates protein kinase C, a protein at the top of several signalling pathways leading to the production of cytokine.

Ionomycin is a calcium ionophore produced by the bacteria Streptomyces conglobatus. It raises intra-cellular calcium concentration causing nuclear factor of activated T-cells (NFAT) signalling. In combination, PMA/Ionomycin cause a non-specific activation of cytokines cells are primed to produce. The BrefeldinA inhibits protein transport from the endoplasmic reticulum to the golgi body, trapping them to facilitate antibody attachment.

The method used to pre-treat the tonsil and peripheral blood cells with the fluorophore bound antibodies for marker identification was as follows:

1. $1x10^6$ cells (tonsil or PBMCs) were added to each well of a 96 well round bottom plate.
2. The pellet was centrifuged at 1500 RPM for 5 minutes at 4^oC and the supernatant discarded.
3. 200ul of the stimulation mix containing PMA, ionomycin and brefeldin A was added to each well. The plate was covered and left in the incubator (37^oC, 5% CO_2) for 3-8 hours.
4. The cells were centrifuged for 5mins at 1500rpm, 4^oC and supernatant discarded.
5. The cells were re-suspended in 200ul of filtered FACS stain buffer, spun and discarded.
6. The cells were re-suspended with 200ul of FACS stain buffer. Spun and supernatants discarded as before.
7. The pre-determined volume of fluorophore bound antibody mixtures made up with FACS buffer were added to each well (for antibody combination, see chapter 5 methods section). The wells were mixed and left covered with foil on ice bath for 20mins.
8. The wells were washed with 200ul of FACS stain buffer before being spun and supernatants discarded as before.
9. The previous step was repeated.
10. 60ul of cell fixation agent was added to each well before being incubated at 4^oC for at least 15mins.
11. The wells were then washed with 150ul of Permeabilisation buffer (ebioscence) and left at room temperature on the bench for 10mins.
12. The plate was spun at 1500rpm for 5mins and supernatant discarded.

13. The pre-determined volume of intracellular fluorophore bound antibodies were mixed with permeable buffer were added to each well. The wells were mixed and incubated for 40mins over ice bath covered with foil.
14. 200ul of permeabilisation buffer was added. The cells were mixed, spun and supernatant discarded as before.
15. The previous step was repeated.
16. Finally, 100ul of stain fixation buffer was added and the plate left at 4^{0}C until processing, which took place within 24 hours.

The BDFortessa was used for these experiments. It has 5 lasers that detect twelve colours as well as an Ultraviolet light. The fluorophores bound to the antibodies used in the experiments are excited by specific wavelengths and emit light that is then recorded by the detectors. There is a degree of overlap between the lights produced resulting in more than one detector recording a signal for the same specific fluorophore. To compensate for this, samples with only one antibody bound fluorophore were run first allowing the analysis software to define a positive signal for each cellular marker. The degree of overlap and the ability to compensate for it needs to be considered when designing a flow cytometry panel.

Once the sample had been run through the flow cytometer, an accompanying set of samples was run to aid analysis. These were the fluorescence minus one samples or FMOs. The samples being analysed were treated with up to thirteen different antibodies for flow cytometry. Thirteen separate samples were prepared which sequentially have one fluorophore excluded, the FMO (Figure 2.2). Therefore, during analysis the FMOs act as a negative indicator of the marker they represent. Positive populations are then identified in a sample using an FMO as a reference for where the negative signals are positioned.

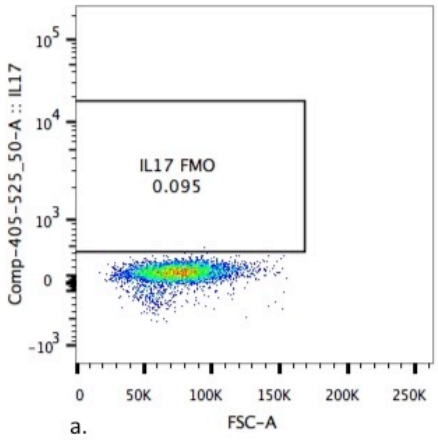


Fig 2.2. The Fluorecence minus one for IL17. The signals of cells with intra-cellular IL17 are represented on the y axis against the signal from the forward scatter laser on the x axis. No positive signals identified on the FMOs thus generating a cut of at which positive signals can be demarcated on a test sample.

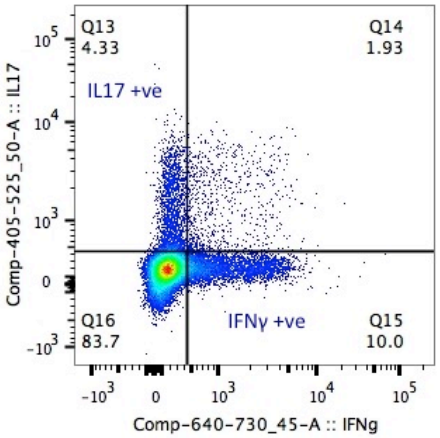


Figure 2.3. Flow cytometry plot of a sample containing antibodies for IL17 and IFNγ displayed against each other (IL17 on y axis and IFNγ on the x axis). Using the values on the y axis from the FMOs above, markers can be set for negative signals (those not possessing IL17) and positive signals. The size of each population can then be expressed as a percentage.

Once the raw data was obtained from the BDFortessa, it was analysed using the flow cytometry data analysis software, *Flowjo* (Flowjo, LLC Oregon USA). The software allows the user to identify populations of signals representing cells with specific markers and quantify cell numbers. The user can then specifically focus on the chosen population of signals and analyse additional markers.

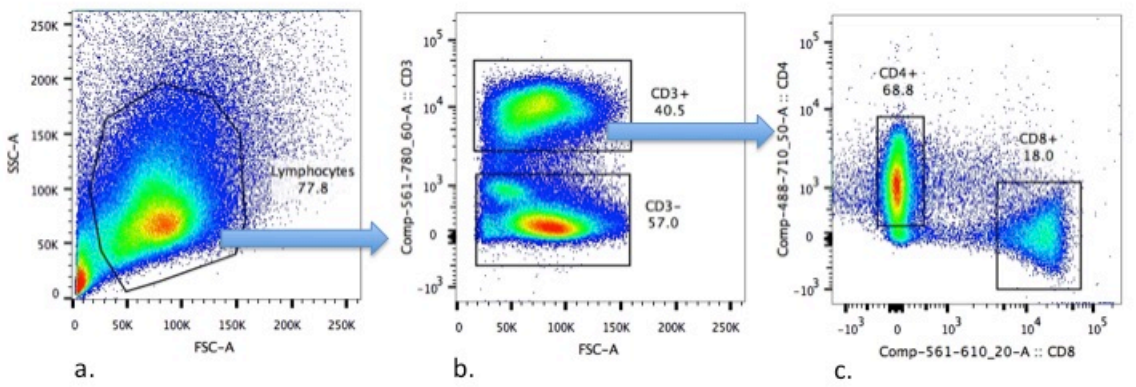


Figure 2.4. An example of a gating strategy used scientifically when flow cytometry plots are analysed using the flowjo software. The population of signals representing cells can be identified and analysed. In this example, the total lymphocytes are first identified using side scatter on the y axis and the forward scatter on the x axis (a.). The population is then isolated and then analysed for the signals negative and positive for the fluorophore attached to the antibody for CD3 (b.). All the CD3+ve signals are then isolated and analysed for those possessing CD4 and CD8 in the same way (c.).

The results of the flow cytometry analysis described in this section can be found in chapters five and six.

2.4 Statistical analysis

All statistical analysis was performed using Graphpad Prism version 5. Categorical data comparisons were made using contingency tables and significance values calculated using Fisher's exact test due to the small samples sizes. None of the continuous data was normally distributed. Therefore all continuous data was represented as medians with interquartile ranges, and comparisons between two groups made using the Mann-Whitney U-test. If more than two group were being compared, the Krukal-Wallis test was used.

Due to the potential bias from multiple comparisons, a significance level of <0.01 was used as a cut off for significant differences. While this may have resulted in the rejection of significant findings (ie. a type II error) the odds of finding significance simply by performing multiple analyses were high. Establishing this significance cut-off controlled for this potential error.

For methods 2.1 referring to the recruitment of STRA children, a power calculation was not performed. The nature of this work involved the description of OSA amongst this rare asthma phenotype. The lack of a control group, an intervention or any previous descriptions

of OSA in STRA children made a statically derived target recruitment number not possible to generate. I therefore aimed to recruit every STRA children during the study period and would take the lack of a recruitment number into consideration when reporting results and developing conclusions.

For methods 2.2 referring to the recruitment of children with OSA and of a disease control group and exploring the incidence of atopy in these children, a power calculation for a target recruitment number was challenging but more achievable. Firstly an incidence of atopy (in my case, peripheral sensitisation to an allergen) within the control group was required. Kim et al [255] reported that in 131 pre-school children, 24.5% were sensitised to at least one aeroallergen. Next the incidence of atopy amongst children with OSA was required. The closest report to my work was McColley et al [231] who published that 36% of children with OSA were peripherally sensitised. Using both these numbers, a significance level of 0.05 and a statistical power of 80%, I would need 246 children in each group to achieve the best chance of a significant finding.

Obviously in a two-year time frame, the recruitment of 492 children would be challenging. One could also criticize both the incidence values used to generate this figure. The incidence of atopy amongst children was a Korean population exposed to different allergens with the authors themselves concluding that sensitisation varied depending on age and was therefore difficult to report. The incidence of atopy amongst OSA children was based on inconsistent allergy testing in a small study population. There are also several studies reporting the incidence is no different between OSA and non-OSA children.

This work was essentially a hypothesis-driven pilot study. Twenty-five children were referred to the local ENT service at Chelsea & Westminster Hospital from September 2012 to September 2013. Based on this, a recruitment aim of 50 children with sleep study confirmed OSA was decided upon as a target recruitment target. The lack of an achievable statistically derived target recruitment number would be taken into consideration when coming to any conclusions based on the data produced.

2.5. Ethics

The STRA children underwent sleep studies for clinically indicated reasons. For the rest of the work laid out above, once all recruitment documentation and a research report had

been produced, an application was submitted to the local research ethics committee. Approval was granted by the NRES committee London- Riverside on 10th February 2014 (Ref 14/LO/0001). Permission was granted to recruit patients, perform the necessary tests, collect and retain the tissue for this and future work.

Chapter 3. OSA in Severe Therapy Resistant Asthma

This chapter focuses on the hypothesis 1, stating that the incidence of obstructive sleep apnoea (OSA) in children with Severe Therapy Resistant Asthma (STRA) is higher than that published in the general paediatric population of 5.2%. The aim of this work was to recruit children aged 6-16 years referred to Royal Brompton Hospital (RBHT), defined as STRA and assess the presence of OSA using cardiorespiratory polygraphy or full polysomnography. Any data not displayed in table form can be found in Appendix 11.

3.1 Introduction

A relationship between OSA and asthma has been reported in children. One of the only studies to use full polysomnography as well as an attempt to utilise a well defined asthmatic study population found up to 63% of a group of 96 poorly controlled asthmatics had OSA and demonstrated a reduction in asthma exacerbations following OSA treatment[241]. This is significantly higher than the incidence published in the general paediatric population of up to 5.7% [5, 8, 10].

Patients with problematic severe asthma have poor control despite high dose prescribed therapy at stage 4/5 of the British Thoracic Society guidelines. They undergo a staged assessment in which 'Difficult Asthmatics' (DA), those with modifiable factors impacting their asthma, are distinguished from those with STRA [256]. Since 2013, those diagnosed as STRA at our institute underwent a sleep study as part of their investigations.

3.2 Research questions

1. Do asthmatic children defined as severe therapy resistant have a higher incidence of OSA compared to the general population?

2. Are the severe therapy resistant asthmatics with evidence of OSA more atopic than those without OSA?

3.4 Methods

A sleep study was performed on all children defined as having STRA between September 2013 and August 2016 (Chapter 2, section 2.1). This investigation was added to the

investigation protocol following the published findings of OSA amongst asthmatics referenced earlier and was therefore clinically indicated. The sleep studies were performed before additional treatment for STRA was instigated such as the intramuscular administration of corticosteroid. OSA was defined as an apnoea-hypopnoea index (AHI) above 1 with 50% or above of the events scored being obstructive in nature as defined by the American Academy of Sleep Medicine (AASM) guidelines [253].

3.5 Results

During the study period, 21 children with STRA underwent a sleep study (Table 3.1). 9 (42%) underwent full polysomnography (PSG) and 12(57%) underwent cardiorespiratory polygraphy (RP). The level of study was determined by the cooperation of the candidate and their tolerance to the electroencephalogram, electromyogram and electro-oclulogram leads. In one instance, equipment failure resulted in one PSG study being down graded to CR-Poly. Good quality data was obtained from all other studies.

Number of male	14 (66%)
Median age in years at time of PSG/ RP (Interquartile range)	**12** (8-13)
Ethnicity:	
Caucasian	**16**
Asian/ Arabic	**3**
Afro Caribbean	**2**
Hispanic	**0**
Other	**0**
Median weight in Kg (Interquartile range)	**45** (30.8-65)
Median height in metres (Interquartile range)	**146** (130.3-157)
Median BMI (Interquartile range)	**21.1** (16.6-26.3)

Table 3.1. Demographics of the 21 children who underwent sleep studies during their assessment as part of the problematic asthma investigations. Median values with interquartile ranges.

12 (57.1%) children had a raised AHI (above 1). 3 (14%) children had evidence of OSA with 50% or more of the events scored being obstructive. All would be classified as mild OSA

with an AHI of 1-5. This accounted for 14% of the children studied and higher than the quoted background incidence of OSA in the general paediatric population (5.7%). However, the 95% confidence interval for the binomial of 3/21 is 3-36% therefore there is no statistically significant difference between the OSA amongst these STRA children and the background incidence of OSA in the general paediatric population.

The 9 (42%) children with a raised AHI but no evidence of an obstructive predominance, had normal central sleep disordered breathing in keeping with physiological events such as post-sigh or post arousal central events with no significant disease. The remaining 9 (42%) children had normal AHI scores (Table 3.2).

	OSA (n=3)	**Non-OSA (n=18)**	**p**
Apnoea-Hypopnea Index (AHI) (Interquartile range)	3.9 (3.5-4.2)	0.9 (0.4-2.3)	0.04
Obstructive AHI (Interquartile range)	2 (2-2.6)	0.1 (0-0.3)	0.007
Central AHI (Interquartile range)	1.1 (0.8-1.8)	0.6 (0.2-1.4)	0.5
Median arterial oxygen saturations (%) (Interquartile range)	96 (96-98)	96 (95-97)	0.64
Median oxygen desaturation index (no. /hour) (Interquartile range)	3.3 (2-5.6)	1.6 (0.6-2.3)	0.08
Median transcutaneous carbon dioxide (CO_2) partial pressure (kPa) (Interquartile range)	5.9 (5.3-5.9)	5.5 (5.3-5.7)	0.56
Median Maximum CO_2 partial pressure (kPa) (Interquartile range)	6.5 (6.3-6.5)	6.5 (6.1-6.9)	0.68
Median Minimum CO_2 partial pressure (kPa) (Interquartile range)	5.3 (4-5.5)	4.8 (4.4-5.3)	0.89

Table 3.2. Quantifiable results obtained from the cardio-respiratory sleep studies undertaken by the STRA children comparing those with OSA to those without. Median values plus interquartile ranges. Significance measures using Mann-Whitney U test.

There were no demographic differences comparing OSA to Non-OSA individuals with the exception of the Non-OSA group having more Caucasians (Table 3.3).

	OSA (n=3)	**Non-OSA (n=18)**	**p**
Number of male	1	13	0.24
Median age in years at time of PSG/ RP (Interquartile range)	12 (10-13)	8.5 (8-12.5)	0.35
Ethnicity:			
Caucasian	**0**	**16**	**0.007**
Asian/ Arabic	2	1	0.04
Black/ Caribbean	1	1	0.18
Hispanic	0	0	-
Other	0	0	-
Median weight in Kg (Interquartile range)	43 (25-76)	45 (33- 64.5)	0.87
Median height in metres (Interquartile range)	143 (124-177)	147.5 (130-156.5)	0.61
Mean BMI (Interquartile range)	22.6 (17.5-26.7)	21.2 (16.6-26.9)	0.7

Table 3.3. Demographics of the 3 children with evidence of OSA and 18 children without evidence of OSA on sleep study. Median values plus interquartile ranges. Significance value using Mann Whitney U test.

3.5.1. Evidence of sleep disordered breathing

The subjects were asked specific questions to elicit evidence of sleep disordered breathing such as evidence of snoring or witnessed apnoea. Answers were mostly obtained from the parent or guardian accompanying them for the sleep study. No difference was identified comparing the answers from those with OSA on sleep study and those without (Table 3.4).

	OSA (n=3)	Non-OSA (n=18)	P
History of snoring.	1	3	0.48
History of witnessed apnoeas.	0	0	-
History of witnessed arousals from sleep.	2	4	0.18
History of mouth breathing.	2	4	0.18
History of morning headaches.	0	2	1.0
History of daytime sleepiness.	1	2	0.38
History of difficulty concentrating during the day.	0	2	1.0
History of behaviour problems during the day.	0	2	1.0

Table 3.4. Comparison of answers to a series of questions focusing on signs and symptoms of sleep disordered breathing between those with sleep study proven evidence of OSA and those without. Data displayed are the numbers who answered yes to each of the specific questions. Significance value using Fisher's exact test.

The subjects were also asked to complete a Modified Epworth Sleepiness Score (mESS), a validated tool used to assess the daytime sleepiness of a child. The median questionnaire score for those with evidence of OSA on sleep study was 5 and for those without, it was 4 showing no significant difference. A score of above 11 is thought to represent excessive sleepiness [249-251].

When comparing the subjects with evidence of OSA on sleep study to those with no evidence of OSA, the OAHI was the only difference identified with a median value of 2 events per hour (IQR 2-2.6) for the OSA subjects and 0.1 events per hour (IQR 0-0.35) for those with no evidence from the sleep studies with a significance *p* value of 0.007 (Figure 3.1, 3.2 & 3.3).

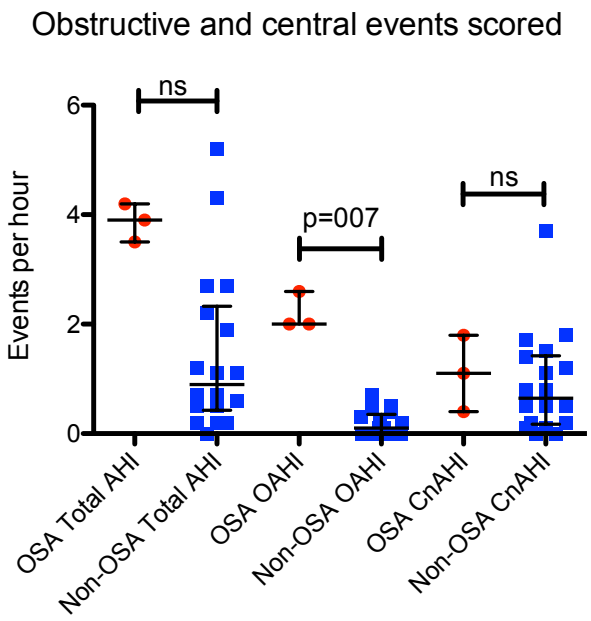


Figure 3.1. Events scored for the STRA subjects sleep studies presented as total AHI, obstructive AHI and central AHI comparing those with confirmed OSA and those whose study did not demonstrate OSA. Median values represented plus interquartile ranges. Only the obstructive AHI values showed a significant difference (p=0.007) comparing OSA to Non-OSA subjects. Significance calculated using Mann-Whitney U-test.

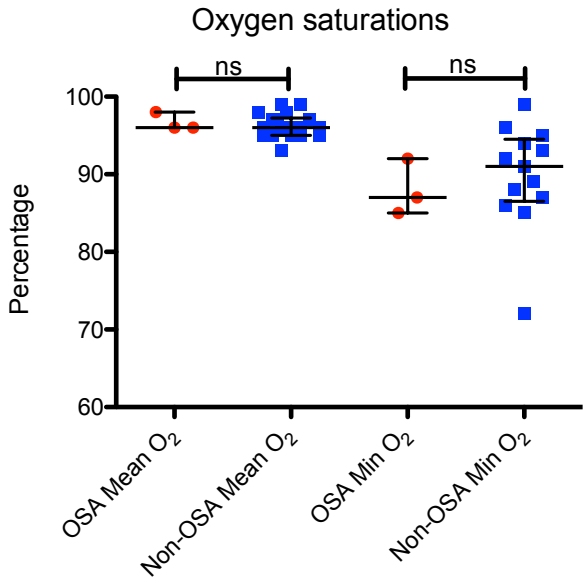


Figure 3.2. Oxygen saturations comparing those with evidence of OSA on sleep study and those without evidence. Median values plus interquartile ranges. No statistically significant difference observed. Significance calculated using Mann-Whitney U test.

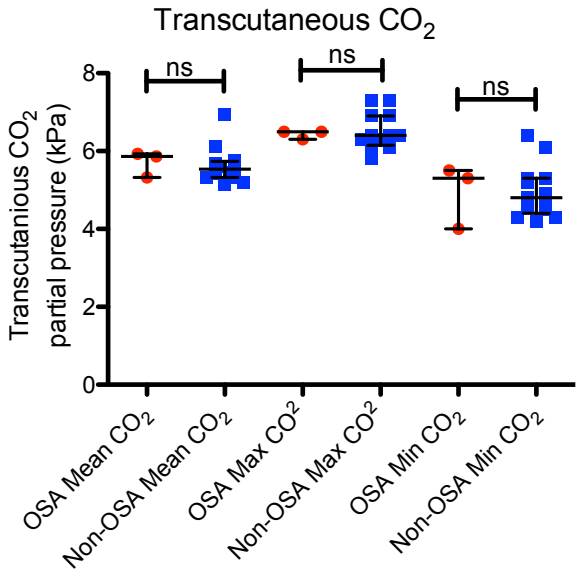


Figure 3.3 Transcutaneous carbon dioxide measured in kPa comparing those with evidence of OSA on sleep study and those without evidence. Median values plus interquartile ranges. No statistically significant difference observed. Significance calculated using Mann-Whitney U test.

Two of the three subjects with evidence of OSA demonstrated flow curve notching/ limitation on sleep study suggestive of upper airway obstruction. Only one of the subjects without evidence of OSA demonstrated this finding. There was also non-statistically significant difference when comparing the oxygen desaturation index for the two groups, with a median value of 3.3 events per hour (IQR 2-5.6) for those with evidence of OSA and 1.6 events per hour (IQR 0.6-2.3) for those without evidence.

As mentioned previously, 8 of the subjects underwent full polysomnography with useable data obtained. This group included the 3 subjects identified as having evidence of OSA. This form of study provides information on the stages of sleep achieved by the subjects and allows a more accurate scoring of hypopnoeas with evidence of arousal on the EEG leads [253]. With regards to the stages of sleep, all 8 subjects demonstrated an appropriate proportion of Rapid eye movement (REM) sleep phase for age with a median proportion of 17.4% (IQR 15.2%- 23.2%) [257].

For this subset of 8 subjects, the median REM associated AHI was higher than the total AHI however this did not achieve significance (total AHI.2.7 (0.2-4) vs REM-AHI 3.7 (IQR 3.1-4.7)). 5 of the 8 subjects studied had higher REM-related AHI than their total AHI,

suggestive of sleep disordered breathing specific to this phase of sleep. Three of these subjects were those already identified as having sleep study evidence of OSA based on the whole study. The remaining 3 of 8 studied had lower or comparable REM AHI (Figure 3.4).

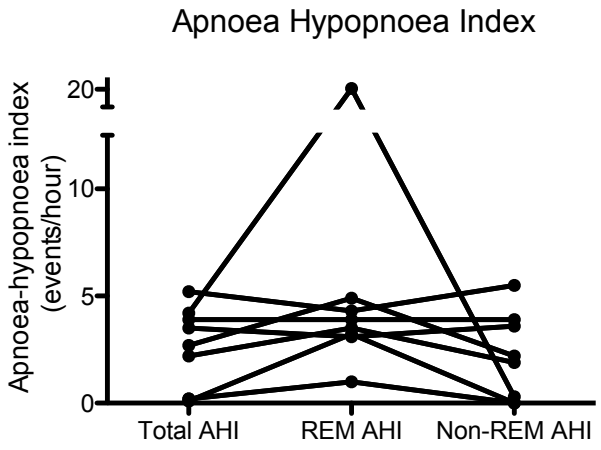


Figure 3.4. Comparison of total AHI, REM related AHI and Non-REM related AHI for STRA children who had full PSG.

For the 3 subjects defined as having OSA based on the whole sleep study, there was no difference in the time spent in REM-sleep or a higher arousal index (Figure 3.5 & 3.6).

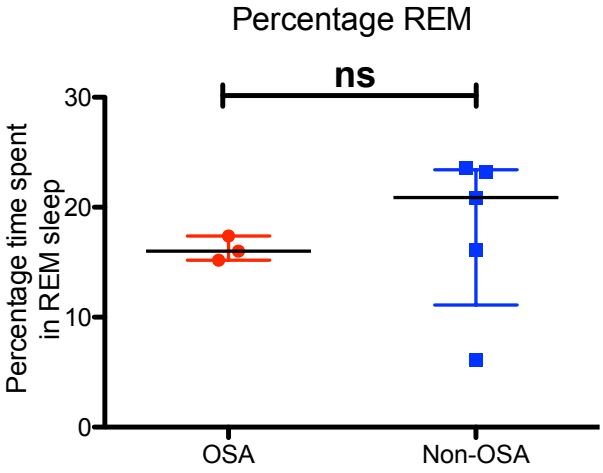


Figure 3.5. Comparison of OSA and Non-OSA STRA patient who had full PSG. Non-significantly lower proportion of time spent in REM sleep. Median values plus interquartile ranges with significance using Mann-Whitney U test.

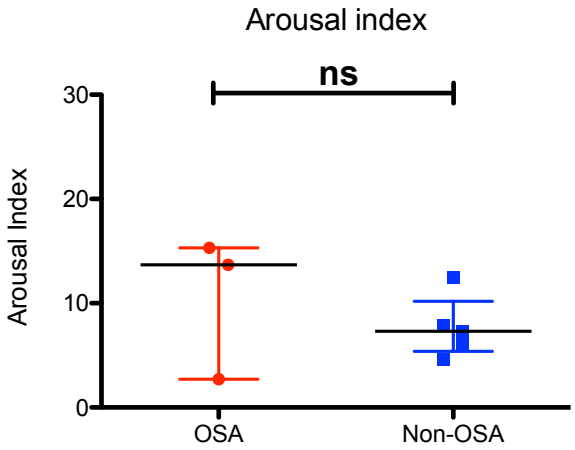


Figure 3.6. Comparison of OSA and Non-OSA STRA patient who had full PSG. A non-significantly higher arousal index within the OSA individuals. Median values plus interquartile ranges with significance using Mann-Whitney U test.

3.5.2. Medical therapy received

There is significant overlap when it comes to medical treatment for asthma and OSA in the form of steroids both administered orally and intranasal as well as leukotriene receptor antagonists, in most cases *Montelukast*. When considering the medication received by both the OSA group and non-OSA groups, no difference was observed regarding doses of inhaled corticosteroids (Figure 3.7).

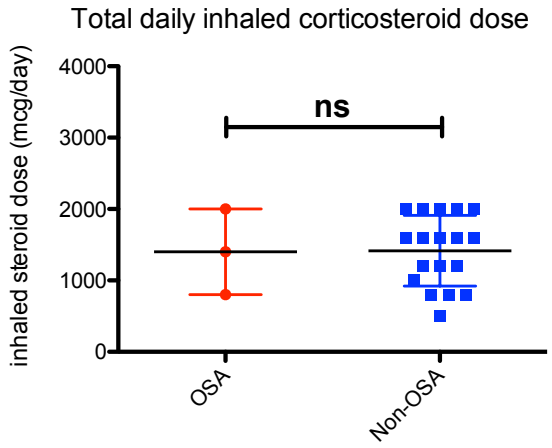


Figure 3.7. Total daily inhaled corticosteroid dose as budesonide equivalent doses comparing OSA and Non-OSA subjects. Median values plus interquartile ranges with significance using Mann-Whitney U test.

Similar proportions of patients in both groups were receiving montelukast (2 OSA subjects (66%) and 12 Non-OSA subjects (57%)) and oral prednisolone (1 OSA subject (33%) and 8 Non-OSA subjects (38%)) when the sleep studies were undertaken. A higher proportion of subjects with evidence of OSA were receiving intranasal steroids for treatment of allergic rhinitis at the time of their sleep studies (2 (66%) vs 3 (14%)) however this was not statistical significant. When the individual subjects' rate of medication pick up from their general practitioner was reviewed, both groups showed a similar high rate suggesting good adherence to treatment in both groups (100% in OSA subjects and 90% in Non-OSA).

3.5.3. Respiratory Health

If we use lung function as a marker for the subject's respiratory health and assess how their asthma and/ or OSA affect this, there was no difference between the two groups (Figure 3.8). Unfortunately, incomplete results for exhaled nitric oxide (eNO) made a comparison using this measure of respiratory health impossible.

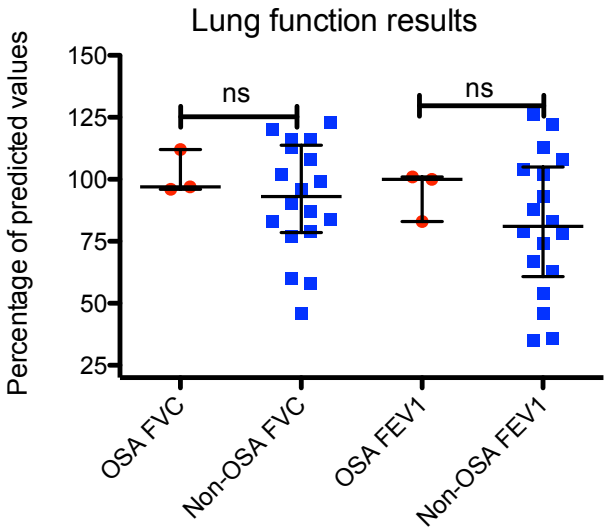


Figure 3.8. Percentage predicted forced vital capacity (FVC) and Forced expiratory value in one second (FEV1) comparing OSA and Non-OSA subjects. Median values demonstrated with interquartile range. No statistically significant differences observed. Significance calculated using Mann Whitney U test.

As part of the problematic asthma assessment used at first presentation and during treatment to monitor progress, two validated questionnaire based tools are used to assess how the individual's disease is impacting their lives as discussed in chapter 2, section 2.1.1.

These are the Paediatric Asthma Quality of Life Questionnaire (PAQLQ) and the Asthma Control Test (ACT). The initial scores for these tools when the subjects were first assessed and before any treatment is instigated were reviewed comparing the OSA and Non-OSA subjects. No significant difference was noted (Figure 3.9 & 3.1.1.).

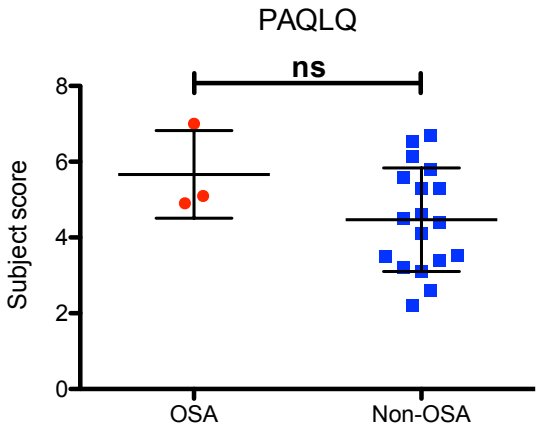


Figure 3.9. Individual subject scores for the Pediatric asthma Quality of Life Questionnaire (PAQLQ) comparing OSA and Non-OSA subjects. Scores taken at the beginning of the problematic asthma assessment. Median vales demonstrated with interquartile ranges. Significance calculated using Mann-Whitney U test.

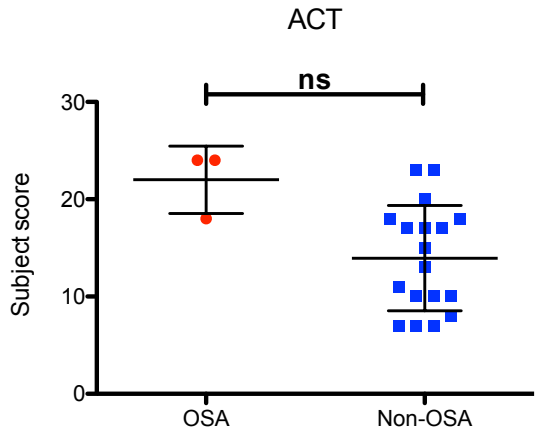


Figure 3.1.1. Individual subject scores for the Asthma Control test (ACT) comparing OSA and Non-OSA subjects. Scores taken at the beginning of the problematic asthma assessment. Median vales with interquartile ranges. Significance using Mann-Whitney U test.

3.5.4. Level of Atopic disease

With the reported association between OSA and atopy, the level or severity of the subjects atopic disease was studied by comparing the total IgE and sum of the specific IgE from the standard aero allergen panel used for the assessment of problematic asthma at RBHT (cat dander, dog dander, tree pollen, grass pollen, house dust mite and aspergillus) for the OSA subjects and Non-OSA subjects. No significant difference was observed. No difference was observed even when converting the total IgE to Z-score for age based on published methods [258] (Figure 3.1.2. & 3.1.3.).

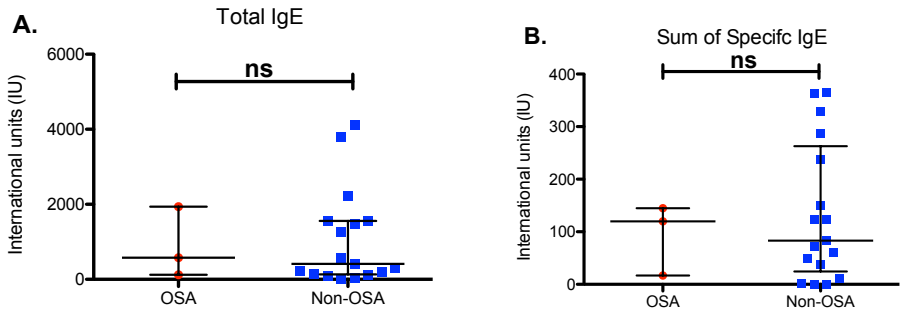


Figure 3.1.2. Total IgE (A.) and the sum of the specific IgE (B.) from the standard aeroallergen panel (cat dander, dog dander, tree pollen, grass pollen, house dust mite and aspergillus) for the OSA and Non-OSA subjects as international units. Median values demonstrated plus interquartile range.

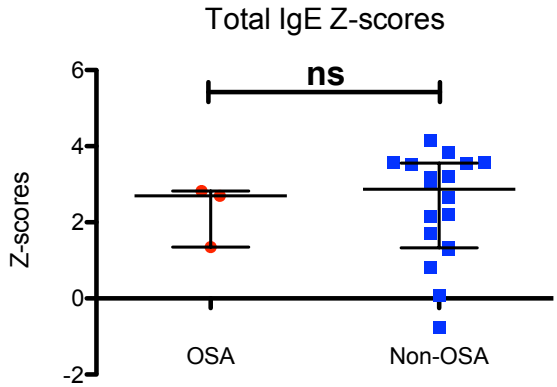


Figure 3.1.3. Total IgE as Z-scores for age for the OSA and Non-OSA subjects as international units. Median values plus interquartile ranges.

The number of peripheral and bronchial eosinophils was also compared for the two groups. As part of the problematic asthma assessment, STRA subjects undergo a bronchoscopy from which the lavage eosinophil levels can be obtained and expressed as a percentage of the

total leukocytes identified on cytological examination. The blood samples were obtained at the same time while the subject was under general anesthesia. For one of the subjects with evidence of OSA, the bronchoalveolar lavage (BAL) was not of sufficient quality to perform a cell count. This was also the case for 6 of the subjects with no evidence of OAS. No difference was observed (Figure 3.1.4.).

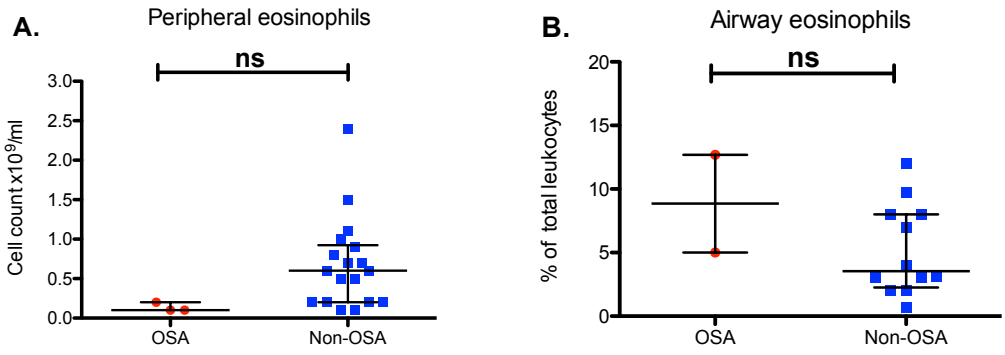


Figure 3.1.4. Peripheral eosinophil counts from blood as cell counter x10^9/ml (A.) and BAL eosinophil counts as a percentage of total leukocytes (B.) identified on cytological examination comparing samples from children with evidence of OSA on sleep study and those without. Median values plus interquartile range with significance calculated using Mann Whitney U-test.

3.6 Discussion

3.6.1. Principle findings

Conclusions drawn from this data only relate to STRA, which is in itself a rare phenotype. Having said that, there appears to be no statistically significant higher incidence of OSA amongst these children compared to the incidence quoted in the general paediatric population. With the exception of the AHI score, there were no differences between those with OSA and those without in sleepiness or clinical features of sleep disordered breathing, respiratory health, medication or levels of atopy. All three of the children with OSA were non-Caucasian. From the sub-set of 8 children who underwent full PSG, which included the 3 children with an OSA diagnosis based on the whole study, the evidence of sleep-disordered breathing was higher during REM sleep for 5 children compared to non-REM sleep.

With regard to the severity of atopy of the individuals studied, no differences were observed based on total IgE, sum of the specific IgE or total IgE Z-scores. The only significant difference noted was that non-OSA STRA children had a higher peripheral blood eosinophil level than the OSA subjects.

No children with STRA to date have undergone surgical treatment for their sleep-disordered breathing. For those with OSA, it would be interesting to see what effect the intramuscular corticosteroid injection has on their sleep-disordered breathing however repeat sleep studies are not currently part of the STRA follow-up and may not be warranted in such mild disease.

3.6.2. Strengths and weaknesses of study

Although the question of whether children with asthma are more prone to OSA has been investigated and reported in the literature, what has been lacking is consistent definitions of both asthma and OSA. This work included both those things. The asthmatic group in question was well defined and had been through a rigorous evaluation process. The diagnosis of OSA was also based on sleep study evidence rather than relying on questionnaire data.

The study population was small which reflects the fact that STRA is a rare asthma subgroup. Efforts were made to collect data on all children defined as STRA during the study period, which yielded only 21 subjects from one of the largest tertiary paediatric respiratory units in the country. With more time and resources, putting all children with problematic asthma (including DA and STRA) through the same evaluation would be interesting and more reflective of the larger asthma population rather than this small subtype.

The definition of OSA used in this study is the same as that used by the European Respiratory Society (ERS) task force consensus statement in 2016 [108]. All three subjects defined as having OSA had clinical symptoms of sleep disordered breathing on direct questioning, an AHI ≥2 and an obstructive AHI ≥1. The direct questions used could have been formalised however by using a recognized assessment tool such as Tucson Children's Assessment of Sleep Apnea Study (TuCASA) questionnaire [100]. The assessment tool used to assess sleepiness, the mESS, has been validated and is reliable however its relevance can be questioned when the non-OSA STRA children showed no difference in scores with the

OSA children. Excessive sleepiness is not a prominent finding in children with OSA therefore consistent with these findings.

The medication burden on the subjects was high as they have severe disease. No difference was observed in the amount or doses of medication used by OSA and non-OSA STRA children. The prescription collection from the general practitioner was used as a marker of compliance and was the same for both groups. The use of a compliance tool is a strength of this work as no other published studies on the subject question compliance. However, collection does not guarantee use. Electronic monitoring for example with a smart-haler that can be downloaded and the amount of medication taken extrapolated would be a more reliable measure of compliance.

Lung function, the PAQLQ and ACT questionnaires were used as measures of respiratory health. Using both allowed me to measure the children's respiratory function but also to have a reading on how their asthma impacted upon their quality of life. An additional measure that could have been used is exhaled nitric oxide, a measure of airway inflammation used to monitor asthma. Raised levels of this test have been reported in OSA[210].

3.6.3. Findings in relation to other studies

The most comparable published study to this work is the Kheirandish-Gozal et al study from 2011[241] when a group of 92 'problematic asthmatics' were recruited and underwent full PSG. This work, which has already been discussed in chapter 1., found 63% of the subjects had evidence of OSA with a mean obstructive AHI of 14.6±8.2. This is obviously a larger study population than mine but what is striking is the high incidence of OSA amongst these children and the severity of the disease identified, both of which are much higher than my results.

The study population in this trial had a higher proportion of African-Americans (25%) whereas only 9% of my recruits defined themselves as African-Caribbean. This ethnicity is a known risk factor for OSA [238], which may be elevating the frequency and severity in the Kheirandish-Gozal study. The authors report a non-statistically significant difference in rates of African-American ethnicity between those with OSA and those without (32% in OSA group vs 14.7% in the non-OSA group). No statistical analysis was performed to take into

account the confounding effects of this ethnicity on their results. Interesting, the only significant demographic detail between the OSA group and non-OSA group in my work was the higher percentage of Caucasian children in the non-OSA group. Therefore, ethnicity should be considered when analyzing my data also.

The incidence of OSA in the STRA children in my study is lower than the above-mentioned study. This may reflect the high doses of anti-inflammatory treatment the children in my study were receiving. These therapies have been shown to improve mild OSA and persistent OSA post surgery [129]. The definition of 'problematic asthmatics' used in this study may also not be comparable to my study population. In the Kheirandish-Gozal work, the children earned their definition based on frequency of exacerbations and a self reported diary of symptoms and bronchodilator use in the three weeks prior to their sleep study. It is our experience that self-reporting of symptoms does not correlate well with true severity. The children in my study have been shown to be truly severe and have good compliance with treatment.

There is evidence from my work, while in a small study group, that the sleep disordered breathing seen in asthma may be associated with REM sleep rather than non-REM. Perez et al 2013 studied asthmatics and non-asthmatics and while they found no overall differences in nocturnal respiratory parameters, the maximum percentage of oxygen desaturations during REM was higher in asthmatics independent of demographic variables [259]. The group raised the possibility of a REM-related vulnerability impacting oxygenation in asthmatics. However, an increase in sleep disordered breathing events during REM sleep irrespective of an asthma diagnosis is a know feature of OSA in children.

3.7 Conclusion

The research question asked of this chapter was whether the incidence of OSA amongst STRA children was higher than reported in the genera population. This chapter also asked if the STRA children with evidence of OSA are more atopic then those without OSA. The hypothesis was that the incidence of OSA would be higher in STRA children. Accepting the small study population and lack of statistically generated target recruitment figure, I have not identified a significantly higher incidence of OSA amongst severe therapy resistant asthmatic children compared to the published incidence in the general paediatric population. I am therefore rejecting my first hypothesis.

There also appeared to be no difference in atopic status amongst the STRA children with OSA and those without OSA in my study. In the next chapter I will address the relation between atopy and OSA in more detail by presenting the data from children who have already been identified as having OSA and exploring how many have a history of atopy or evidence of allergic sensitization. This is a separately recruited group answering a different hypothesis therefore it was appropriate to precede with my work despite the findings of this chapter.

Chapter 4. Atopy in children with OSA

This chapter focuses on hypothesis no. 2, stating that there is a higher prevalence of atopy in children with sleep study proven OSA undergoing adenotonsillectomy compared to those without OSA. The aim was to determine atopic status in children undergoing tonsillectomy for OSA or recurrent tonsillitis using questionnaires and serum specific IgE levels to common aeroallergens. Any data no displayed in table form can be found in Appendix 12.

4.1. Introduction

From the data presented in chapter 3, no difference was identified in the severity of atopic status when comparing STRA children with and without OSA. In this chapter, the question of whether there is a link between atopy and OSA was investigated by recruiting individuals already identified as having OSA and looking for evidence of atopy.

Links between OSA and atopic disease in children and adults have been drawn by many authors. In adult populations, individuals with OSA are more likely to be sensitized to perennial allergens than control groups[260]. In children, studies have showed an association between snoring and parent report allergic rhinitis or allergic dermatitis [224-229] however the majority of published work is based on questionnaire data. An association has been demonstrated between parent reported snoring and OSA diagnosed using polysomnography with atopy based on serum specific IgE in children as young as twelve months of age [230, 231]. In a larger group, Carr et al showed no associated between children with limit channel sleep study evidence of sleep disordered breathing and atopy confirmed in the same manner [242].

4.2. Research Questions

1. Do children with established OSA have higher rates of atopic diseases including asthma, eczema, or allergic rhinitis, or more parental allergies, compared to a disease control group of children free from OSA.

2. Do children with OSA have more objective evidence of atopic sensitisation defined by established laboratory tests compared to a disease control group of children without OSA?

4.3. Methods

Children confirmed as having OSA based on a cardiorespiratory polygraphy (RP) performed at the Royal Brompton Hospital (RBHT) Pediatric sleep and ventilation unit and referred to the local Ear, Nose and Throat (ENT) service were recruited if they were scheduled for adenotonsillectomy at Chelsea and Westminster Hospital (ChelWest) or Royal Brompton Hospital (RBHT). The surgical schedules from ChelWest were also studied on a weekly basis and cross-referenced with the clinical database at RBHT to identify any subjects who had undergone a sleep study.

All recruitment took place during the morning of the child's scheduled surgery. Children listed for adenotonsillectomy for reasons other than OSA were also recruited on the morning of surgery. Blood samples were obtained from all subjects once under anesthesia and before the surgery began (Figure 4.1).

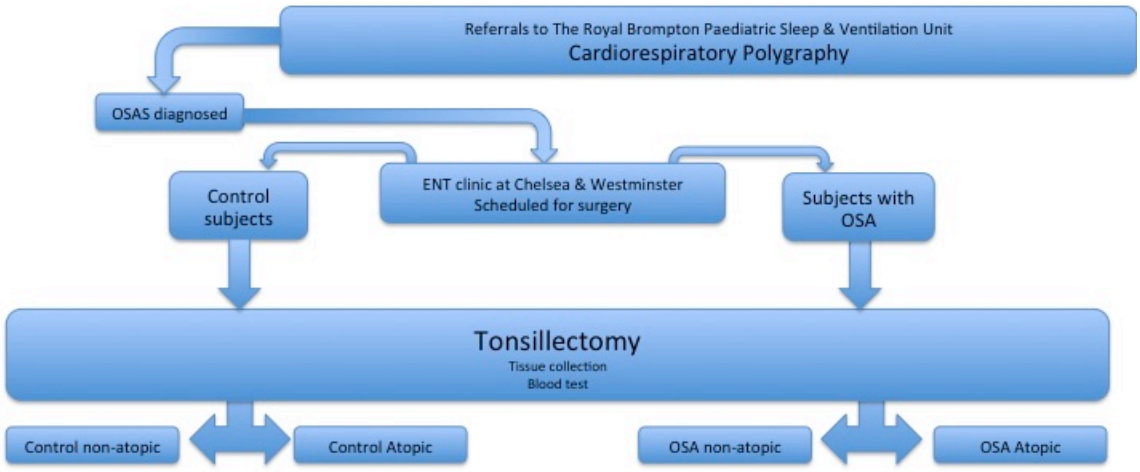


Figure 4.1. Recruitment flow diagram.

Recruitment became problematic for several reasons. Firstly, delays in obtaining ethical approval reduced available time to recruit from twenty-four months to eighteen. Secondly, it transpired that a figure of twenty-five children referred to ENT did not result in twenty-five children being listed and undergoing adenotonsillectomy in a twelve-month period. Some with mild disease opted for medical management. For some, parents were reluctant to have their child undergo surgery. Many children with clinical suspicion of OSA in the ENT

clinic were also scheduled for surgery without a sleep study making them not suitable for recruitment.

It was for these reasons and queries raised about how free from OSA the control group of children undergoing tonsillectomy for indications such as recurrent tonsillitis truly were, that a different approach was taken to recruitment involving the use of home cardiorespiratory polygraphy (Home RP).

4.3.1. Home Cardiorespiratory Polygraphy

Many of the children undergoing adenotonsillectomy for the treatment of OSA did not have a sleep study beforehand. The control group were scheduled for surgery for recurrent tonsillitis and also had not had a sleep study. There was no available funding or time to bring these children into the RBHT Paediatric sleep unit therefore the decision was made to utilise a home cardiorespiratory polygraphy system.

The *Somnotouch* is a mobile device produced by the same company *Somnomedics* who provide and support the equipment for the RBHT in-laboratory sleep. The data obtained provides sufficient information for a RP study with fewer signal measures. Ambulatory sleep studies have been shown to produce comparable data to in-laboratory studies[104] (Table 4.1 & Figure 4.2).

In laboratory Cardiorespiratory Polygraphy	**Home study Cardiorespiratory polygraphy**
Heart rate	Heart rate
Oxygen saturations	Oxygen saturations
Transcutaneous carbon dioxide	Effort bands
Effort bands	Nasal flow
Nasal flow	Movement monitor
Nasal thermistor	Actigraphy
Video	
Limb Movement monitor	

Table 4.1. Comparison of data obtained from in-laboratory cardiorespiratory polygraphy study and home cardiorespiratory polygraphy study.



Figure 4 2. Demonstration of the *Somnotouch* home sleep study device being worn. The red circle enlarged above demonstrates the device while it is being worn which slides onto the thoracic effort band.

The first step was to assess comparability of home with in-laboratory sleep study data. To this end, a short preliminary study was undertaken following amendments to the ethical approval.

4.3.2. Home cardiorespiratory polygraphy preliminary study

Methods: The parents or guardian of children admitted to the RBTH paediatric sleep unit were approached and offered the chance for their child to undergo a sleep study the following night at home. Consent was obtained before the family was introduced to the *Somnotouch* and provided with a demonstration of how it is worn. Written guidance was also provided.

The recruits would leave the sleep unit the following morning with the *Somnotouch* device and perform an unsupervised HRP study that night at home. The device was then collected either by courier or in person the following morning and returned to the hospital for downloading and analysis.

The hypothesis of this sub-study was that the data produced using a home sleep study device would be comparable with that produced from the in-lab sleep study thus making it a reliable research tool. It could then be used on children undergoing tonsillectomy without a prior sleep study making them eligible for recruitment.

Results: Eight children were recruited. Their demographics were as follows (Table 4.2):

Median age (years) Interquartile ranges	5 (3.5-5)
No. Males.	6
Median Height (meters) Interquartile ranges	112 (101.1-114)
Median Weight (kg) Interquartile ranges	19.5 (16.2-23)
Median BMI Interquartile ranges	17.1 (14.7-18)

Table 4.2. Demographics for subjects recruited for sub-study comparing home and in-laboratory cardiorespiratory polygraphy studies. Median values with interquartile ranges.

The limitation of unsupervised sleep studies is that a loss of signal will be undetected until the device is downloaded. Loss of signal due to disconnection of leads or senor malposition for periods of time was to be expected with the home device especially when taking into consideration this was the team's first experience with its use. While there was no loss in signal for the in-lab study owing to the fact nurses were on hand to reconnect or readjust probes if required, loss of signal for the eight home studies resulted in a mean loss of 3 hours and 52 seconds per study. The most common signal to be lost was the flow sensor (occurred in 3 studies) followed by the oxygen saturation monitor (occurred in two studies) and in one study the effort bands. At least four hours of good quality data (all signals recording with no losses) was obtained from each study.

Signal loss resulted in sections of data for which no events could be scored. The effect of this was a non-statistically significant underscoring of the total Apnoea-Hypopnoea Index (AHI), central AHI and obstructive AHI for the home studies compared to the in-lab studies (Figure 4.3).

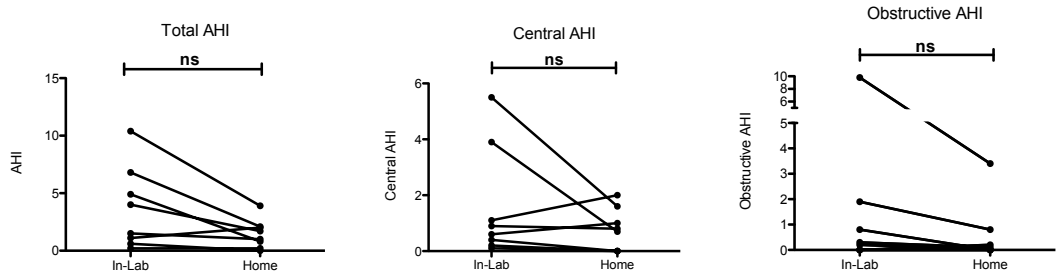


Figure 4.3. Total AHI, central AHI and obstructive AHI comparing in-lab CR-Poly results and home CR-poly results. Non-statistically significant difference observed. ns represents no statistical significance.

Signal loss became less of a problem as I became more familiar with the device and the process of training families was refined. Particularly with the final two recruits, there was no data loss and comparable studies produced both in laboratory and home.

Despite the underscoring of the home studies, based on the total AHI and the obstructive AHI, there would have been no difference in diagnoses for the subjects. Those with a pathological study in laboratory suggestive of OSA would also have been diagnosed with OSA on the home study. Recognizing this to be a very small study group, Bland-Altman comparisons were drawn for the total AHI and obstructive AHI. A linear relationship was observed suggesting that as the event score increases, the difference between the two difference studies becomes more extreme but at the normal end of the scale (AHI <1) there is more likely to be agreement between the two studies (Figure 4.4).

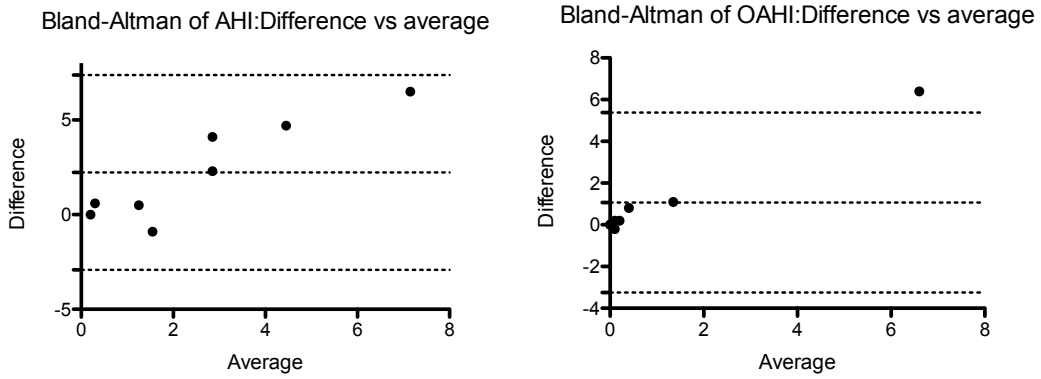


Figure 4.4. Bland-Altman of AHI: difference (home RP generated AHI) vs average and obstructive AHI: Difference (home RP generated AHI) vs average.

Conclusion: For this preliminary study, no power calculation was carried out to generate a recruitment number. Neither was standard operating procedures developed for the home study device. Also, the incidence and severity of disease was by no mean evenly distributed. There are many reasons to criticize this preliminary project. The aim was to try to demonstrate comparability between the two diagnostic tests. It may be perceived as crude but was in fact an attempt to justify the intervention when faced with recruitment difficulties.

While universal use of in-laboratory studies would be the preferred method, the diagnostic criteria to define OSA or not could be used for the home studies and was likely to result in similar outcomes. More importantly, the home study appeared to be as reliable as the in-lab study when no OSA was evident and therefore useful for ruling out OSA in the control subjects. The technique was decided to be acceptable and comparable for use during recruitment.

4.4. Results

45 children were recruited to the study, 24 with OSA and 21 non-OSA controls. All children with OSA had sleep study evidence of disease, two of which were diagnosed based on home sleep study and the remaining by in-laboratory studies. The two diagnosed at home had been listed for adenotonsillectomy based on clinical symptoms of OSA. The home sleep study confirmed the diagnoses.

All 21 controls recruited were children undergoing tonsillectomy due to recurrent tonsillitis. 12 of the controls had sleep studies confirming no evidence of OSA, 7 using home sleep studies. There was no significant difference between the OSA and the non-OSA groups regarding their demographic details (Table 4.3).

	OSA N=24	Non-OSA N=21	p
Median age (years) Interquartile range	4.5 (3-7.7)	6 (4-8.5)	0.3
No. of males	15 (62%)	11 (52%)	0.5
Median Height (cms) Interquartile range	107 (102-132.5)	127 (105-135)	0.7
Median Weight (kg) Interquartile range	19.2 (14.7-33.9)	21.8 (17.3-34)	0.3
Median BMI Interquartile range	17.4 (14.8-19)	17.5 (14.8-25.1)	0.7

Table 4.3. Demographics of recruited subjects. Values demonstrated are median values with interquartile ranges. No statistically significance differences were observed. Significant was calculated using Mann-Whitney U test.

4.4.1. Sleep-disordered breathing within recruited subjects

The subjects were first asked a series of questions focusing on sleep behaviour. The parents for the most part owing to the age of the children answered these questions. There was no statistically significant difference between those with OSA and those without (Table 4.4.). As explained in chapter 2, to avoid potential bias from multiple comparison, a statistical significance cut off of <0.01 was used.

	OSA (n=24)	**Non-OSA (n=21)**	**P**
History of snoring	20 (83%)	17 (80%)	1.0
History of witnessed apneas	12 (50%)	6 (28%)	0.2
History of witnessed arousals from sleep	16 (67%)	8 (38%)	0.02
History of mouth breathing	19 (79%)	17 (80%)	1.0
History of morning headaches	0	4 (19%)	0.04
History of daytime sleepiness	8 (33%)	7 (33%)	1.0
History of difficulty concentrating during the day.	6 (25%)	7 (33%)	0.7
History of behavior problems during the day.	6 (25%)	4 (19%)	0.72
No. of children with fixed bed time	19 (79%)	15 (71%)	0.7
Usual bed time	20:00 (19:00-20:00)	20:00 (20:00-20:45)	0.2

Table 4.4. Answers to direct questioning for features suggestive of sleep disturbance. The values are proportion of subjects answering yes to questioning with percentages. Significance calculated using Fisher's exact test.

The control group was made up of children undergoing tonsillectomy for recurrent tonsillitis. The median number of tonsillitis illnesses was 5 per year (interquartile range 5-6 per year). There was no difference in tonsil grade as per the Brodsky grading scale. The median tonsil grade for both groups was 3 (interquartile range 3-3). There was also no difference in weight of the extracted tonsil tissue between the two groups (Figure 4.5).

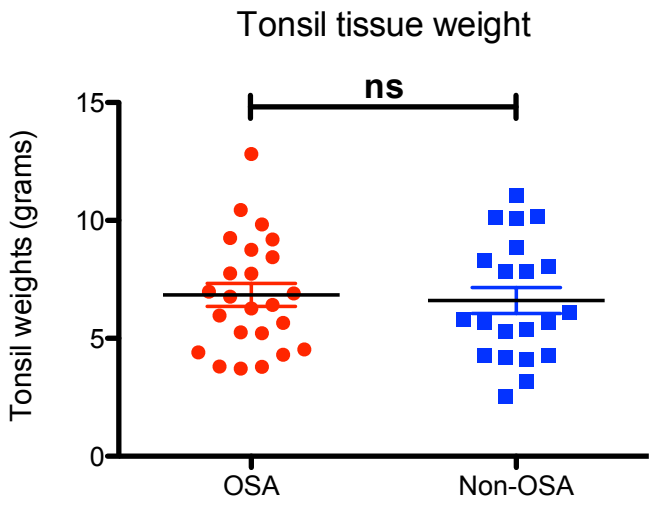


Figure 4.5. Extracted tonsil tissue weight comparing subjects for whom the tissue was extracted to treat OSA against those undergoing surgery for recurrent tonsillitis. Median plus interquartile range with significance using Mann-Whitney U test.

The subjects were also asked to complete the modified Epworth Sleepiness score (mESS), a validated tool used to assess daytime sleepiness in children. There was no significant difference between the two groups (Figure 4.6).

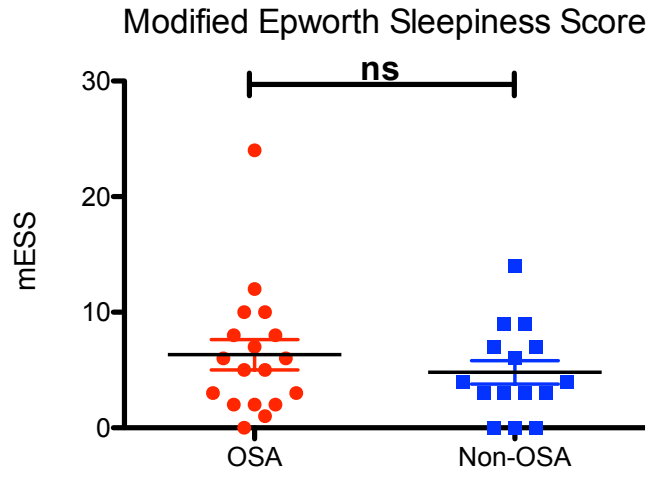


Figure 4.6. The scores generated from the Modified Epworth Sleepiness Questionnaire for those undergoing adenotonsillectomy for OSA compared to those undergoing surgery for other reasons and free from OSA. Median values plus interquartile ranges. Significance calculated using Mann-Whitney U test.

The only difference between the two groups was the cardiorespiratory polygraphy data that defined their group status. The children with OSA obviously had significantly higher AHI and obstructive AHI than the controls who underwent sleep studies (12, 57%) (Figure 4.7. & 4.8.).

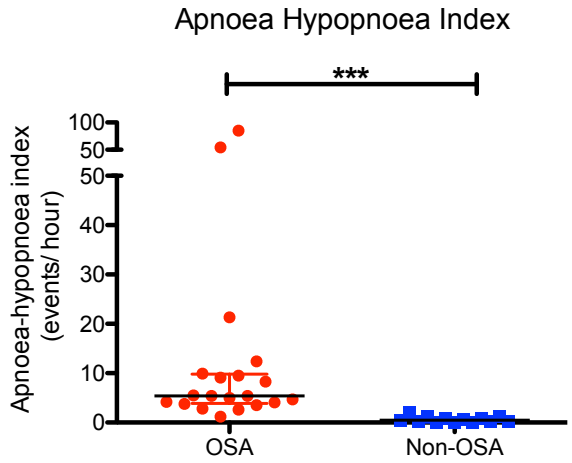


Figure 4.7. Apnoea-hypopnoea index comparison for subjects with and without OSA. Median value plus interquartile ranges. Significant calculated using Mann-Whitney U-test. *** represents a significance level of <0.001.

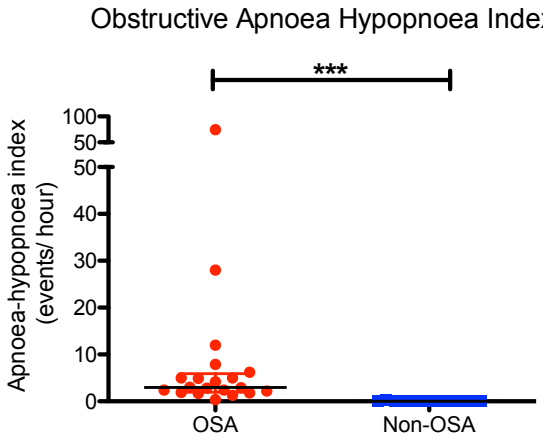


Figure 4.8. Obstructive Apnoea-hypopnoea index comparison for subjects with and without OSA. Median value plus interquartile ranges. Significant calculated using Mann-Whitney U test. *** represents a significance level of <0.001.

4.4.2. Atopy within recruited subjects

The next set of questions focused on symptoms of atopic disease. These were questions commonly asked as part of the difficult asthma assessment and touch on symptoms suggestive of asthma and atopy. As with the previous questions, parents answered, as most children were too young to answer for themselves. No significant differences were observed between children with OSA and controls (Table 4.5 & 4.6.).

	OSA (n=24)	**Non-OSA (n=21)**	**P**
History of Wheeze	8 (33.3%)	5 (23%)	0.5
Only with viral illnesses	4 (50%)	4 (80%)	0.7
With viral illnesses and interval wheezing	4 (50%)	1 (20%)	0.6
History of food allergies	4 (16.6%)	5 (23.8%)	0.7
History of eczema	6 (25%)	7 (33.3%)	0.7
History of hay fever	4 (16.6%)	6 (28.5%)	0.5
History of rhinitis	5 (20.8%)	5 (23.8%)	1.0
Pets at home	3 (12.5%)	4 (19%)	0.7

Table 4.5. Answers to direct questioning for features suggestive of atopic disease. The values are proportion of subjects answering yes to questioning with percentages. Significance calculated using Fisher's exact test.

	OSA (n=24)	**Non-OSA (n=21)**	**P**
Family history asthma	8 (33.3%)	8 (38%)	0.8
Family history eczema	8 (33.3%)	3 (14.3%)	0.2
Family history hay fever	10 (41.6%)	12 (57.1%)	0.4

Table 4.6. Answers to direct questioning for features suggestive of a family history of atopic disease. The values are proportion of subjects answering yes to questioning with percentages. Significance calculated using Fisher's exact test.

Finally, with proposed relationships between birth history and early years feeding practice and rates of atopy, questions were asked about the subject's infant life. No significant difference was observed (Figure 4.7.).

	OSA	Non-OSA	p
Median Gestation at birth (weeks) (Interquartile range)	40 (38-40)	40 (39.5-40.5)	0.2
Proportion of vaginal births (Percentage)	19 (79%)	11 (50%)	0.06
Median birth weight (Kg) (Interquartile range)	3.49 (3.1-3.8)	3.2 (2.9-3.6)	0.3
Proportion breast fed (Percentage)	9 (37%)	7 (33%)	1.0

Table 4.7. Infant birth details and feeding patterns comparing subjects with OSA and controls. Gestation at birth and birth weight as median values with interquartile ranges and significance using Mann-Whitney U test. Proportion of vaginal births and breast-fed children quoted as total and percentages and significance calculated using Fisher's exact test.

In an effort to define atopy with laboratory evidence, the blood samples from the recruited individuals were analysed by testing for the aeroallergen specific IgE panel. The total IgE was also investigated. The most common positive specific IgE was to cat dander but house dust mite provided higher values measured in international units (Figure 4.9).

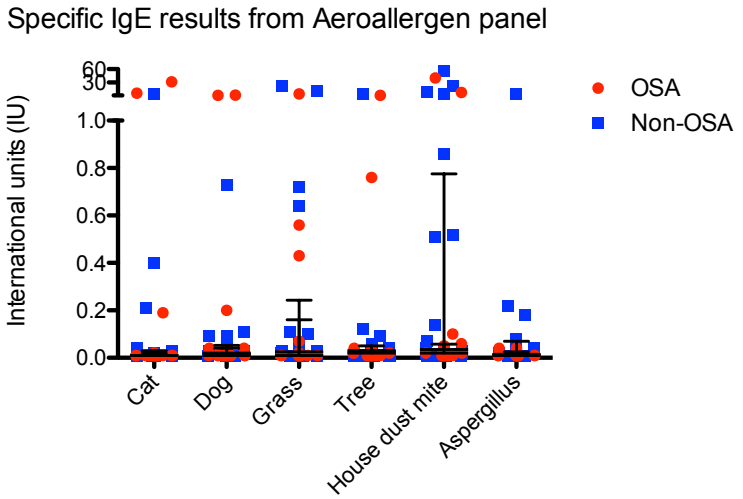


Figure 4.9. Results from the aeroallergen panel of all subjects recruited. Subjects undergoing adenotonsillectomy for OSA and those undergoing adenotonsillectomy for other reasons. Median values demonstrated with interquartile ranges.

Of the individuals diagnosed with OSA, 5 (20%) had raised specific IgE suggestive of atopic disease. In the control group 10 (48%) had raised specific IgE suggesting there was more evidence of atopy in controls compared to the OSA subjects. The sum of the specific IgE values for the OSA and control individuals also suggested higher atopy prevalence amongst the controls (Figure 4.1.1.).

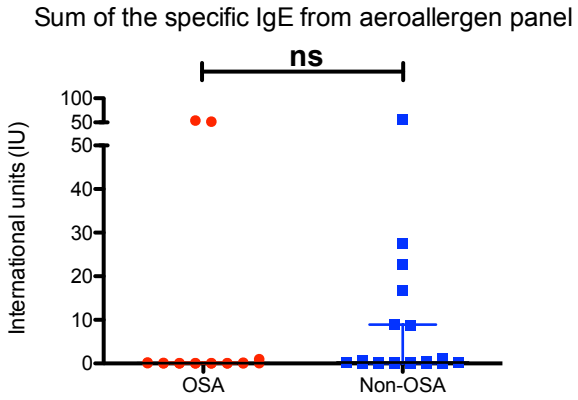


Figure 4.1.1. Sum of the specific IgE test results for subjects undergoing adenotonsillectomy for OSA compared to those undergoing adenotonsillectomy for other reasons. Median values demonstrated with interquartile ranges. Significance using Mann-Whitney U-test.

There was no significant difference in total serum IgE or when expressing the total IgE as Z–scores for age between the OSA and control subjects (Figure 4.1.2. & 4.1.3).

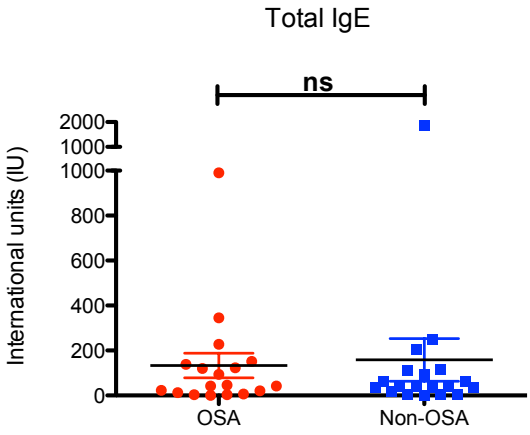


Figure 4.1.2. Total IgE for subjects undergoing adenotonsillectomy for OSA compared to those undergoing adenotonsillectomy for other reasons. Median values demonstrated with interquartile ranges. Significance calculated using Mann Whitney U-test.

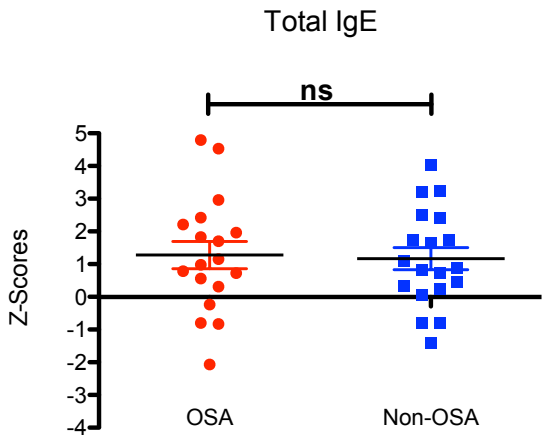


Figure 4.1.3. Total serum IgE as Z-scores for subjects undergoing adenotonsillectomy for OSA compared to those undergoing adenotonsillectomy for other reasons. Median values demonstrated with interquartile ranges. Significance calculated using Mann Whitney calculation.

4.6 Discussion

4.6.1. Principle findings

The sleep study evidence was used to confirm OSA, namely the AHI, was the only significant difference detected between those with OSA and those without. There was no difference in symptoms of sleep disordered breathing or daytime sleepiness based on mESS in either group.

There was no difference in tonsil size between the groups. All extractions were carried out by the same group of surgeons who all practice the same technique of extra-capsular cauterization removal with total tonsil removal. If tonsil hypertrophy was solely the cause of the child's sleep disordered breathing, one would have expected those tonsils to be larger either as a grade prior to surgery or as the total tissue weight.

No difference was demonstrated between the subjects with OSA and those without based on features of atopy. No differences were apparent when questions were asked about atopy and atopic disease, or when objective assessments of atopy were made using total and specific serum IgE. Based on this data, children undergoing tonsillectomy for OSA do not appear to have higher rates of atopy or atopic disease compared to non-atopic controls.

4.6.2. Strengths and weaknesses of study

The study group is small in comparison to previously published work on the subject. For example, the study group in the Carr et al publication [242] contained over 100 children. Therefore any conclusions drawn should be done with caution. Every effort was made to maximise recruitment. What had not been appreciated prior to starting this work was the high number of children undergoing tonsillectomy without sleep study evidence of OSA. One of the goals of this work was to recruit only children with sleep study evidence of OSA, which therefore limited the recruitment potential and resulted in a failure to recruit the target of 50 children with OSA.

The use of the home sleep study equipment was one example of an attempt to maximise recruitment. These devices are comparable with in-laboratory study equipment as discussed in chapter 1 and the preliminary results of my study suggest while these studies may potentially under-score events, when there is no evidence of sleep disordered breathing, there was good agreement between the two types of studies. Despite this, the fact that two different methods were used to diagnose sleep disordered breathing would be a criticism of the data produced. There is also a limitation in the fact that only half the subjects in the control group underwent any form of sleep study. However, an attempt to exclude OSA in the control group is an improvement on much of the literature as most authors did not use sleep studies on control groups of recurrent tonsillitis.

As discussed in the previous chapter, the direct questions used to evaluate sleep-disordered breathing could have been formalised by using a recognized assessment tool such as Tucson Children's Assessment of Sleep Apnea Study (TuCASA) questionnaire [100]. The assessment tool used to assess sleepiness, the mESS, has been validated however its relevance can be questioned when the non-OSA children showed no difference in scores.

A weakness of this work was also the lack of assessment of the adenoids. These tissues also contribute to the obstruction of the upper airway and have a higher ability to regrow post removal. Modern extraction techniques liquefy the tissue making collection and assessment of size or cellularity impossible. The lack of difference in palatine tonsil grade or weight despite sleep study evidence of or lack of OSA may be due to the adenoids explaining why the tonsil dimensions showed no difference.

Every effort was made to assess the children recruited for evidence of atopy and atopic disease. The questions used were taken from the problematic asthma protocol discussed in chapter 2 and 3. Formal assessment tools are available of grading individual features examined such as the ISAAC questionnaire or video based questionnaires to identify and correctly define wheeze. However, as the number of children answering positively to those questions was so low, they were not used.

The use of specific IgE and total IgE as a defining marker of atopy is frequently used in the literature on this subject in similar aged children [230, 231, 242]. One explanation for the disproval of the hypothesis could be that this is not the correct method for defining atopy, that peripheral sensitization will be greater at an older age or that peripheral blood is not the correct compartment to use when looking for evidence of atopy. Tonsil tissue itself has been shown to display evidence of localised allergic sensitisation, inconsistently correlating with serum specific IgE. Zhang and colleagues demonstrated a higher frequency of positive reactions to specific IgE test from homogenised tonsil tissue supernatant compared to peripheral blood suggesting a local atopic sensitization [261]. Future work may involve homogenising the saved and frozen tonsil tissue. However, if the tissue itself was sensitised and that was in some way compromising airflow resulting in OSA, one might expect the tissue mass to be greater than the control subject. This was not the case with my subjects.

4.6.3. Findings in relation to other studies

A summary of published work addressing the link between OSA and atopy can be found in chapter 1. Of the studies listed, only two can be directly compared to my work. Firstly, McColley et all 1997 [231] looked at 39 children referred to John Hopkins paediatric sleep unit with a history of habitual snoring and found a high prevalence (36%) of sensitisation to an aeroallergen panel amongst them. Similar rates of OSA were diagnosed in the atopic and non-atopic groups based on full PSG analysis. This work lacked a control group as all children recruited had sleep-disordered breathing but suggests that atopy is associated with primary snoring and not OSA.

The second study to discuss is from Carr et al 2007 [242]. This team of ear, nose and throat surgeons looked at peripheral sensitization based on specific IgE to an aeroallergen panel in over 100 children undergoing tonsillectomy for OSA based on a limited channel oximetry study and witnessed evidence of snoring and apneas. There was inconsistent testing of all

aeroallergens with some children missing out on one or more allergens however 20% tested positive for house dust mite and small proportions (<10%) for the rest of the panel suggesting there is not a high rate of atopy amongst these children. Again, a control group was lacking.

The data presented in this chapter along with the studies named above do not provide strong evidence of a link between OSA and atopy. The other studies listed in chapter 1 have based the association predominantly on questionnaire-collected data. It stands to reason that a child with atopic disease such as dermatitis or rhinitis will suffer disturbed sleep or demonstrate sleep disordered breathing as a result of their illness causing upper airway congestion. Better management of their atopic disease is likely to improve their sleep quality.

4.7 Conclusion

The research questions asked of this chapter were whether children with OSA have more evidence of atopy both clinically and objectively compared to children without OSA. The hypothesis was that those with OSA will be more atopic. From the data presented in this chapter and accepting that this is a small study population lacking a statistically powered recruitment target, there appears to be no difference in atopic status between children undergoing tonsillectomy for OSA or recurrent tonsillitis based on direct questioning and assessment of peripheral sensitisation. In this context, I have therefore rejected hypothesis no. 2.

The next two chapters will focus on the extracted tonsil tissue and evaluation of whether there is a difference in lymphoid cell populations in those with and without OSA or atopy. These are description of the tissue composition and therefore independent of the findings from the previous two chapters therefore it was appropriate to progress with the investigation.

Chapter 5. Lymphoid cell populations in tonsils and peripheral blood from children with obstructive sleep apnoea

This chapter investigates my hypothesis no. 3, stating that tonsils from children with sleep study proven OSA have more Th17 cells and fewer Treg cells. The aims of this chapter were to recruit children scheduled for tonsillectomy and collect the extracted tonsil tissue for flow cytometry analysis to phenotype T-cell subsets and ILCs. All data displayed in table form can be found in appendix 13.

5.1. Introduction

The previous chapter showed, taking into account the limitations of a small sample size, when comparing children undergoing tonsillectomy as a treatment for OSA against those undergoing the same procedure as a treatment for recurrent tonsillitis, there was no difference in the number of children who were atopic based on answers to clinical questionnaire at recruitment or by objective confirmation of atopy using specific allergen testing. Atopy was not associated with OSA as initially hypothesised.

While adenotonillar hypertrophy is the most common cause of OSA, the underlying mechanisms resulting in hypertrophy are unclear. Differences in regulatory T-cells (Treg) and T helper 17 cells (Th17) have been associated with OSA. Higher proportions of Th17 and lower proportions of Treg cells have been reported in the adenoidal tissue of children with suspected OSA [44]. Similar findings in peripheral blood Th17 and Treg cell counts were found for adults and children with full polysomnography (PSG) confirmed OSA[58, 62]. These results suggest a skewing from a regulatory phenotype to an IL-17 signature in subjects with OSA. In this chapter I present the results of my investigation of tonsil tissue and peripheral blood from children with OSA including a control group free from disease, looking at Th17 and Treg proportions. Since data in the literature has drawn links between OSA, asthma and atopy, I have also investigated the role of the type 2 cytokines, interleukin (IL) 4, 5 and 13 which are thought to drive symptoms in asthma[262]. If there is an association between OSA and allergic disease, the samples from children may have a stronger Th2 phenotype compared to controls.

Th2 cells secrete IL-4, IL-5 and IL-13. ILC2 cells also produce type 2 cytokines (IL-4, IL-5, IL-9 & IL -13) and have been found at barrier surfaces such as tonsils as well as in peripheral blood[144]. ILC2s have been linked to allergy in mouse models[147, 148, 263] and allergic disease in humans with their discovery in the nasal polyps of patients with chronic rhinosinusitis[154]. ILC2s have also been assigned critical roles in asthma and atopic dermatitis [155, 156] and therefore may play a role in OSA pathology too.

5.2. Research questions

1. How does the total number of $CD4^+$ cells as cell counts and/ or percentage of total Tcells in the tonsils and peripheral blood of children with OSA compare to a disease control group with recurrent tonsillitis?
2. Are Th2 and Th17 cells present in a higher proportion (cell counts and/ or percentage of total Tcells) than Treg cells in the tonsils and peripheral blood of subjects with OSA compared to those with recurrent tonsillitis?
3. How do ILC populations differ both in cell counts and percentage of lymphoid cells, in the presence of OSA compared to a disease control group of children with recurrent tonsillitis?

5.4. Methods

5.4.1. Preliminary work.

5.4.1.a. Antibody choice and titration

While waiting for ethical approval, several techniques were tested and refined prior to recruitment. The first was to ascertain the appropriate concentration of antibody to use when staining human tonsil cells. The commercially available anti-human directly conjugated antibodies required titration to determine the optimum concentration for staining cells. This titration was performed on all the antibodies used in these experiments. Blood was obtained from healthy volunteers and PBMCs extracted using standard laboratory techniques (Chapter 2, section 2.3.2.). The cells were counted and $1x10^6$ cells per well used for staining. After testing serial dilutions starting with those recommended by the antibody provider, a concentration of 1:100 was found to achieve successful staining for all antibodies I planned to use determined by changes in MFI (mean fluorescence intensity).

The next step of preparatory work involved developing a flow cytometry staining protocol and panel that would allow staining of multiple cell markers from the same sample utilising the full extent of the laser detection filters on the BD Fortessa. A 12 colour panel was developed (Appendix 10) using commercially available antibodies and tested on lymphocytes extracted from mouse spleens. This tissue was chosen as it required similar preparatory steps as tonsil tissue. The tissue was also easily available as ethical approval for the work was already in place.

In an effort to save resources before my experiments could begin, the twelve fluorophore panel was developed (Appendix 10) using commercially available anti-mouse antibodies already purchased by the laboratory group. These were predominantly B-cell markers, which was appropriate as the test tissue was spleen. The twelve fluorophores were chosen with florescent wavelengths that would not overlap allowing the FACS machine to distinguish between different cell markers. For this trial run, eleven colours were tested as the twelfth colour to be used utilised the ultraviolet laser was not available at the time. This was utilised for a viability cell dye in the panel and the only marker used in this laser. Its absence in the trial run was therefore not detrimental. Full details about the processing of samples, the functions of a flow cytometer and the interpretation of results were discussed in Chapter 2., section 2.3.3.

Adequate compensation was achieved using 11 colours with the chosen staining strategy. These preliminary experiments enabled me to determine a working flow cytometry panel, with optimal antibody concentrations and an optimised method for preparation of the cells for staining with extracellular, intracellular, and intra-nuclear markers. Panels containing the preferred cell markers for T-cells and ILCs on the fluorescent colours trialled previously could then be generated (Table 5.1, 5.2, 5.3 & 5.4.).

Laser colour	Antibody name and wavelength	**Lineage Panel 1**	**FMO1**	**FMO2**	**FMO3**	**FMO4**	**FMO5**	**FMO6**	**FMO7**	**FMO8**	**FMO9**	**FMO10**	**FM11**	**Compensation**
Ultra violet	Zombie UV	**L/D**	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	CD3
Violet	BV421 (PB) 450/50	**IL-4**		IL-4	IL-4	IL-4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	CD3
Violet	BV525 525/50	**IL-17**	IL-17		IL-17	IL=17	IL-17	IL-17	IL-17	IL-17	IL-17	IL-17	IL-17	CD3
Violet	BV605 610/20	**CD19**	CD19	CD19		CD19	CD19	CD19	CD19	CD19	CD19	CD19	CD19	CD3
Blue	FITC 530/30	**Lin**	Lin	Lin	Lin		Lin	Lin	Lin	Lin	Lin	Lin	Lin	CD3
Blue	PerCP-Cy5.5 695/40	**CD127**	CD127	CD127	CD127	CD127		CD127	CD127	CD127	CD127	CD127	CD127	CD13
Yellow	PE 585/15	**IL-5**	IL-5	IL-5	IL-5	IL-5	IL-5		IL-5	IL-5	IL-5	IL-5	IL-5	CD3
Yellow	PE Texas Red 610/20	**CD45**	CD45	CD45	CD45	CD45	CD45	CD45		CD45	CD45	CD45	CD45	CD3
Yellow	PE Cy7 780/60	**CD3**	CD3	CD3	CD3	CD3	CD3	CD3	CD3		CD3	CD3	CD3	CD3
Red	APC 670/14	**IL-13**	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13		IL-13	IL-13	CD3
Red	Alexa700 730/45	**IFNγ**	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ		IFNγ	CD3
Red	APC Cy7 780/60	**CRTH2**	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2	CRTH2		CRTH2

Table 5.1. Lineage panel 1 for identifying ILCs. One cell dye utilising the ultraviolet laser and used to identify viable cells, three antibodies utilising the violet laser (BV421, BV525 and BV605), two utilising the blue laser (FITC and PerCP-Cy 5.5), three utilising the yellow laser (PE, PE Texas red and PE-Cy7) and three utilising the red laser (APC, Alexafluor 700 and APC-CY7). The cell surface markers and intracellular cytokines were chosen based on current published literature on cell markers of ILCs at the time. A series of fluorescence minus 1 (FMO) were developed to use as measures when identifying the absence or presence of the markers on the cells. The compensations used for each antibody are listed on the right of the table.

Laser colour	Antibody name and wavelength	**Lineage Panel 2**	**FMO1**	**FMO2**	**FMO3**	**Compensation**
Ultra violet	Zombie UV	**L/D**	L/D	L/D	L/D	CD3
Violet	BV421 (PB) 450/50	**IL-4**	IL-4	IL-4	IL-4	CD3
Violet	BV525 525/50	**IL-17**	IL-17	IL-17	IL-17	CD3
Violet	BV605 610/20	**CD19**	CD19	CD19	CD19	CD3
Violet	BV710	**CD127**		CD127	CD127	CD3
Blue	FITC 530/30	**Lin**	Lin	Lin	Lin	CD3
Blue	PerCP-Cy5.5 695/40	**CD3**	CD3		CD3	CD3
Yellow	PE 585/15	**IL-5**	IL-5	IL-5	IL-5	CD3
Yellow	PE Texas Red 610/20	**CD45**	CD45	CD45	CD45	CD3
Yellow	PE Cy7 780/60	**Gata3**	CD3	CD3		CD3
Red	APC 670/14	**IL-13**	IL-13	IL-13	IL-13	CD3
Red	Alexa700 730/45	**IFNγ**	IFNγ	IFNγ	IFNγ	CD3
Red	APC Cy7 780/60	**CRTH2**	CRTH2	CRTH2	CRTH2	CD3

Table 5. 2. Lineage panel 2 for identifying ILC group 2 cells. One cell dye utilising the ultraviolet laser and used to identify viable cells, three antibodies utilising the violet laser (BV421, BV525 and BV605), two utilising the blue laser (FITC and PerCP-Cy 5.5), three utilising the yellow laser (PE, PE Texas red and PE-Cy7) and three utilising the red laser (APC, Alexafluor 700 and APC-CY7). The cell surface markers, intracellular cytokines and intranuclear transcription factors were chosen based on current published literature on cell markers of ILC group 2 cells at the time. A series of fluorescence minus 1 (FMO) where developed to use as measures when identifying the absence or presence of the markers on the cells. The compensations used for each antibody are listed on the right of the table.

Laser colour	Antibody name and wavelength	**T-cell Panel 1**	**FMO1**	**FMO2**	**FMO3**	**FMO4**	**FMO5**	**FMO6**	**FMO7**	**FMO8**	**FMO9**	**FMO10**	**FM11**	**Compensation**
Ultra violet	Zombie UV	**L/D**	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	L/D	CD3
Violet	BV421 (PB) 450/50	**IL-4**		IL-4	IL-4	IL-4	IL4	IL4	IL4	IL4	IL4	IL4	IL4	CD3
Violet	BV525 525/50	**IL-17**	IL-17		IL-17	IL=17	IL-17	IL-17	IL-17	IL-17	IL-17	IL-17	IL-17	CD3
Violet	BV605 610/20	**CD19**	CD19	CD19		CD19	CD19	CD19	CD19	CD19	CD19	CD19	CD19	CD3
Blue	FITC 530/30	**CD56**	Lin	Lin	Lin		Lin	Lin	Lin	Lin	Lin	Lin	Lin	CD3
Blue	PerCP-Cy5.5 695/40	**CD4**	CD127	CD127	CD127	CD127		CD127	CD127	CD127	CD127	CD127	CD127	CD13
Yellow	PE 585/15	**IL-5**	IL-5	IL-5	IL-5	IL-5	IL-5		IL-5	IL-5	IL-5	IL-5	IL-5	CD3
Yellow	PE Texas Red 610/20	**CD8**	CD45	CD45	CD45	CD45	CD45	CD45		CD45	CD45	CD45	CD45	CD3
Yellow	PE Cy7 780/60	**CD3**	CD3	CD3	CD3	CD3	CD3	CD3	CD3		CD3	CD3	CD3	CD3
Red	APC 670/14	**IL-13**	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13	IL-13		IL-13	IL-13	CD3
Red	Alexa700 730/45	**IFNγ**	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ		IFNγ	CD3
Red	APC Cy7 780/60													

Table 5.3. Tcell panel 1 for identifying T helper cells. One cell dye utilising the ultraviolet laser and used to identify viable cells, three antibodies utilising the violet laser (BV421, BV525 and BV605), two utilising the blue laser (FITC and PerCP-Cy 5.5), three utilising the yellow laser (PE, PE Texas red and PE-Cy7) and two utilising the red laser (APC and Alexafluor 700). The cell surface markers and intracellular cytokines were chosen based on current published literature on cell markers of T helper cells. A series of fluorescence minus 1 (FMO) where developed to use as measures when identifying the absence or presence of the markers on the cells. The compensations used for each antibody are listed on the right of the table.

Laser colour	Antibody name and wavelength	**T-cell Panel 2**	**FMO1**	**FMO2**	**Compensation**
Ultra violet	Zombie UV	**L/D**	L/D	L/D	CD3
Violet	BV421 (PB) 450/50				
Violet	BV525 525/50				
Violet	BV605 610/20	**CD19**	CD19	CD19	CD3
Blue	FITC 530/30	**CD56**	Lin	Lin	CD3
Blue	PerCP-Cy5.5 695/40	**CD4**	CD127	CD127	CD13
Yellow	PE 585/15	**Foxp3**		Foxp3	CD3
Yellow	PE Texas Red 610/20	**CD8**	CD45	CD45	CD3
Yellow	PE Cy7 780/60	**CD3**	CD3	CD3	CD3
Red	APC 670/14				
Red	Alexa700 730/45				
Red	APC Cy7 780/60	**CD25**	CD25		CD3

Table 5.4. Tcell panel 2 for identifying T regulatory cells. One cell dye utilising the ultraviolet laser and used to identify viable cells, three antibodies utilising the violet laser (BV421, BV525 and BV605), two utilising the blue laser (FITC and PerCP-Cy 5.5), two utilising the yellow laser (PE Texas red and PE-Cy7) and one utilising the red laser (APC-CY7). The cell surface markers, intracellular cytokines and intranuclear transcription factor's were chosen based on current published literature on cell markers of T regulatory cells. A series of fluorescence minus 1 (FMO) where developed to use as measures when identifying the absence or presence of the markers on the cells. The compensations used for each antibody are listed on the right of the table

5.4.1.b. Gating strategies

Once ethical approval had been obtained to recruit and collect human tonsil tissue, flow cytometry was utilised to identify T-cells and ILCs. Initially cells were identified by their extracellular markers (Figure. 5.1).

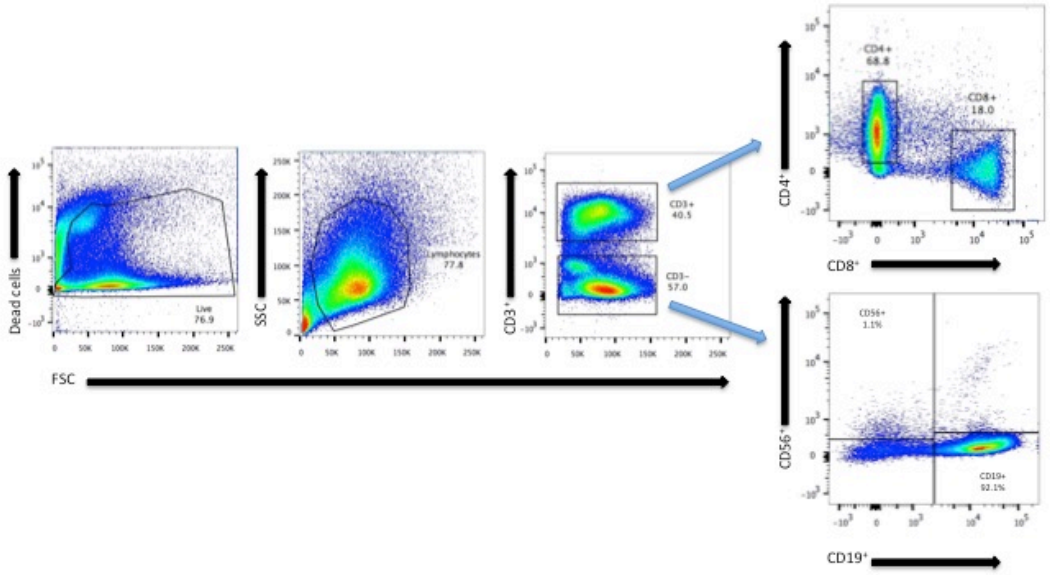


Fig 5.1. Flow cytometry gating strategy for identifying T-cells, NK-cells and B-cells in human tonsil tissue and PBMCs.

Once the process of identifying CD4+ T-cells had successfully been established, the cytokine profile of those cells could be elucidated as well as those possessing the transcription factor Foxp3. By this method, T-cell subsets could be identified (Table5.5. & Figure 5.2.).

Type 1 T helper cell (Th1)	$CD4^+IFN\gamma^+$
Type 2 T helper cell (Th2)	$CD4^+IL4^+$
	$CD4^+IL5^+$
	$CD4^+IL13^+$
T helper 17 cells (Th17)	$CD4^+IL17^+$
T-regulatory cell (Treg)	$CD4^+CD25^+Foxp3^+$

Table 5.5. CD4+ T-cell subsets and the definitions used to identify them through flow cytometry.

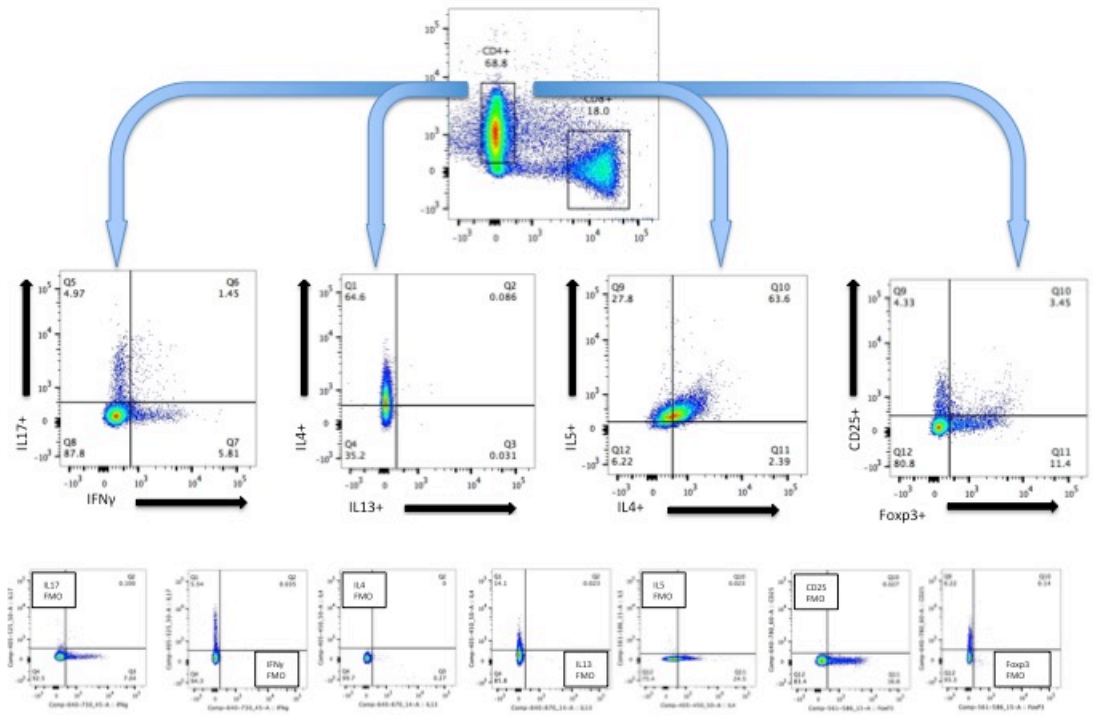


Fig 5.2. Flow cytometry strategy for identifying sub-types of T-cells: Th1, Th2, Th17 and T-reg cells along with the FMOs used to delineate the location of the population of the population of interest.

Developing a gating strategy for ILCs was more challenging as they are a rare population and the limited work published at the time on their identification. The first step entailed identifying cells that lacked known lineage markers. For this, I utilised a commercially available lineage cocktail containing a combination of known cell markers, referred to as 'Lin' on the flow cytometry templates (Table 5.1 & 5.2.). This antibody cocktail contained antibodies for CD2 (T-cells), CD3 (T-cells), CD14 (Macrophages, neutrophils), CD16 (NK-cells, neutrophils, macrophages, monocytes), CD19 (B-cells), CD56 (NK-cells), and CD235a(erythrocytes) (Figure 5.3.).

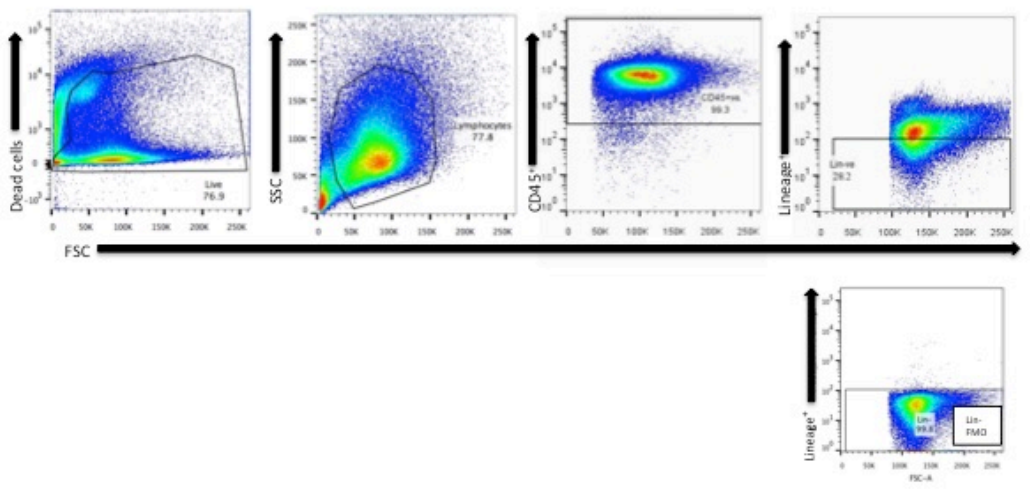


Figure 5.3. Flow cytometry gating strategy to identify lineage negative cells and the FMO for lineage negative cells.

Unfortunately the lineage cocktail was not sufficient to identify the lineage negative cells effectively in tonsil cells. After several attempts to perfect the technique, I discovered that by adding additional CD3 and CD19 antibodies into the staining protocol, we were able to effectively gate out the T cells and B cells before applying the lineage cocktail to the CD3 and CD19 negative cells to gate more accurately around lineage negative cells (Figure 5.4.).

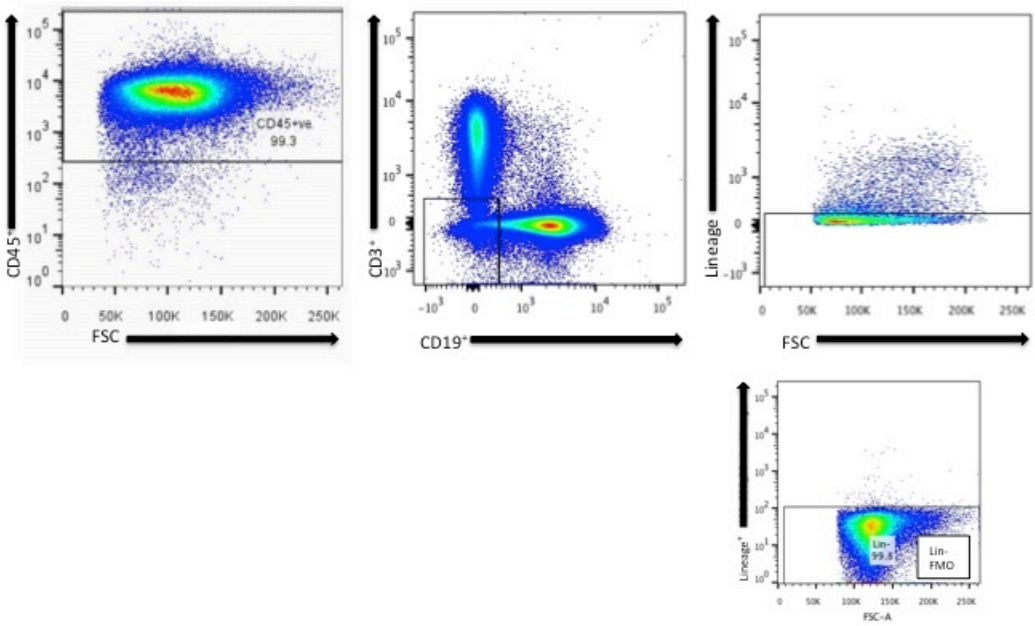


Figure 5.4. Improved antibody staining and gating resulting in a more accurate lineage negative cell population defined as a population more easily identifiable using the FMO data for location. The FMO for lineage negative cells displayed again.

With this improved antibody staining and gating strategy, more accurate cytokine producing lineage negative cells and those with transcription factors could be quantified in the samples (Figure 5.5, 5.6, & 5.7.). Table 5.6 demonstrates the combinations of cell surface markers, cytokines and transcription factors used to define cells as belonging to the three groups of ILCs (ILC1, ILC2 and ILC3) based on published literature at the time.

Total Lineage negative cells	Lin^-
Innate lymphoid cell group 1	$Lin^-IFN\gamma^+$
	$Lin^-CD127^+IFN\gamma^+$
Innate lymphoid cell group 2	Lin^-IL4^+
	$Lin^-CD127^+IL4^+$
	Lin^-IL5^+
	$Lin^-CD127^+IL15^+$
	Lin^-IL13^+
	$Lin^-CD127^+IL13^+$
	Lin^-CRTH2^+
	Lin^-Gata3^+
Innate lymphoid cell group 3	Lin^-IL17^+
	$Lin^-CD127^+IL17^+$

Table 5.6. Lineage negative ILC groups and the extracellular and cytokine markers used to identify them.

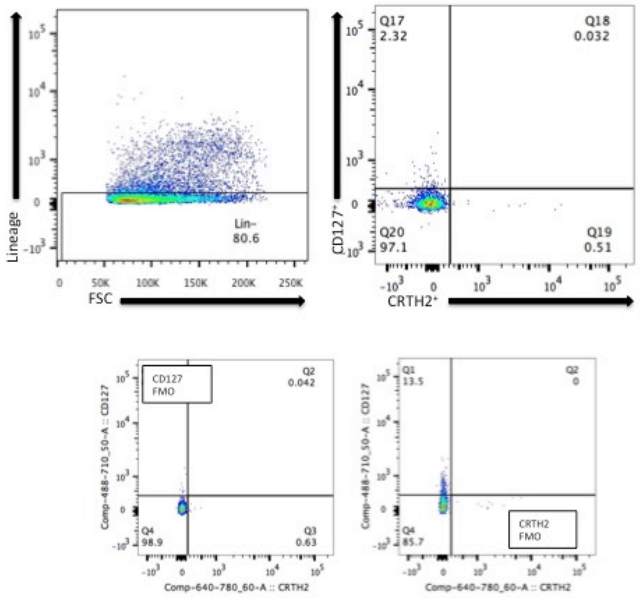


Figure 5.5. Lineage negative cells that display the extracellular marker CD127+ and the ILC2 marker CRTH2+ along with the FMO for CD127 and CRTH2.

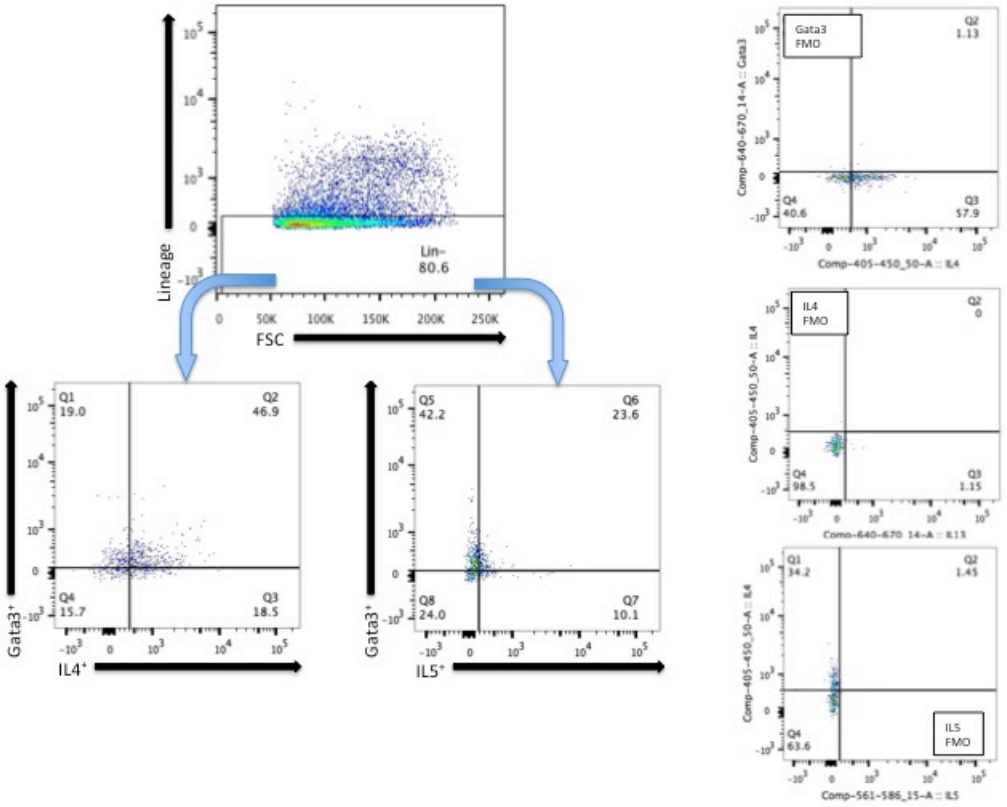


Figure 5.6. Lineage negative cells that stain positive for the transcription factor Gata3 and secrete IL-4 and IL-5 along with the FMOs for Gata3, IL4 and IL5. All lineage negative cells were gated comparing Gata3+ positive cells against IL-4+ positive cells then Gata3+ and IL-5+.

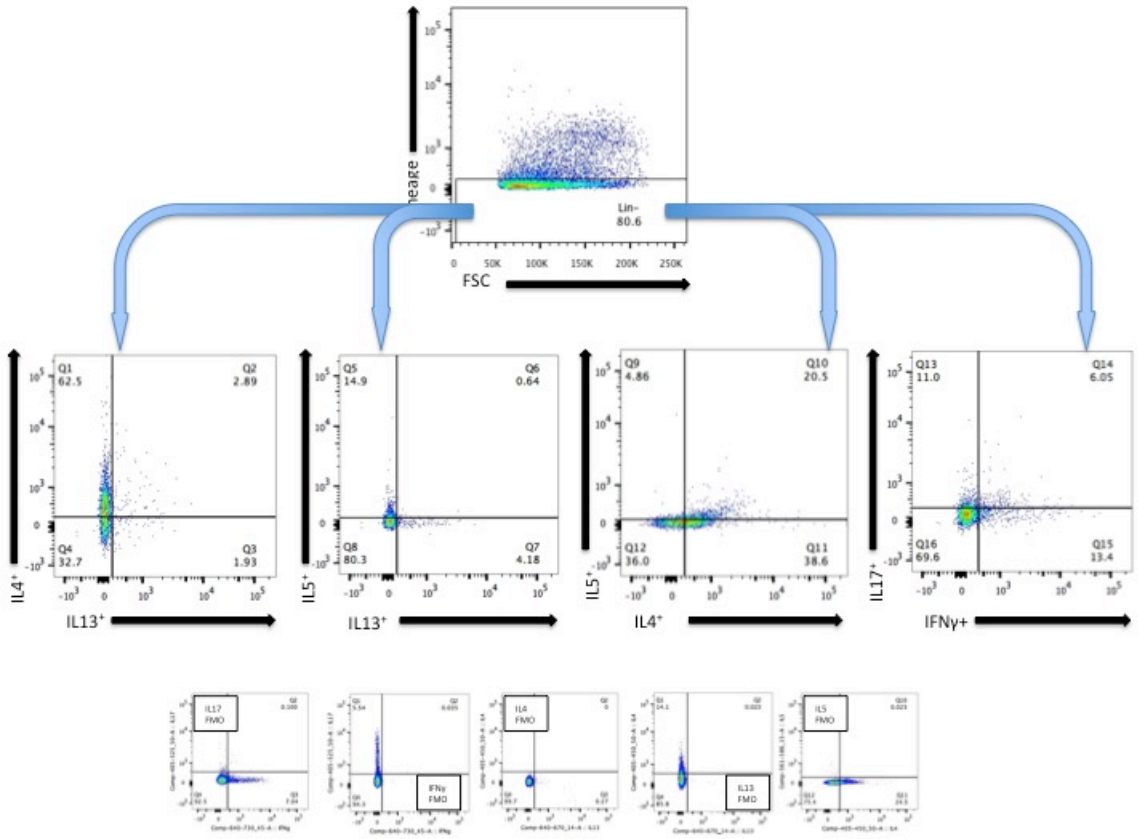


Figure 5.7. Lineage negative cells produce IL-4, IL-13, IL-5, IL-17 and IFNγ when stimulated in preparation for FACS analysis and the FMOs for each of the cytokines. All lineage cells were gated using the combinations listed above.

5.4.2. Subjects.

Tonsil tissue was obtained from all 45 children recruited for assessment of atopic status (Chapter 4). These were children with sleep study proven OSA (n=24) and 21 children undergoing tonsillectomy for recurrent tonsillitis (over half of which had a normal sleep study ruling out disease as covered in the previous chapter) (Figure 5.8.).

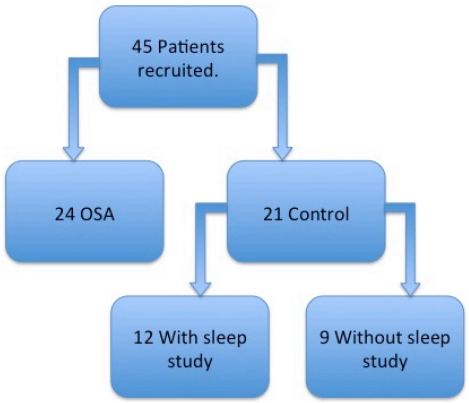


Figure 5.8. Recruitment diagram demonstrating group numbers when analysing for differences based on OSA diagnosis in the tonsil cell samples.

The T-cell panel was developed and refined alongside subject recruitment. To this end, initial samples were not analysed for cytokine profile until confident extracellular marker identification had been optimised. Following findings of low numbers of IL-13 producing $CD4^+$ T-cells, IL-4 was added and then IL-5 to improve detection of Th2 cells. Once refined, the transcription factor Foxp3 was also added to identify Tregs (Figure 5.9.).

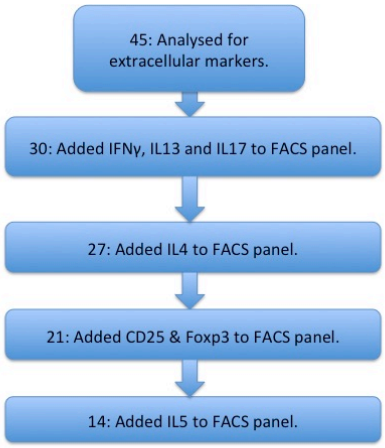


Figure 5.9. Flow diagram showing potential number of tonsil samples analysed for T helper cells at each stage of the FACS panel development.

The ILC FACS panel required several adjustments and alterations to develop a reliable lineage negative population from tonsil tissue. As a result, it was not possible to obtain cytokine data from the first 10 samples. As with the T-cell panel, other markers were added

as recruitment progressed prompted by changes and developments in the literature (Figure 5.1.2.).

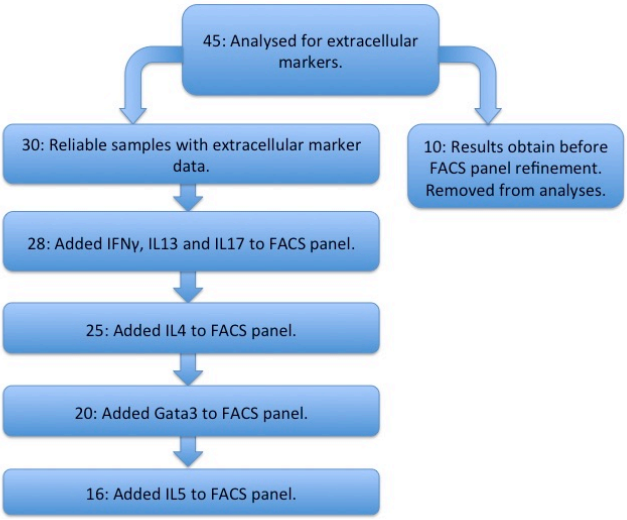


Fig 5.1.2. Flow diagram showing potential number of tonsil samples analysed for ILCs at each stage of the FACS panel development.

For the peripheral blood samples, priority was given to the specific IgE tests required to identify atopic and non-atopic subjects. When sufficient blood was available after these tests were carried out, these samples were processed in the same way as the tonsil tissue to identify T cells and ILCs. Unfortunately this resulted in fewer PBMC samples available to analyse (Fig 5.1.3.).

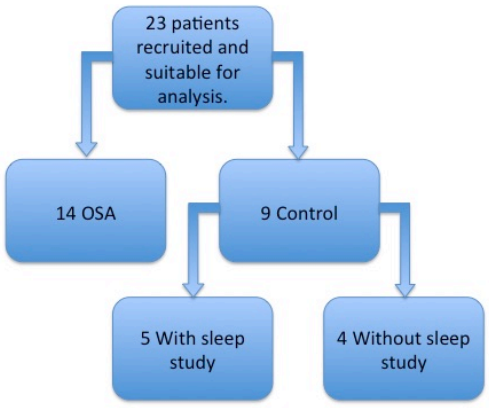


Figure 5.1.3. Recruitment diagram demonstrating group numbers for analysis of cells from peripheral blood when analysing for differences based on OSA diagnosis.

Intracellular cytokine and transcription factor analysis of PBMC samples changed as the FACS panel was being developed (Figure 5.1.4& 5.1.5.).

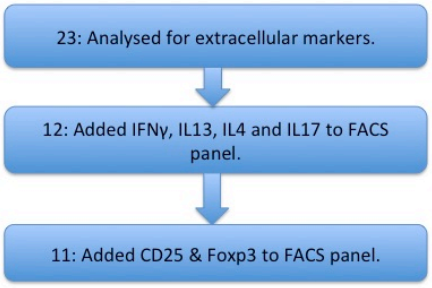


Figure 5.1.4. Flow diagram showing potential number of peripheral blood samples analysed for T helper cells at each stage of the FACS panel development.

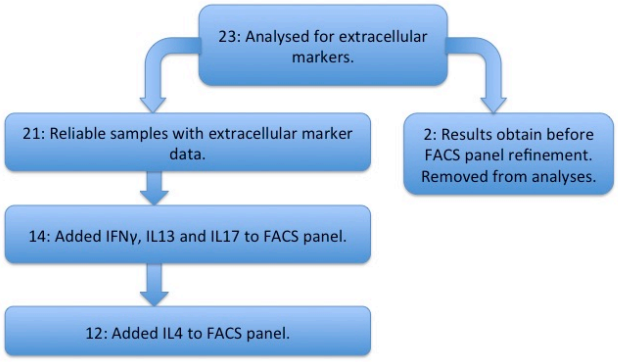


Figure 5.1.5. Flow diagram showing potential number of peripheral blood samples analysed for ILCs at each stage of the FACS panel development.

5.5. Results

5.5.1 Total Cells

The median total leukocyte cell count obtained from a pair of tonsils was $1.8x10^9$ cells (interquartile range $1.1x10^9$ to $2.6x10^9$) which correlates with published data [254]. The predominant cell-type in tonsils was B-cells and T-cells. Very few granulocytes were present and not formally counted. ILCs were a small proportion of the cells identified (Figure 5.1.6.). In peripheral blood, the predominant cell type was T-cells with significantly smaller proportions of ILCs (Figure 5.1.7).

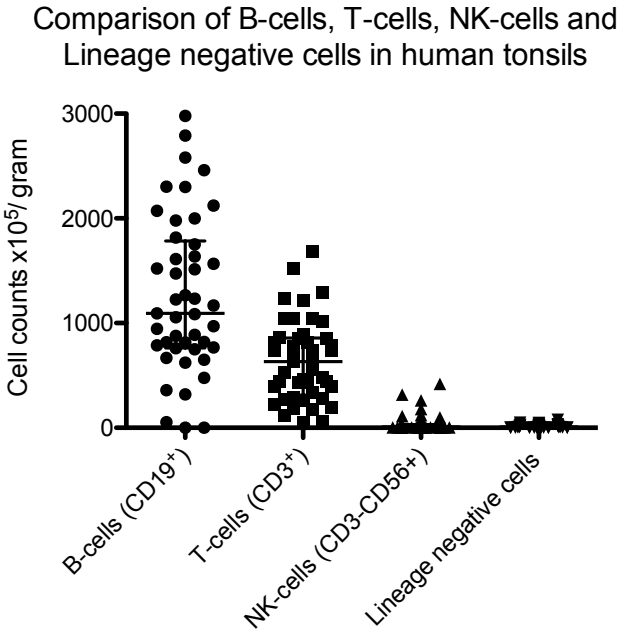


Figure 5.1.6. Differential cell counts per gram of tonsil tissue for B-cells, T-cells, NK-cells and lineage negative cells. Median values with inter-quartile ranges.

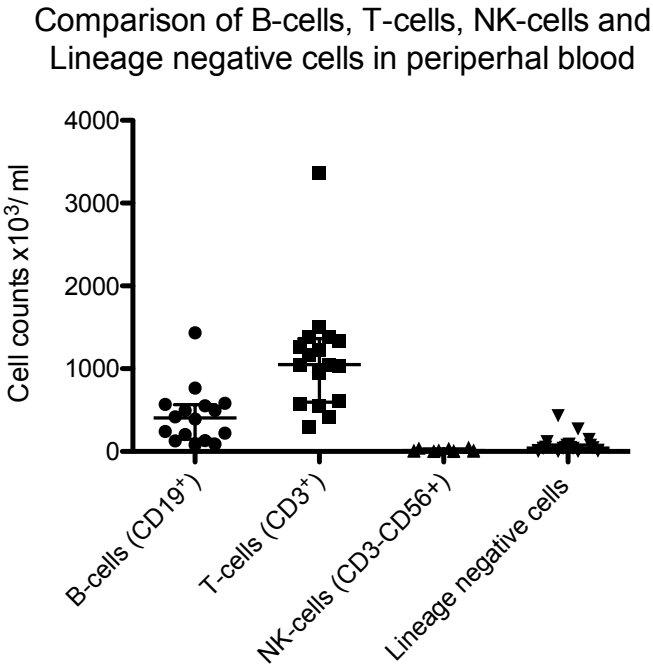


Figure 5.1.7. Differential cell counts per ml of blood for B-cells, T-cells, NK-cells and lineage negative cells. Median values with inter-quartile ranges.

When comparing the total cell counts obtained from a pair of pharyngeal tonsils from children with OSA to control subjects, no difference was observed (Figure 5.1.8.).

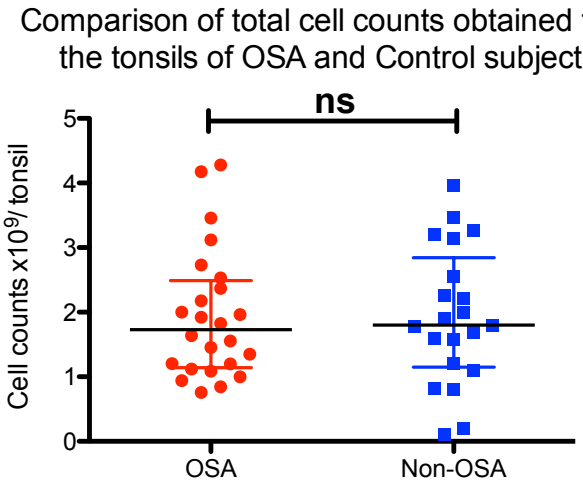


Figure 5.1.8. Similar total cell counts obtained from a pair of pharyngeal tonsils comparing OSA subjects to non-OSA. Median cell counts displayed with interquartile range. Significant calculated using Mann-Whitney U test.

5.5.2 T-cells in tonsils and peripheral blood from children with OSA compared to controls

5.5.2.a Tonsil T-cell tissue results: OSA vs Non-OSA subjects.

Tonsils were first analysed by grouping the subjects into OSA and Non-OSA. For the control subjects, although just under half of the recruits did not have sleep study confirmation of absent OSA, no difference was observed when those with and without sleep studies were compared. Therefore all controls were grouped together. There were no differences in numbers or proportions of T-cells, B-cells or NK-cells in tonsils from children with and without OSA (Figure 5.1.9. & 5.2.1.).

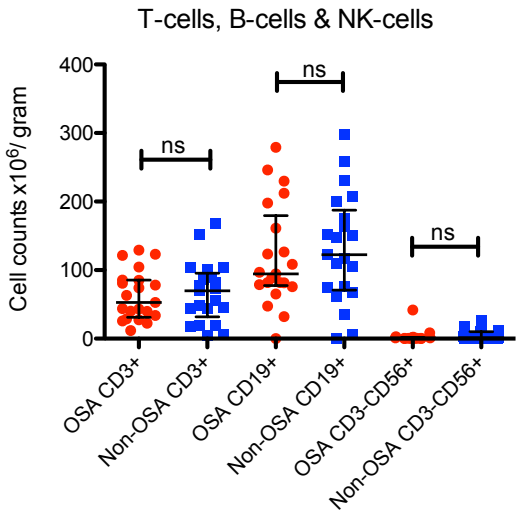


Figure 5.1.9. T-cells, B-cells and NK cell counts per gram of tonsil tissue comparing OSA subjects to Non-OSA subjects. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann-Whitney U test.

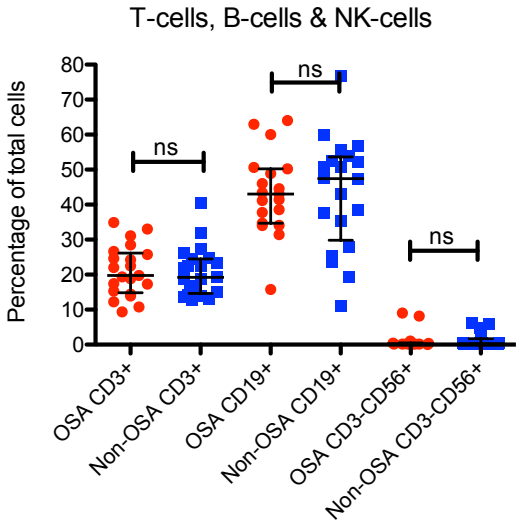


Figure 5.2.1. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing OSA subjects to Non-OSA subjects. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann-Whitney U test.

T-cell sub-sets were analysed based on the hypothesis that OSA tonsils will demonstrate a Th2 phenotype. There were no differences in $CD4^+$ cells in tonsils from children with and without OSA whether assessed as cell counts per gram of tissue (Figure 5.2.2.) or percentage of total T-cells ($CD3^+$) (Figure 5.2.3.).

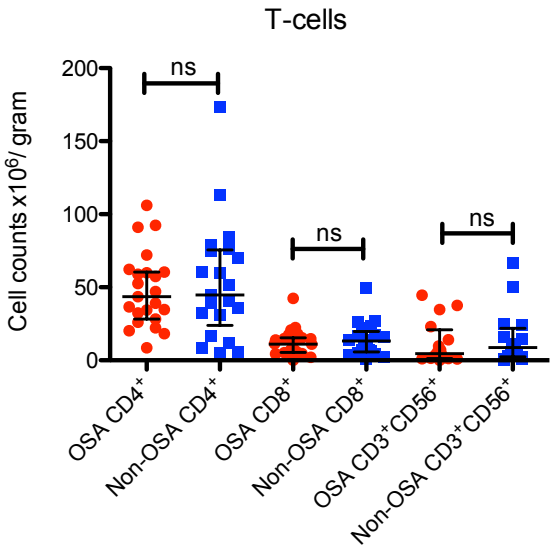


Figure 5.2.2. T-cell counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann-Whitney U test.

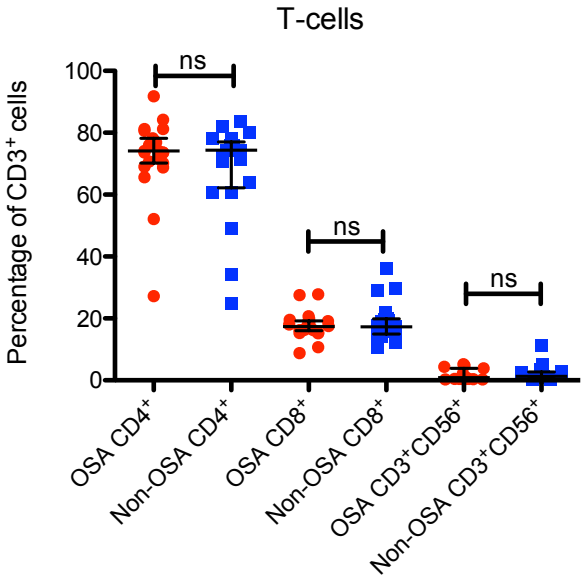


Figure 5.2.3. T-cells as a percentage of total cell counts comparing OSA subjects to Non-OSA subjects. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann-Whitney U test.

Consistent with previously published findings of a reduction in Treg numbers in peripheral blood and adenoidal tissue, the Tregs ($CD4^+CD25^+FoxP3^+$) were found to be significantly reduced in OSA subjects when analysed as total cells per gram of tissue (median cell count

of 5.7x10^5 cells/ gram of tissue (IQR 3.5x10^5-9.7x10^5) in OSA subjects vs 19.7x10^{5}cells / gram of tissue (IQR 11.5x10^5-29.2x10^5) in controls, p=0.002) and as a percentage of the total CD4$^+$ cells compared to non OSA subjects (median 1.3% (IQR 1%-1.3%) in OSA subjects vs 3.2% (IQR 2.3%-4.4%) in controls, p=0.002) (Figure 5.2.4).

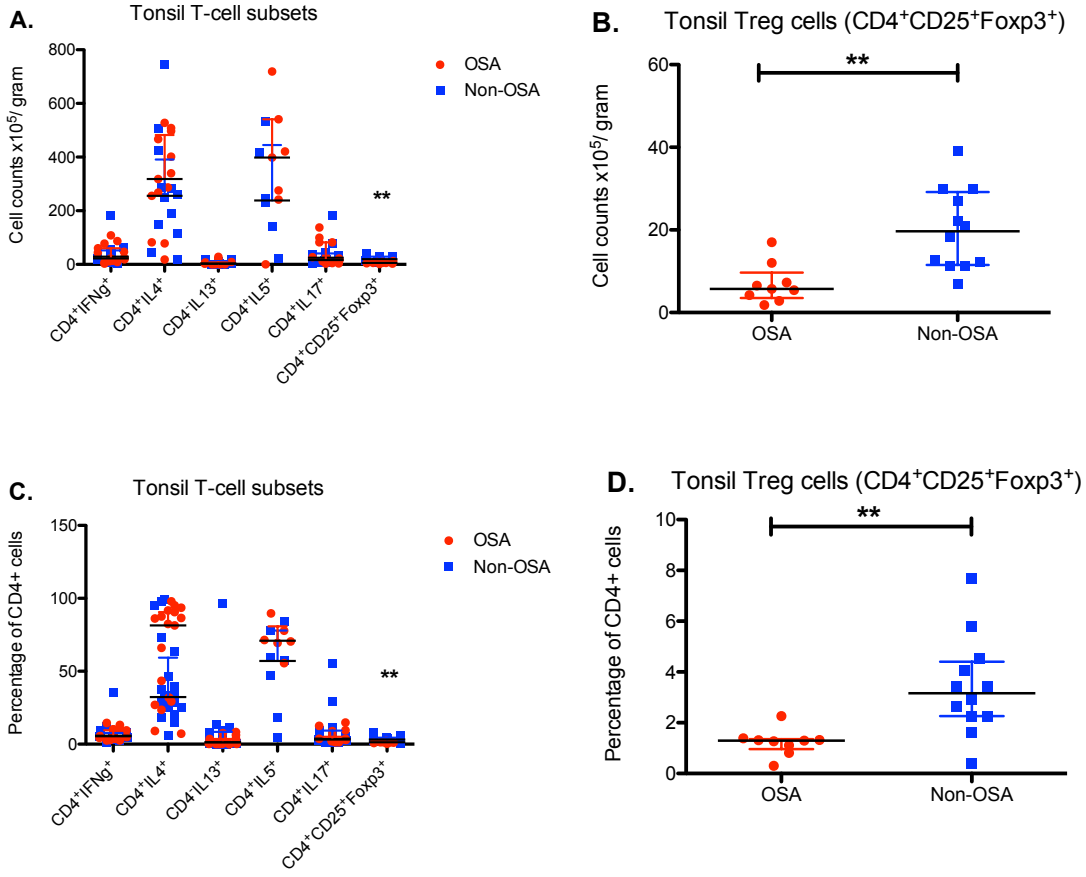


Figure 5.2.4. A comparison of Tonsil Th cell subsets defined by cytokine production and Tregs as cells per gram of tissue (A.) and as percentage of total Th cells (CD4+) (C.) between subjects with OSA and control subjects without. The tonsil Tregs (CD4+CD25+Foxp3+) are lower as both cells per gram of tissue (B.) and percentage of total Th cells (D.) in subjects with OSA compared to controls. All values demonstrated are medians with interquartile ranges. Significance using the Mann-Whitney U test. ** represents significance equal to 0.002.

5.5.2.b Peripheral blood T-cell results- OSA vs Non-OSA subjects.

No differences were observed between OSA and non-OSA children in numbers of T-cells, B-cells or NK-cells (CD3$^+$CD56$^+$) in PBMCs as cells per ml of blood (Figure 5.2.5.) or as a percentage of the total cells (Figure 5.2.6.).

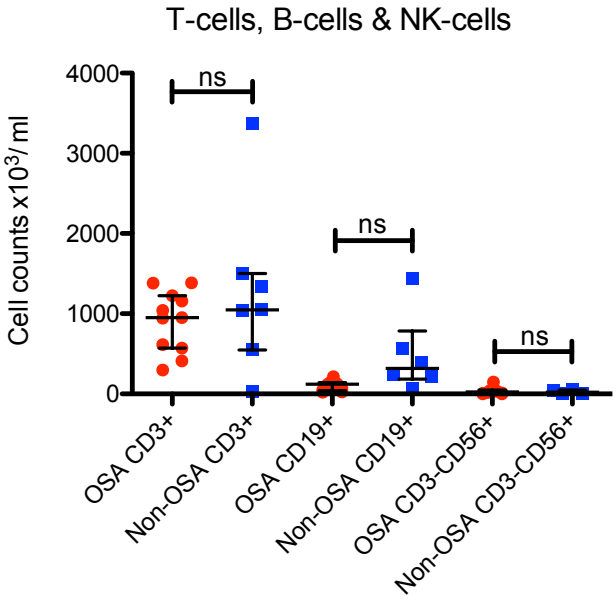


Figure 5.2.5. T-cells, B-cells and NK cell counts per ml of blood comparing OSA subjects to Non-OSA subjects. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann-Whitney U test.

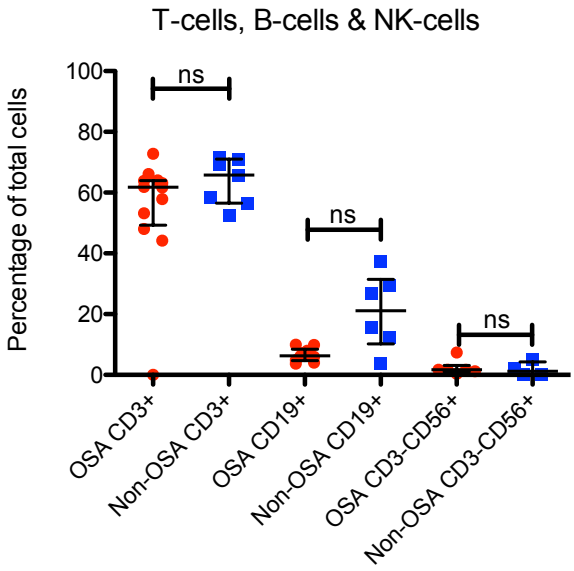


Figure 5.2.6. T-cells, B-cells and NK cell median percentages of total cells comparing OSA subjects to Non-OSA subjects. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann-Whitney U test.

As with the tonsil tissue, the same results were found when focusing on the T-cells alone with no difference observed when comparing the cell counts per ml of blood (Figure 5.2.7.)

or percentages of total T-cells (CD3+) (Figure 5.2.8.) for subjects with OSA compared to Non-OSA.

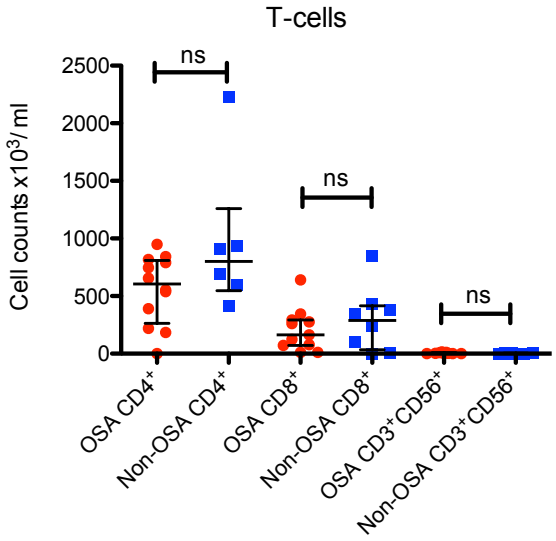


Figure 5.2.7. T-cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann-Whitney U test.

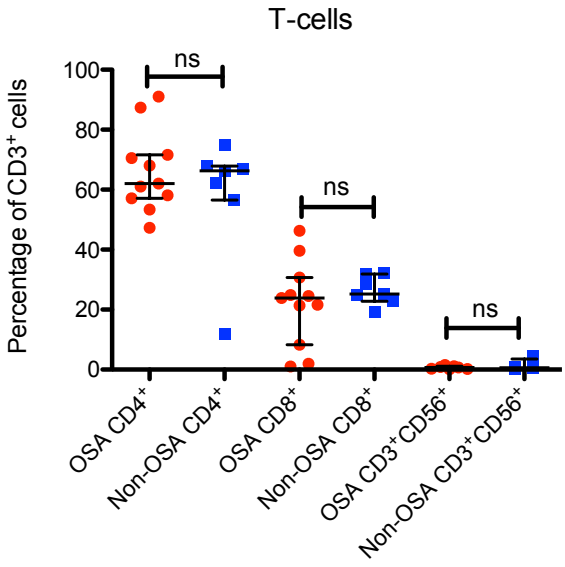


Figure 5.2.8. T-cells median percentages of total T-cells ($CD3^+$) comparing OSA subjects to Non-OSA subjects. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann-Whitney U test.

In a similar fashion to tonsil tissue, Tregs ($CD4^+CD25^+Foxp3^+$) were significantly lower in OSA compared to control subjects as cells counts per ml of blood collected (median cell count $1x10^3$ cell/ ml of blood (IQR $0.2x10^3$-$0.6x10^3$) in OSA subjects vs $6.3x10^3$ (IQR $2.5x10^3$-$13.7x10^3$) in controls, p=0.004) but not as percentage of total $CD4^+$ cells (Figure 5.2.9.).

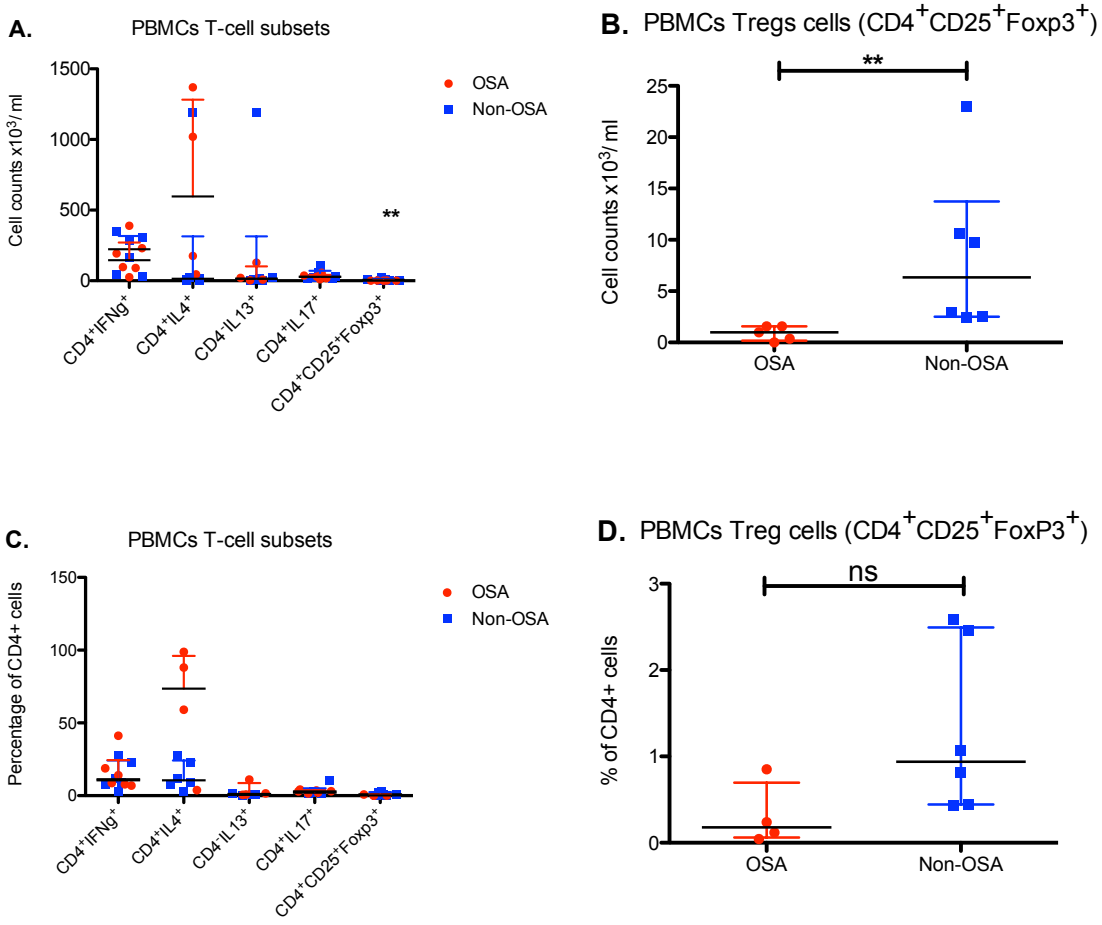


Figure 5.2.9. A comparison of peripheral blood Th cell subsets defined by cytokine production and Tregs as cells per ml of blood (A.) and as percentage of total Th cells (CD4+) (C.) between subjects with OSA and control subjects without. The peripheral Tregs (CD4+CD25+Foxp3+) are lower as cells per ml of blood (B.) but not statistically as percentage of total Th cells (D.) in subjects with OSA compared to controls. All values demonstrated are medians with interquartile ranges. Significance using the Mann-Whitney U test. ** represents significance equal to 0.002. ns represents no statistical significance.

5.5.3. ILCs in tonsils and peripheral blood from children with OSA compared to controls

5.5.3.a. ILCs in tonsil tissue- OSA vs Non-OSA.

The subjects were analysed comparing those with OSA to those without. In the first instance comparisons were made based on the presence of the extracellular marker CD127 and the ILC2 marker CRTH2. No differences were observed in numbers of ILCs in tonsils from OSA and non-OSA subjects (Figure 5.3.1.).

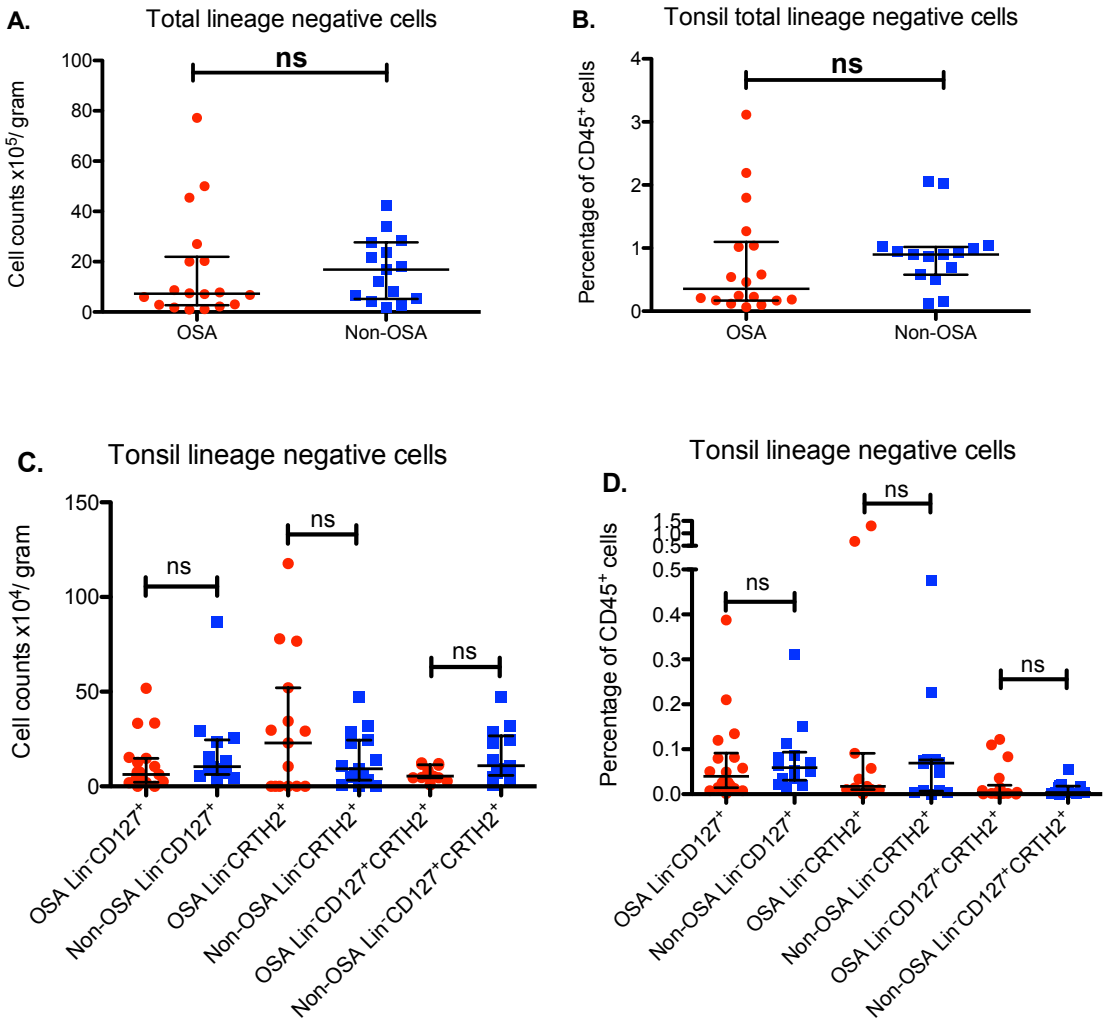


Figure 5.3.1. A comparison of total tonsil lineage negative cells as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) (B.) comparing OSA and non-OSA subjects. The same comparison this time with lineage negative cells with published extracellular marks for ILCs (CD127+) and ILC2s (CRTH2+) as cells per gram of tissue (C.) and percentage

of total lymphoid cells (D.) All data displayed as median values and interquartile ranges. Significance calculated using Mann-Whitney U test.

For the next step in analysis, cytokine expressing lineage negative cells were assessed. These included the type 1 cytokine IFN-γ, type 2 cytokines IL4, IL5 and IL13 and the ILC3 cytokine IL17. Numbers of ILCs were not different as cell counts per gram of tissue or as percentage of total CD45⁺ cells between OSA and non-OSA subjects (Figure 5.3.2.).

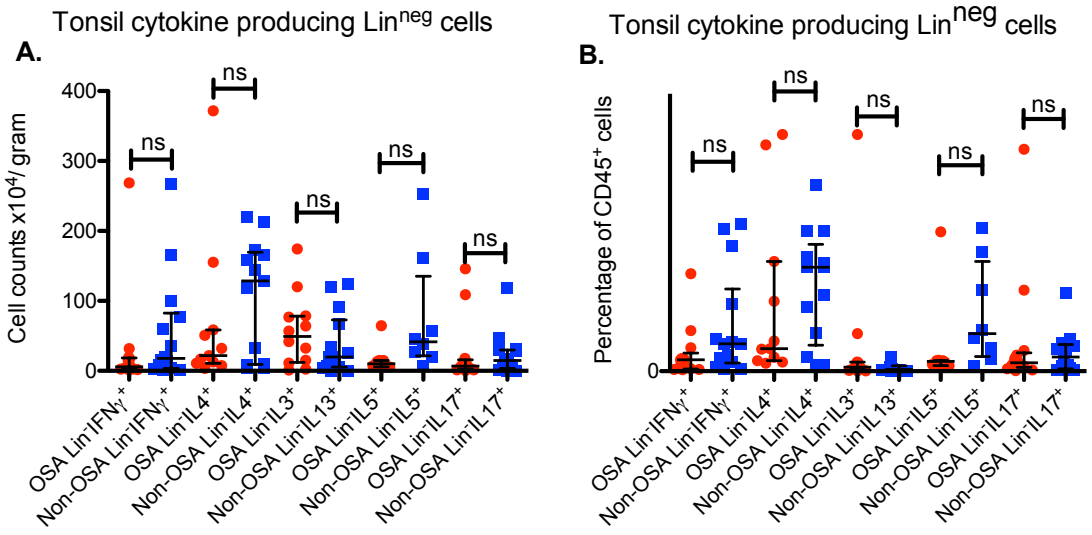


Figure 5.3.2. A. Tonsil cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. B. Cytokine producing lineage negative cells as percentage of lymphoid cells (CD45+) comparing OSA subjects to Non-OSA subjects. All data displayed as median values with interquartile ranges. Significance calculated using Mann-Whitney U test.

With no differences observed in the previous comparisons, Lin- cells that expressed CD127 and intra-cellular cytokines were quantified. This significantly reduced the number of cells both as cell counts per gram of tissue and as a percentage of the CD45⁺. The population of Lin⁻CD127⁺IL13⁺ cells was too small to reliably analyse and was excluded. However, no differences were seen for any cell type between OSA and non-OSA (Figure 5.3.3.).

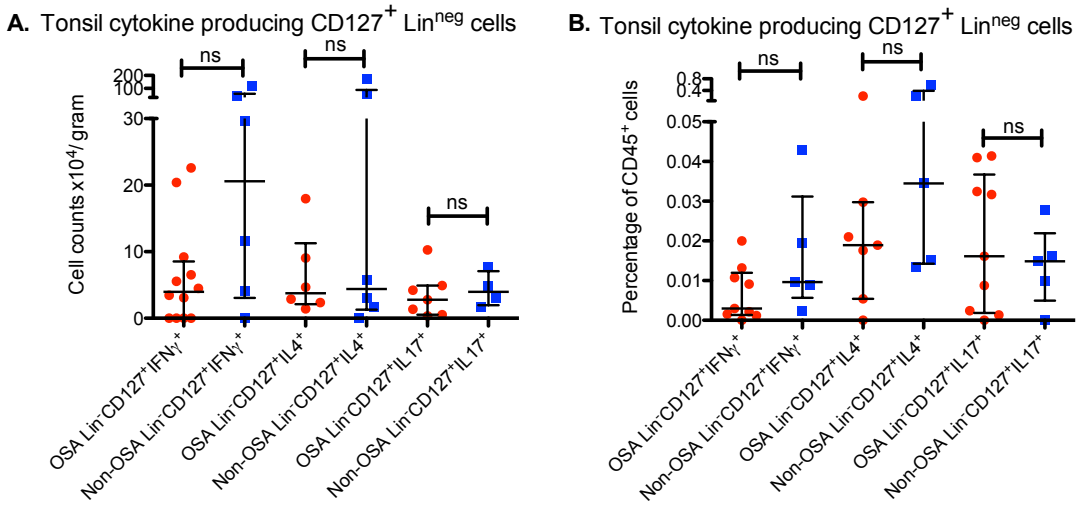


Figure 5.3.3. A. CD127⁺ cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. B. CD127⁺ cytokine producing lineage negative cells as percentages of CD45⁺ cells comparing OSA subjects to Non-OSA subjects. All data displayed as median values and interquartile ranges. Significance calculated using Mann-Whitney u test.

Finally, I focused specifically on the group 2 ILCs utilizing the transcription factor Gata3. This followed the hypothesis that the samples from individuals with OSA have an ILC2 phenotype in keeping with the association between type 2 cytokines (in T-cells). Once again, no difference was observed as cells per gram of tissue or as a percentage of total CD45+ cells (Figure 5.3.4.).

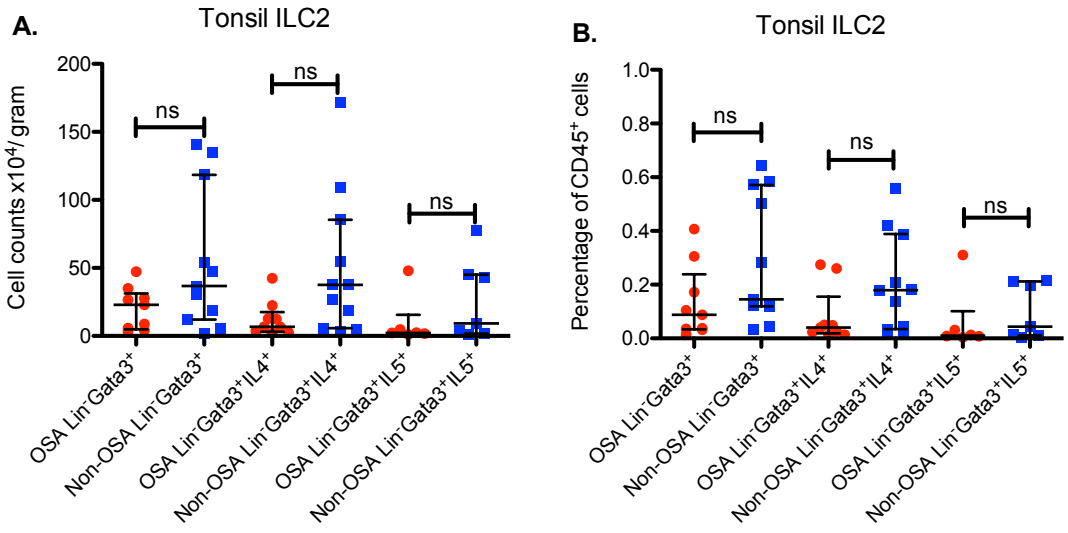


Figure 5.3.4. A. Gata3⁺ lineage negative cells and cytokine producing Gata3⁺ lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. B. Gata3⁺ lineage negative cells and cytokine producing Gata3⁺ lineage negative cells as percentage of CD45⁺ cells comparing OSA subjects to Non-OSA subjects. All data displayed as Median values with interquartile ranges. Significance calculated using Mann-Whitney u test.

5.5.3.b. ILCs in peripheral blood- OSA vs Non-OSA

The presence of ILCs in PBMCs was analysed using the same regime as for tonsils. The first comparisons were therefore focused on the presence of the extracellular marker CD127 and the ILC2 marker CRTH2. No difference was observed when analysing the samples comparing OSA and non-OSA subjects (Figure 5.3.5.).

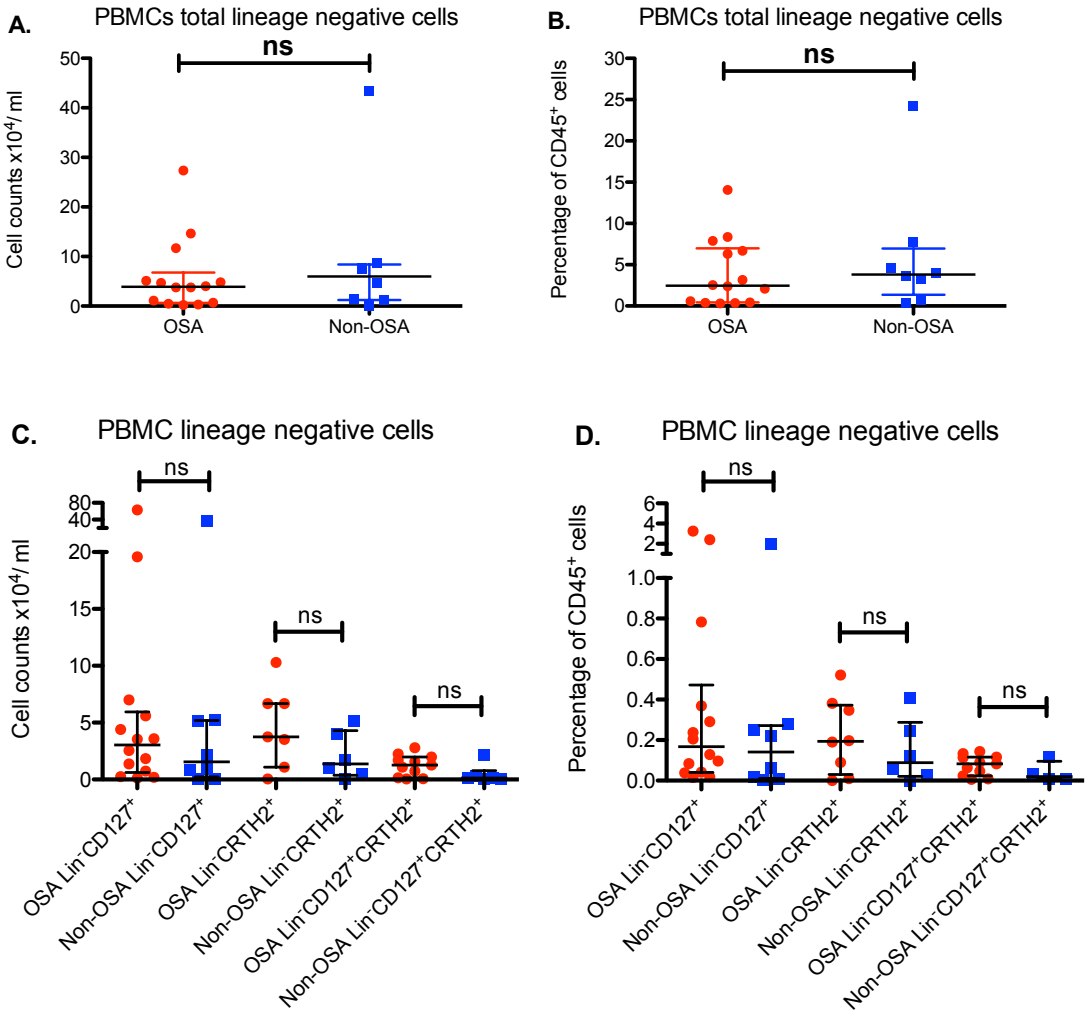


Figure 5.3.5. A comparison of total peripheral blood lineage negative cells as cells per ml of blood (A.) and as percentage of total lymphoid cells (CD45+) (B.) comparing OSA and non-OSA subjects. The same comparison this time with lineage negative cells with published extracellular marks for ILCs (CD127+) and ILC2s (CRTH2+) as cells per ml of blood (C.) and percentage of total lymphoid cells (D.) All data displayed as median values and interquartile ranges.

The numbers of cells identified as lineage negative with extra-cellular markers and cytokine producing became too small to analyse. The only analysis possible was of the cytokine producing lineage negative cells. These included the type 1 cytokine IFN-γ, the type 2 cytokines IL4 and IL13 and the type 3 cytokine IL17. No differences were apparent when comparing OSA and non-OSA subjects either as cells per ml of blood or percentage of total CD45+ cells (Figure 5.3.6.).

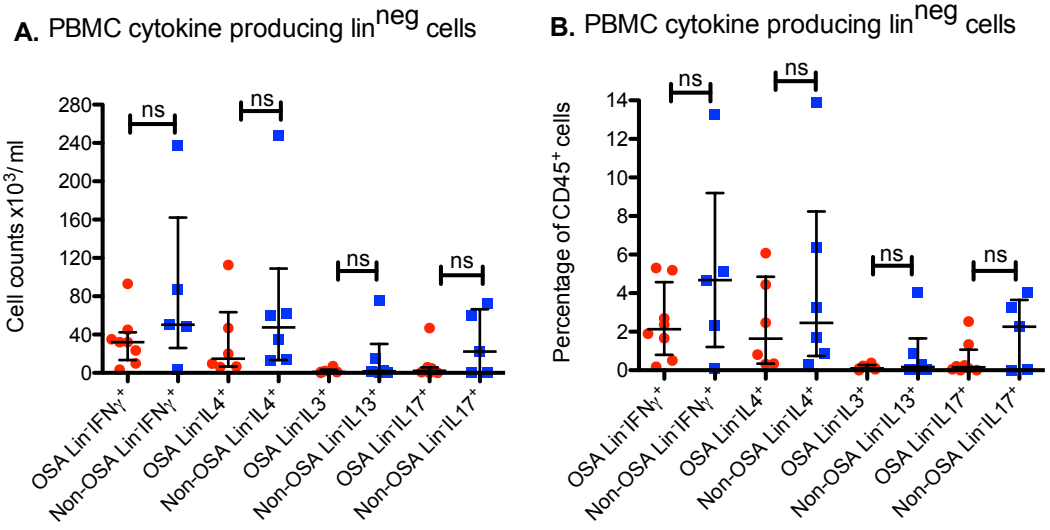


Figure 5.3.6. A. Cytokine producing lineage negative cells as median cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. B. Cytokine producing lineage negative cells as percentage of lymphoid cells (CD45+) comparing OSA subjects to Non-OSA subjects.. All data displayed as median values with interquartile ranges.

5.6 Discussion

5.6.1. Principle findings

With regard to the cellular phenotype of tonsil tissue from children with OSA, there was no significant difference in the proportion of Th17 cells in OSA compared to non-OSA patients. However, Treg cells were significantly reduced in children with OSA compared to controls. This finding was also reflected, in part, in the peripheral blood samples collected. This

suggests a possible underlying pathology for OSA that involves a reduction in anti-inflammatory regulation that may result in a partially uncontrolled proliferation of tissue, and airway compromise resulting in OSA.

In support of the results from chapter 4 where there was no increase in the rates of atopy amongst children with OSA, there was also no significant Th2 cell predominance in the tonsils or peripheral blood of children with OSA. This is further evidence that there is no link between OSA and atopy. As suggested previously, an association between atopy, asthma and sleep disordered breathing may be true owing to the effects the diseases have on the upper airway but there does not appear to be a direct relationship with OSA.

There were no significant differences in ILCs in the tonsils or peripheral blood based on the definitions used in this work, between children with OSA and the control subjects. In my hypothesis, I had suggested that the ILC2 cells were more likely to be raised as these cells have been implicated in asthma and allergy pathology but as it is becoming more evident from my work that a link between asthma, allergy and OSA is unlikely. A relationship with ILC2 cells is also therefore unlikely and is confirmed by the results in this chapter.

5.6.2. Strengths and weaknesses of study

The first criticism of this work is the element of analysis bias. As the flow cytometry technique for these samples was developed and refined alongside recruitment, not all samples underwent the full analysis, specifically cytokine production and transcription factor identification. In retrospect, practice and refinement with tonsil tissue that was ultimately not included in the results would have been better however, time and resources did not allow for this to happen.

The same method for extracting the single cell suspension from the tonsil tissue has been published and followed by authors in the literature and is therefore standard practice. The same can be said for the processing the samples prior to flow cytometry analysis. These methods are the same used throughout the laboratory group. The definitions of the cell types used however is not universally recognized. While the identification of the T helper cells was not new, the ILC group definitions were and are therefore open to criticism. The inclusion of more transcription factors such as Tbet for the ILC1s could add additional strength to the cell identification.

The definition of Tregs used in this work was a cell with the helper T-cell marker CD4, the interleukin 2 receptor alpha (IL2RA) CD25 and the transcription factor fork-head box protein 3 (Foxp3). This was based on previously published data also relating a deficiency in Treg cells with OSA used these markers. These are naturally occurring Tregs that are thymic in origin[264]. What has not been discussed is the adaptive inducible regulatory T-cell capable of producing the anti-inflammatory cytokine IL10 along with other cytokines (to a lesser degree than other Th cells) but not IL4. The $CD4^+CD25^+$ Tregs are recruited and activated early during an immune response to control its magnitude where as these $CD4^+IL\text{-}10^+$ cells are induced by repeated allergen stimulation and may act later to dampen the immune response and restore tolerance [265].

While IL-10 has been investigated and appears reduced in the plasma and tonsils of OSA subjects, cells identified as $CD4^+IL10^+$ using flow cytometry have not. Any future work relating OSA to Treg function or presence needs to take into account all subtypes exploring whether the naturally occurring Tregs are the only population to be depleted. Consideration is also needed to take into account other sources of inhibitory, immune controlling cytokines. Considerable focus has been placed on $CD4^+$ Treg cells however any cell with the capacity to secrete inhibitory cytokines may have "regulatory" potential, including γδ T-cells, B-cells, mast cells, and various APC populations [266]. If T-cell regulation is a component of OSA pathology, other forms of inhibition need to be explored.

As discussed, this reduction in Tregs could be evidence of an underlying predisposition to a pro-inflammatory state in subjects resulting in tissue proliferation, airway luminal reduction and resulting OSA. However, if this were the case, one would expect the tissue mass to be larger in OSA subjects compared to the controls. The results from chapter 4 have already demonstrated no difference in tonsil size both on direct vision, size grading or in weight of the extracted tissue. In this chapter, I now have evidence that the number of cells obtained from the tonsils of these children does not differ either. As discussed in chapter 4, this pathology may be present in the tonsils of the upper airway but more evident in the adenoids which are difficult to assess in this manner as modern extraction techniques do not result in collectable material.

The reliability of the ILC definitions and proportions of identified cells will be discussed in relation to the results in chapter 6.

The results for this chapter were obtained using flow cytometry. This is a recognised method of immune phenotyping. However, my choice of using FMOs to guide gate placement and therefore generate cellular proportions could be criticised. While FMOs are a recognised guide, the use of cells in these control measures raised the possibility of innate fluorescence, as displayed by macrophages and eosinophils. Cells emit similar light wavelengths to a flurophore used as part of my complex flow cytometry panel may provide false data. This could be overcome by using isotype controls instead which would generate a true negative population to base judgement on and should be considered in any future work.

The cellular cytokine profile of the samples did not demonstrate any significant difference between the groups. However, the methods of cellular cytokine production can also be criticised. The use of PMA/Ionomyicn as described in chapter 2 is a recognised method of inducing a cell to produce what it is primed to produce but necessarily what it would or is producing. Therefore the cytokines present when processed may be so artificially and not a reliable example of the tissues cytokine activity.

5.6.3. Findings in relation to other studies

Tregs can be categorised as naturally occurring or adaptive. They exert their immune mediating properties by suppressing the function of Th1, Th2, Th17, and allergen specific IgE. They have also been found to suppress basophils, eosinophils, and mast cells but induce levels of specific IgG4 [267]. It has been suggested that they adopt different mechanisms to suppress immune responses: directly via cell contact and indirectly via reducing the capacity of antigen presentation on antigen presenting cells [268]or via anti-inflammatory cytokines[269, 270]. Tregs are therefore key players in the control of all immune responses including responses to self, tumors, microbes and grafts as well as inflammation [45-47].

An insufficiency or dysfunctional Tregs have been implicated in autoimmune and inflammatory diseases such as X-linked disease, Immunodysregulation, polyendocrinopathy, enteropathy (IPEX) as well as type 1 diabetes, multiple sclerosis, systemic lupus erythematous (SLE), rheumatoid arthritis, IBD and psoriasis [48] (figure 5.3.6.).

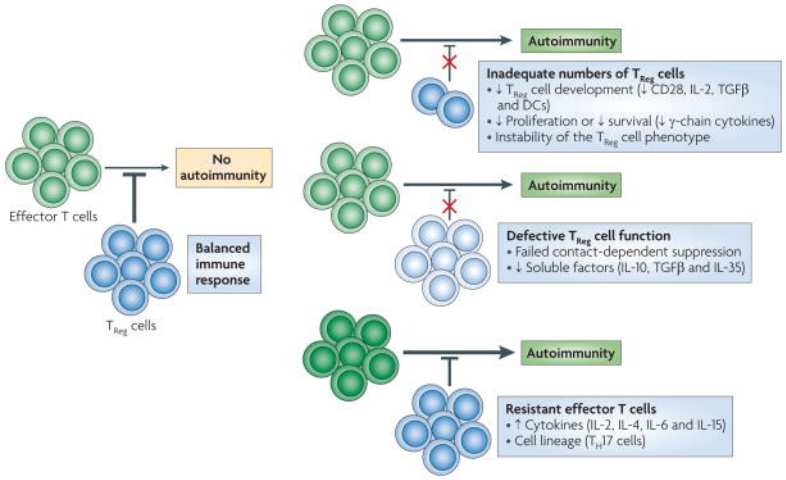


Figure 5.3.6. Immunopathology of Treg depletion, dysfunction or resistance resulting in autoimmunity. depetion from Nature reviews: Immunology, Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases, 2010 [48]

Some studies have suggested that pulmonary $CD4^{+}CD25^{high}$ Tregs are impaired in paediatric asthma[271]. Imbalance in Th17/Tregs, elevated IL-17 and IL-4 response and down-regulation of FOXP3 were found in a group of allergic asthmatic children with symptoms uncontrolled by inhaled corticosteroids [272]. While my results in this and the previous two chapters imply that a relationship between OSA and asthma is unlikely, it is quite plausible that both diseases may in-part be the result of a dysfunction or reduction in Tregs but not linked to each other.

My work is the first identification of a reduction in Tregs in the palatine tonsils of children with OSA compared to a disease control group however a reduction within other body compartments has been identified. Sada et al 2011 [44] found a negative linear correlation for Th17/Treg ratio from children's extracted adenoidal tissue and a patient clinical symptom score for sleep disordered breathing suggesting the patients with higher Th17 and lower Treg cells in their adenoids will be more symptomatic. Ye et al 2012 [62] reported a similar finding in adults newly diagnosed with OSA based on polysomnography. Peripheral blood samples demonstrated a higher proportion of Th17 cells and reduction in Tregs with a higher ratio of both in severe OSA and lower ratio in milder disease.

Ni et al 2015 [61] also looked at peripheral Th17 and Treg cells in children, taking into account adenoidal size and the presence of clinical history of allergic rhinitis. They found a higher Th17/Treg ratio was present in the adenoids and peripheral blood of children with OSA with changes in this ratio being associated with the level of adenoidal hypertrophy. Children with higher proportions of Th17 cells and lower Tregs had more significant nasal obstruction. Also, those children with allergic rhinitis and OSA demonstrated a higher Th17/Treg ratio than those with OSA alone.

OSA has been associated with endothelial dysfunction in children measured by a modified hyperaemic test which involves the occlusion of the radial and ulnar arteries with a blood pressure cuff and doppler measurement of a cessation and return of blood flow, quantified as the peak occlusive hyperaemia (TMax). Tan et al 2013 [58] demonstrated an inverse correlation between percentage of circulating T-regs and TMax. They also showed a negative correlation between Treg suppression and a sleep pressure score, a surrogate measure for sleep fragmentation but not to the children's sleep study derived apnoea-hypopnoea index.

5.7. Conclusions

The research questions asked of this chapter were whether the tonsils and peripheral blood of children with OSA had a higher Th2/ ILC2 phenotype than those of children without OSA. I also asked whether the Treg and Th17 cell proportions were altered in the face of OSA with the hypothesis being that Tregs would be diminished and Th17 cells in a higher proportion compared to the OSA free controls. T-regulatory cells were lower in the OSA subjects however the Th17 cells demonstrated no difference. There was no other difference in T-cell, B-cell, NK-cell or lineage negative cells that distinguished one group from the other. I have therefore proven hypothesis no. 3 in part as I did not find a higher proportion of Th17 cells as expected based on the literature.

In the next chapter, I will focus on differences in the same tissue between subjects who are atopic or not to see if the genetic predisposition to allergy and allergic asthma dictates changes in cellular phenotype independent of OSA.

Chapter 6. Lymphoid cell phenotype of tonsils and peripheral blood from atopic and non-atopic children

This chapter focuses on the fourth of my hypotheses, stating that the tonsils from atopic subjects contain a skewed Th2 cellular phenotype. As with the previous chapter, the aim was to recruit children scheduled for tonsillectomy and that have atopic characterisation, and collect tonsil tissue for flow cytometry analysis to phenotype T-cell subsets and ILCs. All data can be found in table form in Appendix 14. This chapter will also investigate hypothesis no.5 that the tonsils of atopic children will contain a greater proportion of ILC group 2 cells compared to the tissue from non-atopic children.

6.1 Introduction

As demonstrated in the previous chapter, the only immunological difference identified between subjects with and without OSA was a lower proportion of Tregs in individuals with OSA. While chapter 4 demonstrated no difference in atopic states between individuals with or without OSA, the question was raised whether the samples collected (tonsil tissue and peripheral blood) may show a difference, specifically in a functional Th2 phenotype in cells with the capacity to produce Th2 associated cytokines.

As group 2 innate lymphoid cells have a cytokine signature similar to Th2 cells I also postulated that the samples from atopic individuals will contain more ILC2s than other subtypes. These cells have been implicated in allergic disease both alongside and independent of T-cells [273].

6.2. Research questions

1. Are the numbers of $CD4^+$ cells (as cell counts and percentage of total T-cells) in the tonsils and peripheral blood of atopic subjects greater compared to the same tissue from non-atopic subjects?
2. Are the numbers of ILC group 2 cells (as cell counts and percentage of total ILCs) in the tonsils and peripheral blood of atopic subjects greater compared to the same tissue from non-atopic subjects?

6.3. Methods

6.3.1 Recruitment

The definition of atopy used in this project was at least one elevated specific IgE to a panel of common aeroallergens. This definition however relies on successful results obtained from the specific IgE tests. These test results can be affected if the samples provided are insufficient for example, resulted in an incomplete aeroallergen panel result set. An individual with an incomplete panel could therefore be defined as non-atopic when in fact the missing specific IgE test could have yielded a positive result. Therefore, initially the samples were segregated into those with a positive result to the specific IgE panel and those with a complete, but negative panel, excluding those with two or more insufficient tests. This resulted in 15 atopic and 20 non-atopic individuals, the latter referred to as **non-atopic (complete aeroallergen panel)** (Figure 1.6).

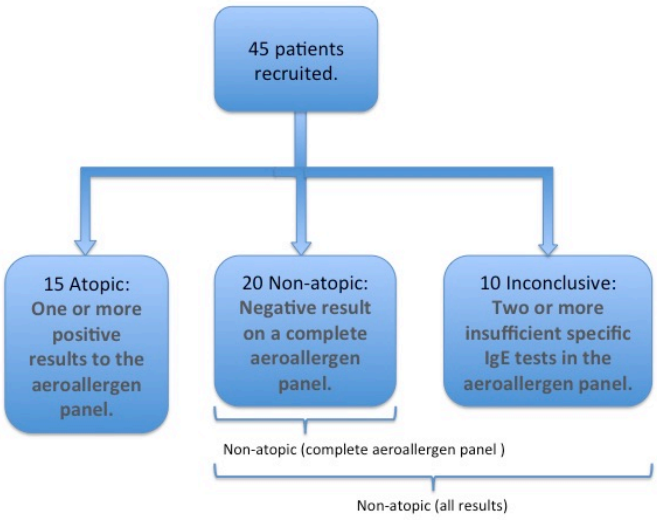


Figure 6.1. A recruitment diagram, demonstrating group proportions when analysing for differences based on atopic status considering those with and without a complete set of specific IgE tests.

The excluded results were then included in the analysis along with the samples from the non-atopic group with a complete set of negative aeroallergen specific IgE results. This group will be referred to as **non-atopic (all results)** (Figure 6.1.).

One criticism of the method of defining atopic status based on an aeroallergen panel could be that perhaps the correct allergens were not tested and that a child may have a positive result to a non-aeroallergen. Every effort was made to overcome this by asking patients/ guardians at recruitment whether an allergy to anything was known about and then including said allergen in the test panel. However, in an effort to analyse any possible differences, subjects were also grouped depending on the presence or absence of a raised total serum IgE above the normal laboratory reference range of 62 international units (IU). This cut-off value is based on adult data for normal and raised total IgE [274]. In this situation, those with insufficient total IgE were excluded. Interestingly, only 7 children had both a raised total IgE (above 62 IU) and a positive result to the aeroallergen panel therefore qualifying as atopic by both definitions (Figure 6.2.).

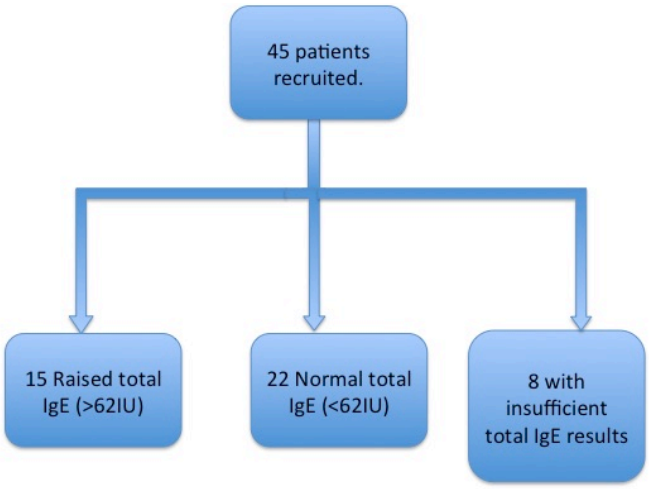


Figure 6.2. A recruitment diagram demonstrating group proportions when analysing for differences based on the presence or absence of a raised total IgE results.

As discussed in the previous chapter, the flow cytometry panels were developed and refined as tissue became available therefore not all samples were analysed for all cellular markers or cytokine production. The following diagrams show the proportion of samples available for analysis and where numbers were lost (Figure 6.3& 6.4.).

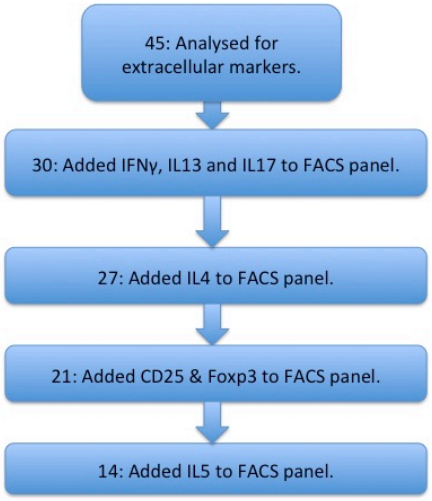


Figure 6.3. A flow diagram showing potential number of samples analysed for T helper cells at each stage of the FACS panel development.

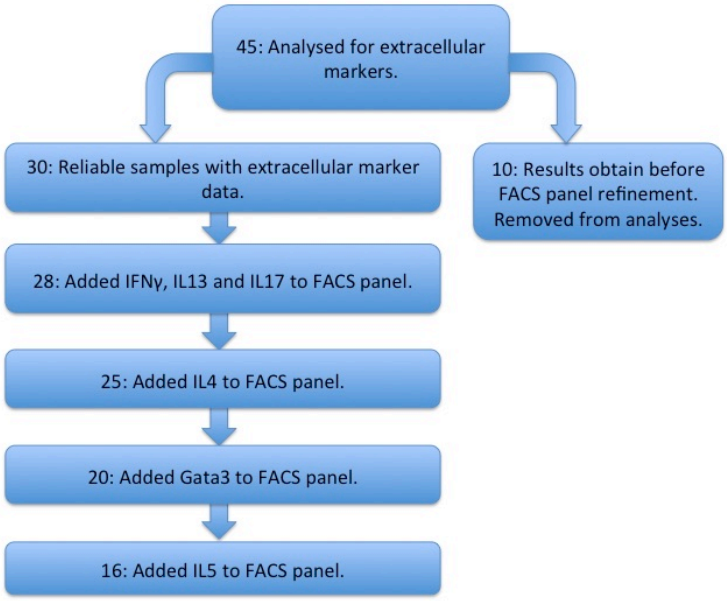


Figure 6.4. Flow diagram showing potential number of samples analysed for innate lymphoid cells at each stage of the FACS panel development.

For peripheral blood samples, priority was given to specific IgE tests required to identify atopic and non-atopic subjects. When sufficient blood was available after these tests were carried out, these samples were processed to identify T-cells and ILCs. Unfortunately this resulted in a smaller number of PBMC samples available to analyse (Figure 6.5& 6.6).

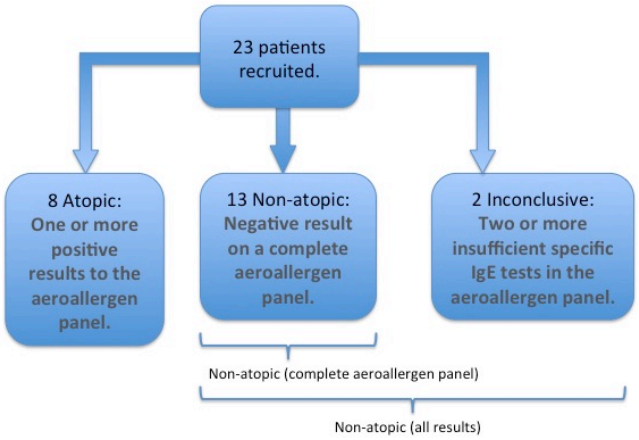


Figure 6.5. A recruitment diagram demonstrating group proportions of peripheral blood samples available to analyse when dividing samples based on atopic status.

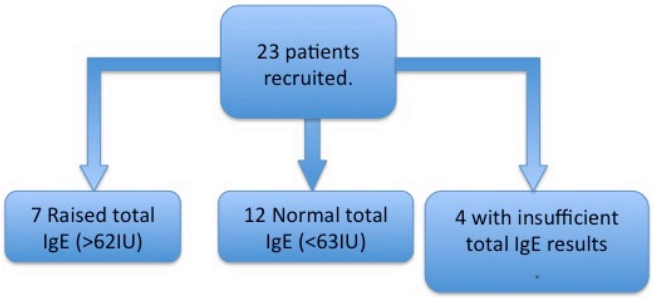


Figure 6.6 A recruitment diagram demonstrating group proportions of peripheral blood samples available to analyse when dividing samples based on presence or absence of a raised total IgE.

As with the tonsil tissue, the refinement of the FACS panels resulted in a reduction of those samples analysed for extracellular markers or cytokine production (Figure 6.7& 6.8.).

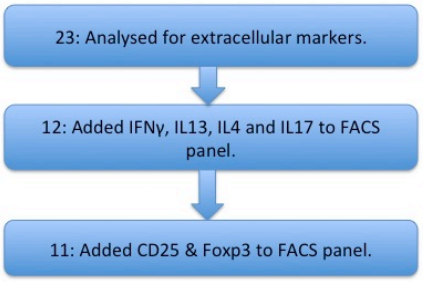


Figure 6.7. A flow diagram showing potential number of samples analysed for T helper cells at each stage of the FACS panel development.

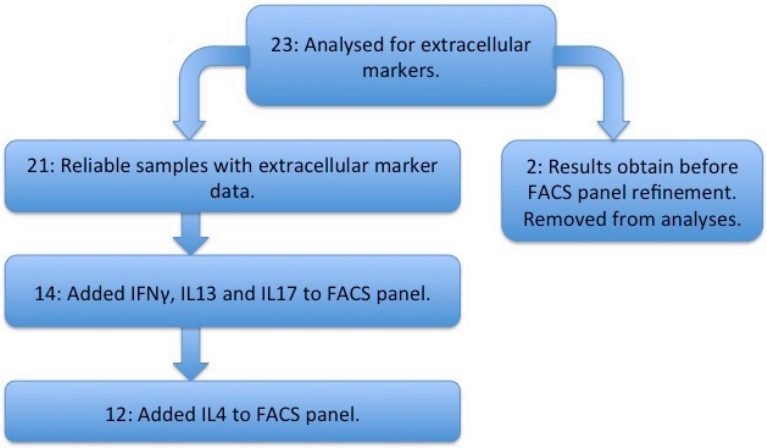


Figure 6.8. A flow diagram showing potential number of samples analysed for ILCs at each stage of the FACS panel development.

6.5 Results

6.5.1. T-cells

6.5.1.a. Lymphoid cell composition of tonsil tissue and peripheral blood from atopic and non-atopic (complete aeroallergen panel) children.

Firstly, atopic subjects were compared to non-atopic subjects with a complete and negative aeroallergen panel and therefore defined as Non-atopic (complete aeroallergen panel). The first comparison made was between total T-cells ($CD3^+$), B-cells ($CD19^+$) and Natural killer cells ($CD3^-CD56^+$). B-cells were the largest group in the tonsil samples while T-cells were more abundant in peripheral blood. No statistically significant difference was observed between atopic and non-atopic (complete aeroallergen panel) children from these

comparisons as cell counts or as a percentage of the total cells in either the tonsil samples or the peripheral blood (PBMCs)(Figure 6.9.).

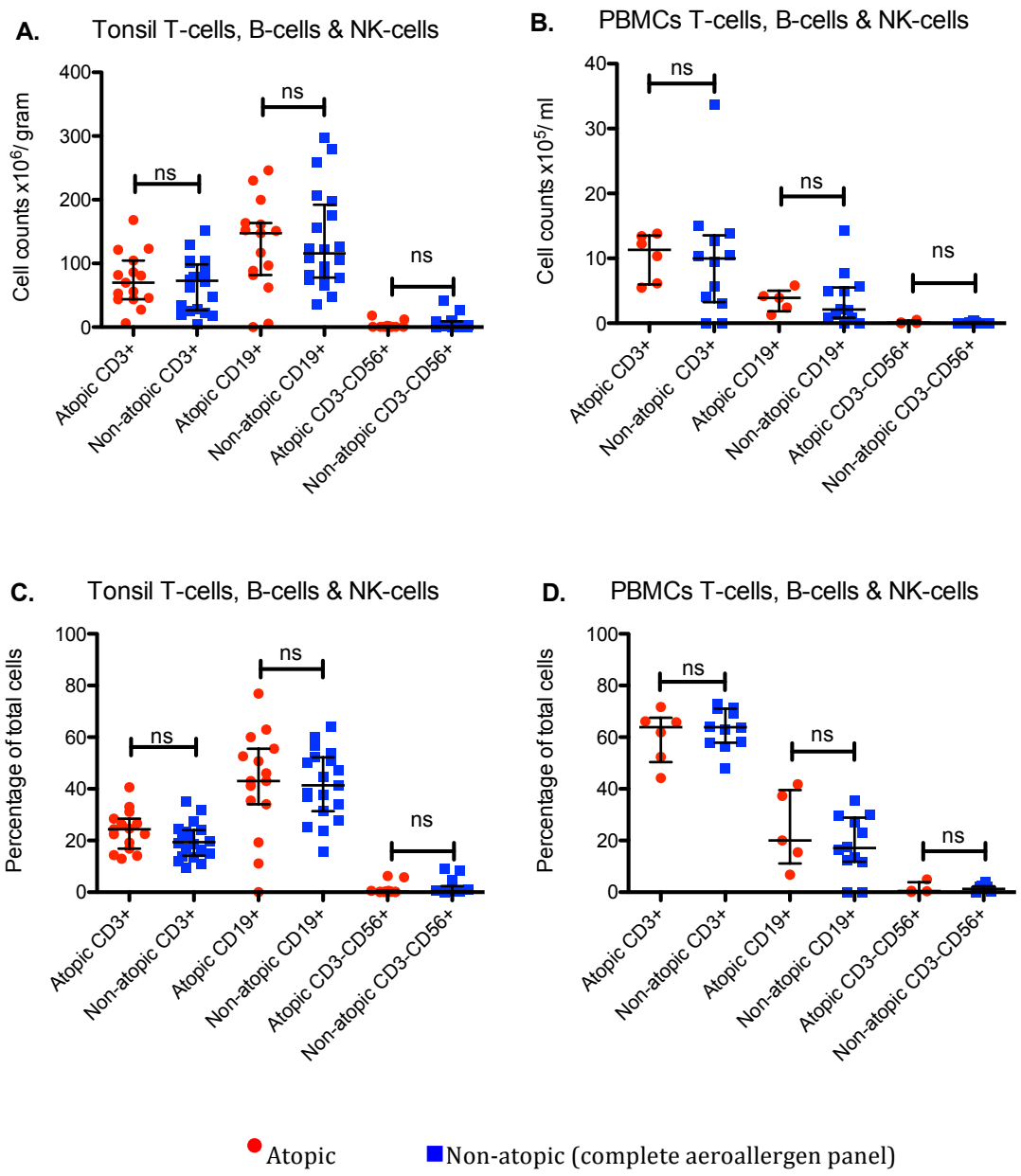


Figure 6.9. A comparison of the T-cells, B-cells and NK-cells in tonsil tissue as cells per gram of tissue (A.) and as percentage of total cells (C.) between atopic (red) and non-atopic(complete aeroallergen panel) (blue) subjects. The same comparison this time in peripheral blood samples (PBMCs) as cells per ml of blood (B.) and as percentage of total cells (D.). All displayed values are medians with interquartile ranges.

Next the analysis focused on T-cells comparing the $CD4^+$ cells, $CD8^+$ cells and natural killer T-cells ($CD3^+CD56^+$). More $CD4^+$ T-cells were present in both sample types compared to the

other T-cell subsets.No difference was observed between atopic and non-atopic (complete aeroallergen panel) subjects for either tonsil tissue or for blood (Figure 6.1.1.).

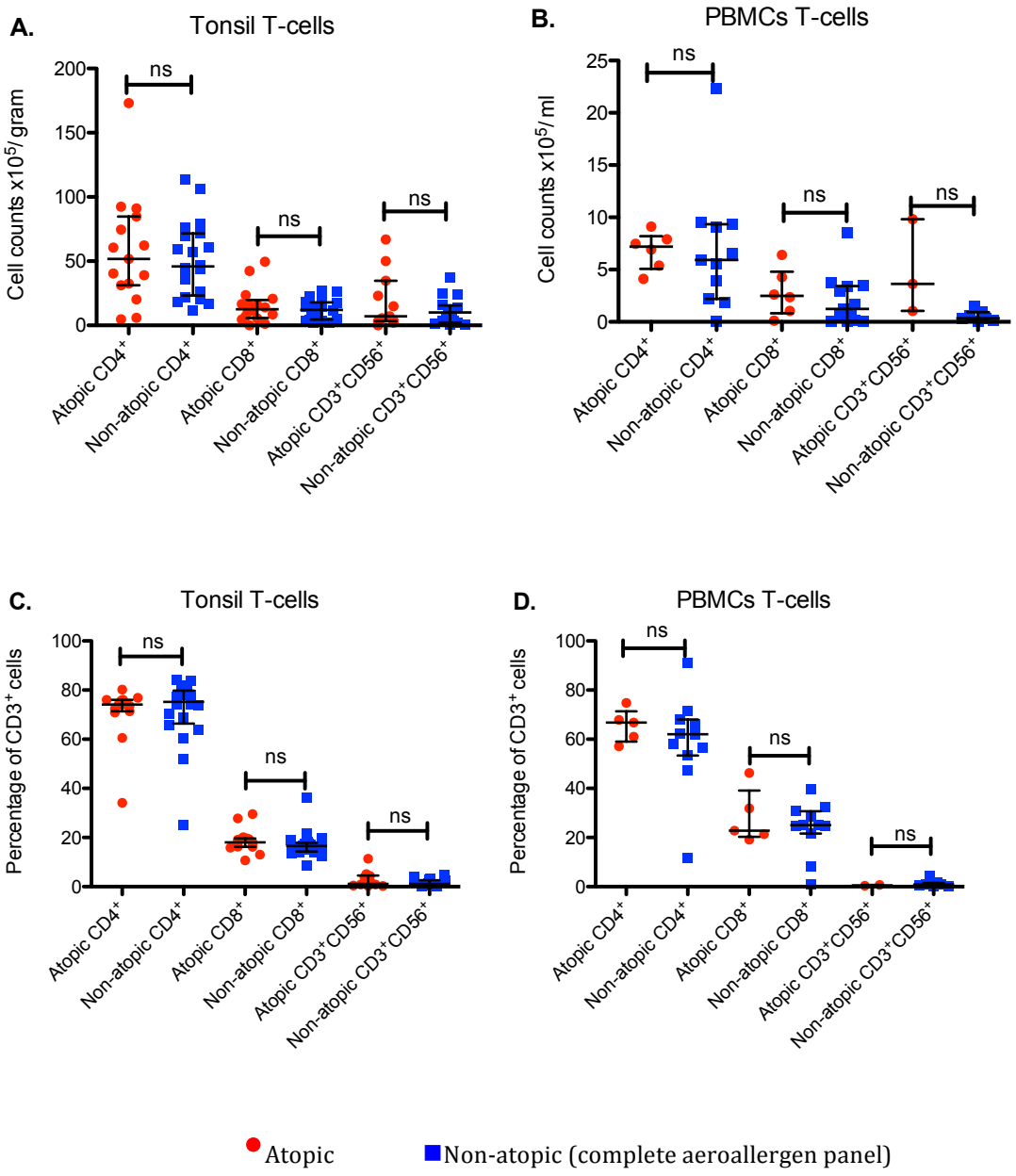


Figure 6.1.1. A comparison of T-cells in tonsil tissue as cells per gram of tissue (A.) and as percentage of total T-cells (C.) between atopic (red) and non-atopic (complete aeroallergen panel) (blue) subjects. The same comparison this time in peripheral blood (PBMCs) as cells per ml of blood (B.) and as percentage of total T-cells. All displayed values as medians with interquartile ranges.

Finally, the defined groups were compared focusing on the CD4+ T-cell subsets based on capacity for cytokine production and including Tregs using the same definitions described

in chapter 5. No statistically significant difference was observed when comparing the samples from atopic individuals with the non-atopic (complete aeroallergen panel) individuals in both tonsil tissue and peripheral blood (Figure 6.1.2.)

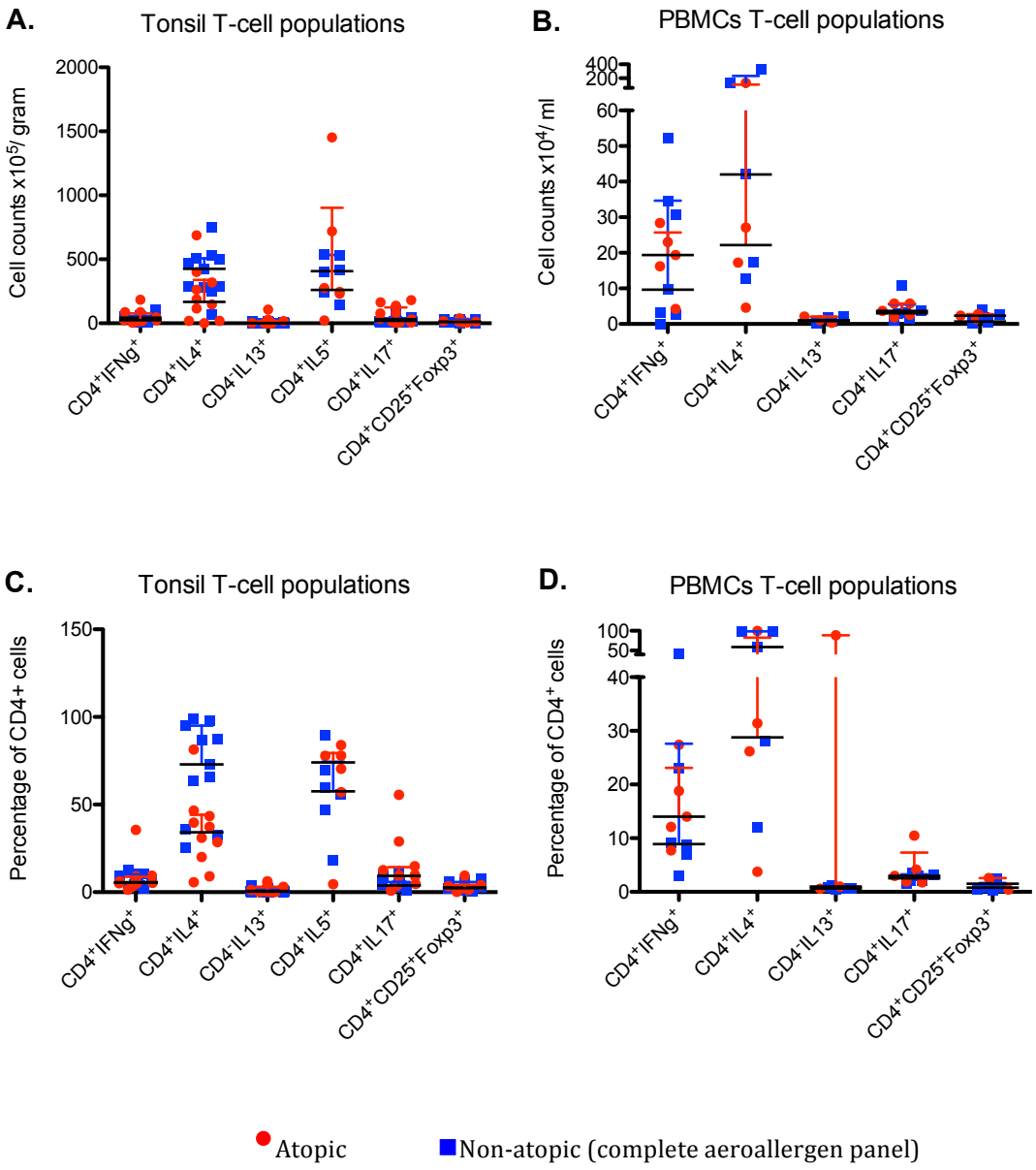


Figure 6.1.2. A comparison of Th cells defined by their cytokine production in tonsil tissue as cells per gram of tissue (A.) and as percentage of total T-cells (C.) between atopic (red) and non-atopic (complete aeroallergen panel) (blue) subjects. The same comparison this time in peripheral blood (PBMCs) as cells per ml of blood (B.) and as percentage of total T-cells. All displayed values as medians with interquartile ranges.

6.5.1.b. Lymphoid cell composition of tonsil tissue and peripheral blood from children comparing atopic vs non-atopic (all results) subjects.

The next set of comparisons included all samples from subjects designated as non-atopic whether this was the result of a complete negative aeroallergen panel or by default due to an incomplete set of results. The same comparisons were made starting with the T-cells, B-cells and NK-cells and no difference was observed for both tonsil tissue or peripheral blood samples comparing atopic individuals with the non-atopic (all results) (Figure 6.1.3.).

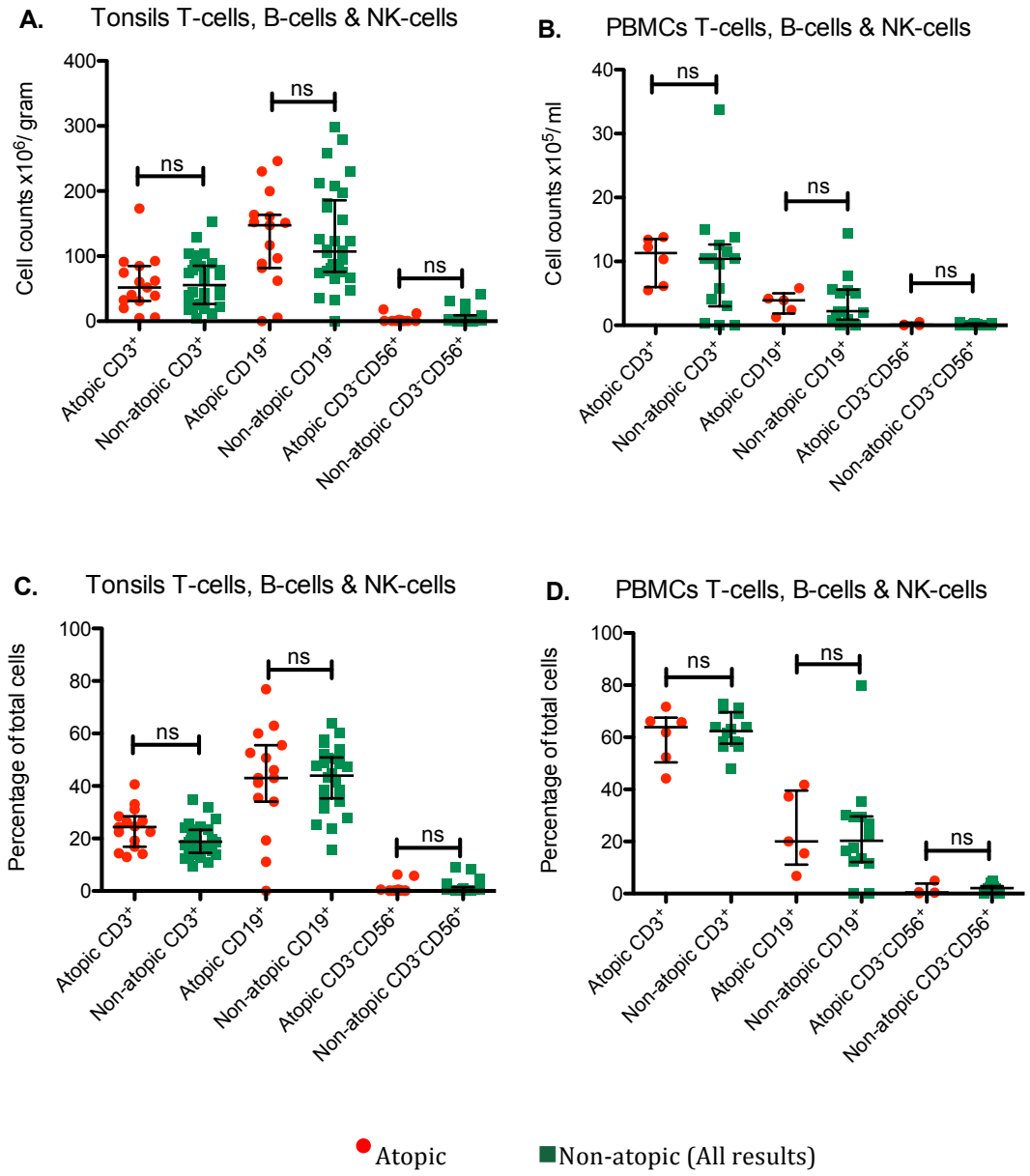


Figure 6.1.3. A comparison of the T-cells, B-cells and NK-cells in tonsil tissue as cells per gram of tissue (A.) and as percentage of total cells (C.) between atopic (red) and non-atopic(all results) (green) subjects. The same comparison this time in peripheral blood samples (PBMCs) as cells per ml of blood (B.) and as percentage of total cells (D.). All displayed values are medians with interquartile ranges.

The next comparison was made to see if the addition of non-atopic subjects with an incomplete set of aeroallergen results would alter the results when focusing on T-cell subsets. This made no difference for either tonsil or blood sample results (Figure 6.1.4.).

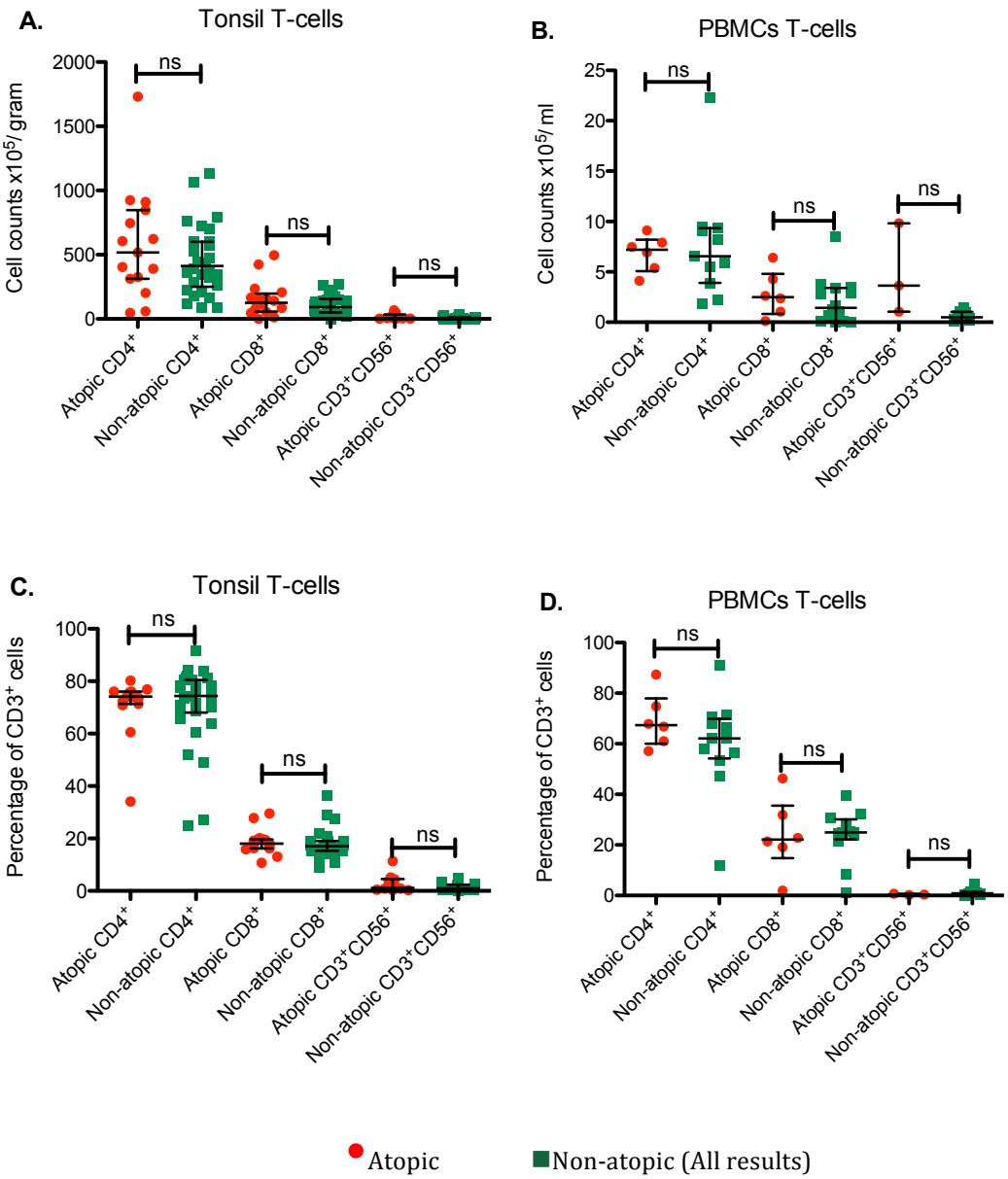


Figure 6.1.4. A comparison of T-cells in tonsil tissue as cells per gram of tissue (A.) and as percentage of total T-cells (C.) between atopic (red) and non-atopic (all results) (green) subjects. The same comparison this time in peripheral blood (PBMCs) as cells per ml of blood (B.) and as percentage of total T-cells. All displayed values as medians with interquartile ranges.

Finally, comparisons were made by assessing intra-cellular cytokines in T-cells. With the addition of the non-atopic subjects with incomplete results panels, one comparison did show a significant difference. In the tonsil samples only, when displaying results as a percentage of the total $CD4^+$ cells, tonsils from non-atopic (all results) children had a higher proportion of $CD3^+CD4^+IL\text{-}4^+$ cells compared to tonsils from non-atopic children (median value for atopic subjects 34.1% (IQR 17.3-44.2) vs 86.2% (IQR 64.1-95.2) for non-atopic (all results), p=0.002). This was the only significant difference between atopic and non-atopic subjects in both tonsils and the peripheral blood (Graph 6.1.5 & 6.1.6.).

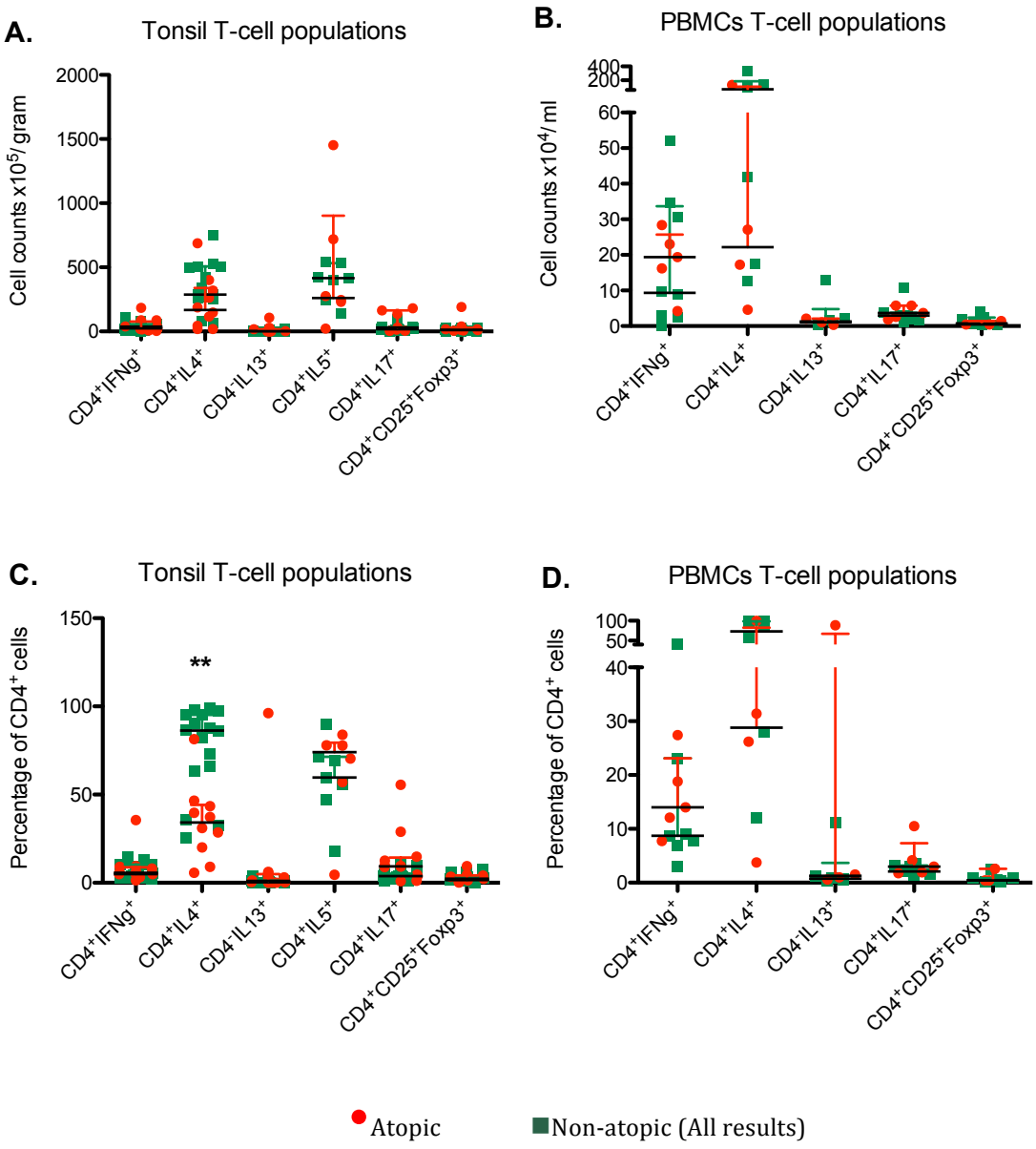


Figure 6.1.5. A comparison of Th cells defined by their cytokine production in tonsil tissue as cells per gram of tissue (A.) and as percentage of total Th cells (C.) between atopic (red) and non-atopic (all results) (blue) subjects. The same comparison this time in peripheral blood (PBMCs) as cells per ml of blood (B.) and as percentage of total Th cells. All displayed values as medians with interquartile ranges. Mann-Whitney U-test). **: p=0.002

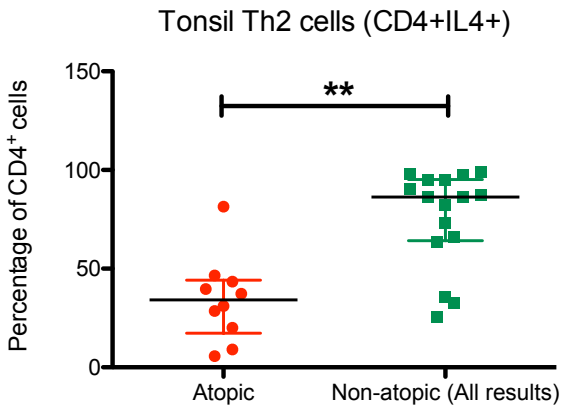


Figure 6.1.6. Comparison of Th2 cells ($CD4^+Ll4^+$) as cell counts per gram of tonsil tissue between atopic and Non-atopic (all results) subjects. Median values with interquartile ranges and significance using Mann-Whitney U-test. ** represents statistical significant level of 0.002.

6.5.1.c. Lymphoid cell composition of tonsil tissue and peripheral blood from children with raised serum IgE and normal serum IgE

The third group of comparisons made were between subjects with a raised total IgE (over the laboratory standard normal cut-off >62 international units) compared to those with normal total IgE. Firstly, comparisons were made between T-cell, B-cell and NK-cells. No differences were observed for tonsil or PMBCs as cell counts or percentage of total cells (Figure 6.1.7).

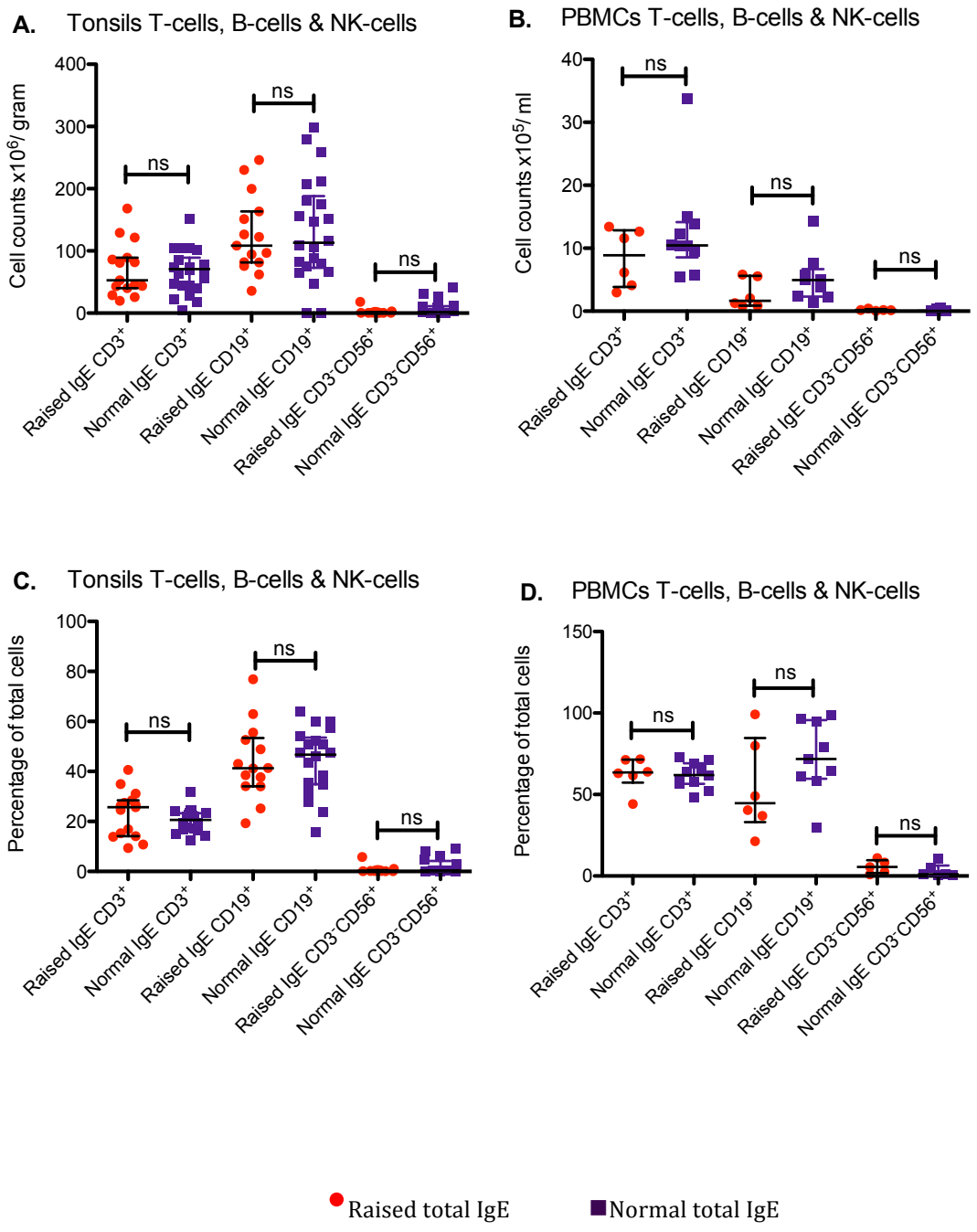


Figure 6.1.7. A comparison of Tonsil T-cells, B-cells and NK-cells as cells per gram of tissue (A.) and as percentage of total cells (C.) between subjects with raised IgE (Red) to those with normal IgE (Purple). The same comparison this time using peripheral blood cells (PBMCs) as cells per ml of blood (B.) and as percentage of total cells (D.). All values displayed as median values with interquartile ranges.

When T-cell subtypes were examined, no differences were apparent in either tonsil tissue or peripheral blood in children with elevated compared to normal IgE (Figure 6.1.8).

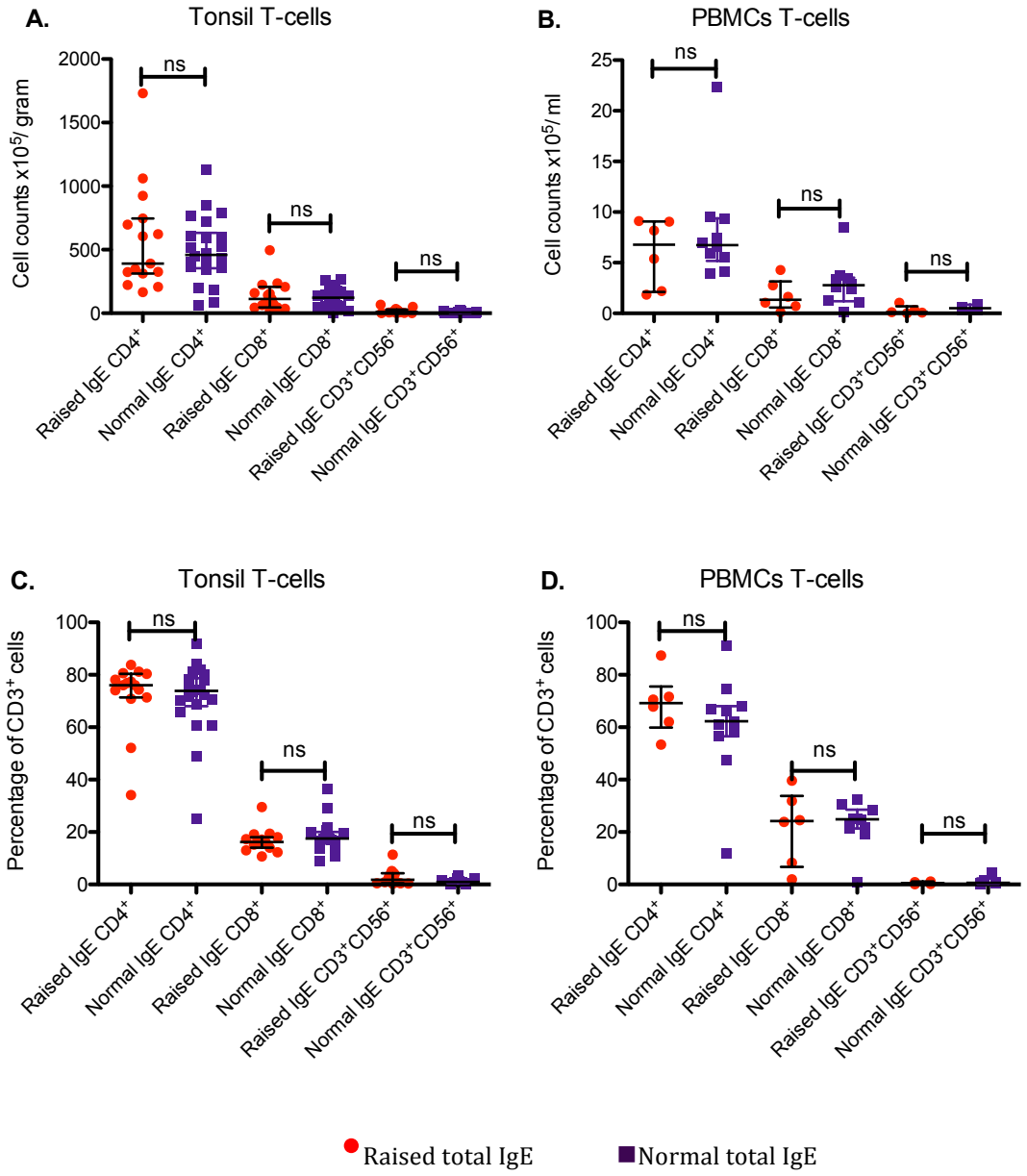


Figure 6.1.8. A comparison of Tonsil T-cells as cells per gram of tissue (A.) and as percentage of total T-cells (C.) between subjects with raised IgE (Red) to those with normal IgE (Purple). The same comparison this time using peripheral blood cells (PBMCs) as cells per ml of blood (B.) and as percentage of total T-cells (D.). All values displayed as median values with interquartile ranges.

Finally, analysing the intra-cellular cytokine profile of the $CD4^+$ cells to see if a difference between the subjects with raised total IgE and those with a normal total IgE could be observed, no differences were found. The results for $CD4^+IL4^+$ cells in the peripheral blood as cell counts per ml of blood and as a percentage of total $CD4^+$ cells did show a higher

proportion in the atopic individuals however this did not achieve a statistically significant difference (Figure 6.1.9.).

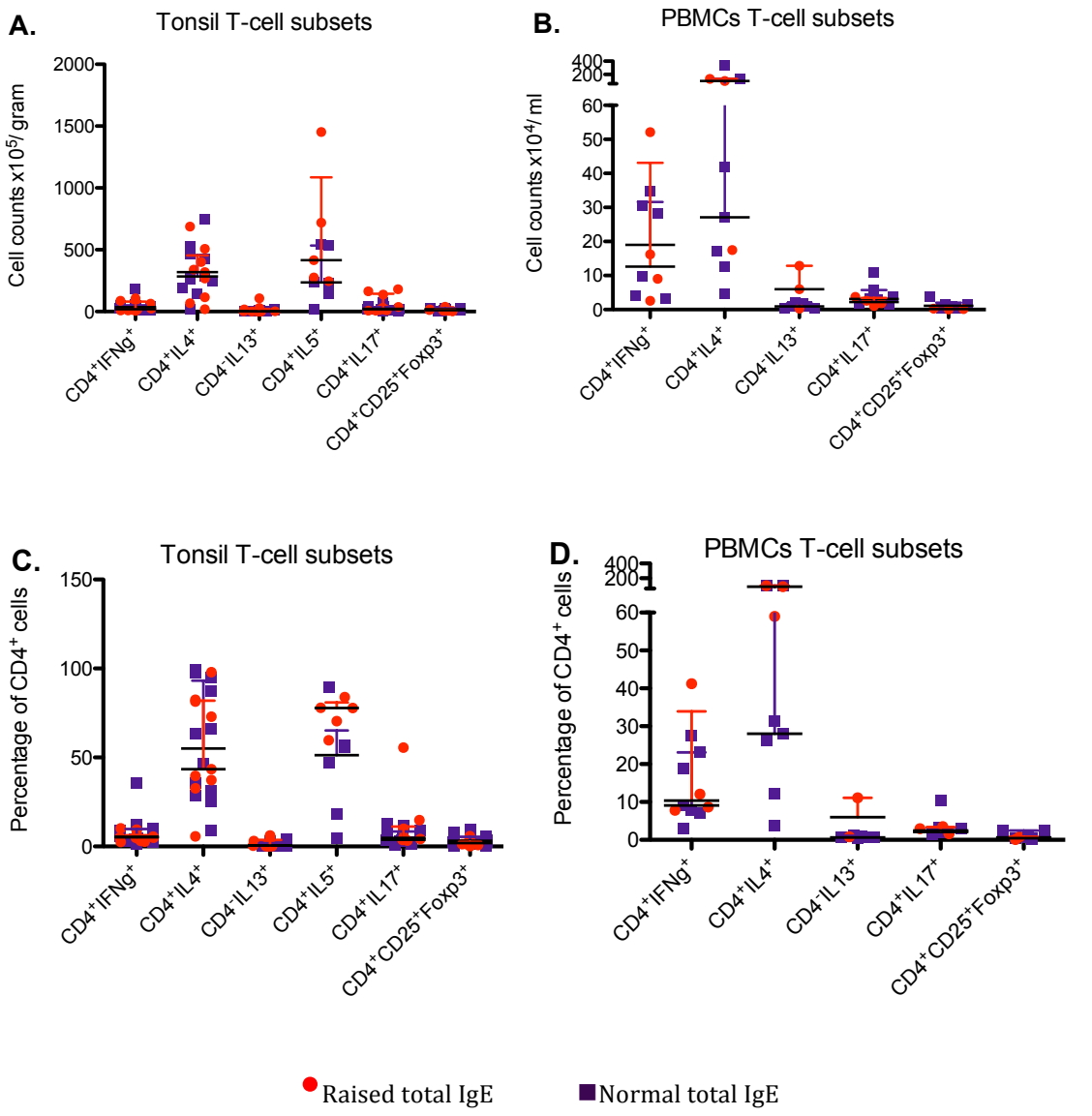


Figure 6.1.9. A comparison of Tonsil Th cells defined by cytokine secretons and Tregs as cells per gram of tissue (A.) and as percentage of total Th cells (C.) between subjects with raised IgE (Red) to those with normal IgE (Purple). The same comparison this time using peripheral blood cells (PBMCs) as cells per ml of blood (B.) and as percentage of total T-cells (D.). All values displayed as median values with interquartile ranges.

6.5.1.d. T-cell counts vs total IgE

Categorising the samples into distinct groups (atopic or non-atopic) did not yield any promising significant differences despite using three different definitions of atopy. Instead of using the value of >62IU as a cut off for a raised total IgE, I next plotted the actual value for IgE against cell counts from tonsils to see if any correlation was evident. This was not the case for the cell counts per gram of tonsil tissue results or when plotting the actual value for total IgE against cell counts as a percentage of total cells, total T-cells or total $CD4^+$ cells.

6.5.2 Innate Lymphoid Cells

6.5.2.a. ILCs in tonsil tissue and PBMCs comparing atopic and non-atopic (complete aeroallergen panel) children.

Innate lymphoid cells (ILCs), were initially identified using only extracellular markers: total lineage negative cells, CD127+ and those with the chemoattractant receptor expressed in Th2 cells (CRTH2) defined as ILC group 2 cells. The first comparison was between subjects with a complete set of aeroallergen tests to define atopic status.

Significantly higher $Lin^{neg}CD127^+$ cells were present in tonsils from atopic subjects compared to non-atopic (complete aeroallergen panel) subjects (median value $52.7x10^4$ (IQR $37.2x10^4$-$127.2x10^4$) in atopic samples vs $7.6x10^4$ (IQR $4.6x10^4$-$18x10^4$) for non-atopic (complete aeroallergen panel), p=0.0004) (Figure 6.2.1). Lin^{neg} $CD127^+CRTH2^+$ cells (ILC2s) expressed as percentage of $CD45^+$ cells were also elevated in atopic individuals compared to non-atopic (complete aeroallergen panel) (Median percentage 0.008% (IQR 0.003%-0.02%) in atopic samples vs 0.001% (IQR 0.0006%-0.004%) in non-atopic (complete aeroallergen panel), p=0.005) (Figure 6.2.1).

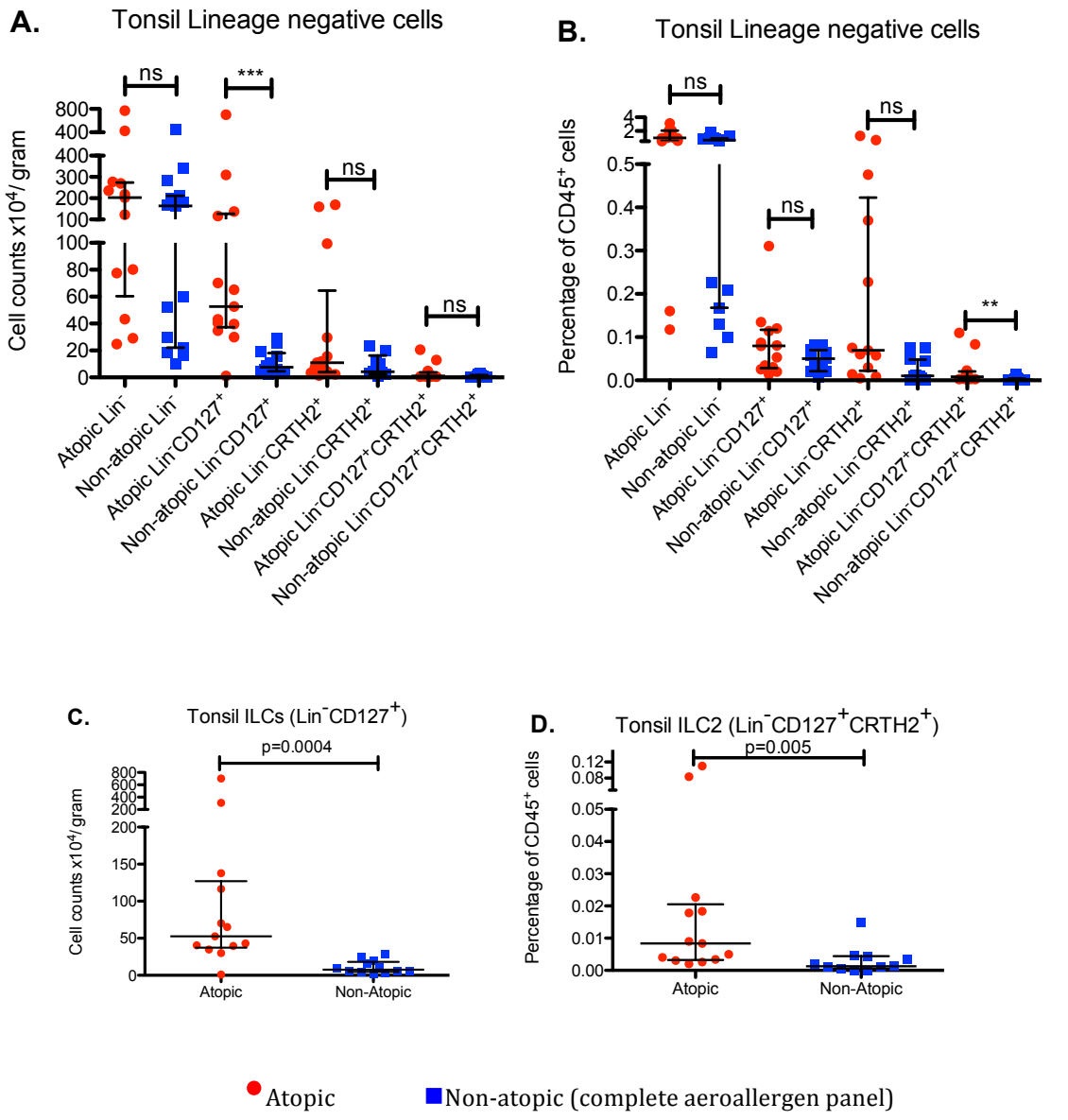


Figure 6.2.1. A comparison of tonsil lineage negative cells identified by published extracellular markers as cell counts per gram of tissue (A.) and as percentage of total lymphoid cells ($CD45^+$) (B.) between atopic (red) with non-atopic (complete aeroallergen panel) (Blue). The $Lin^{neg}CD27^+$ cells are significantly higher in atopic subject as cells per gram of tissue compared to the non-atopic subjects (p=0.0004) (C.). The ILC2s ($Lin^{neg}CD127^+CRTH2^+$) are significantly higher in the atopic subjects compared to non-atopic as percentage of total lymphoid cells ($CD45^+$) ((p=0.005) (D.). All values displayed as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

PBMCs were analysed by the same protocol. No significant differences were present in ILC2 cells, but Lin^{neg} cells from atopic individuals were proportionally higher than the non-atopic children (Figure 6.2.2.).

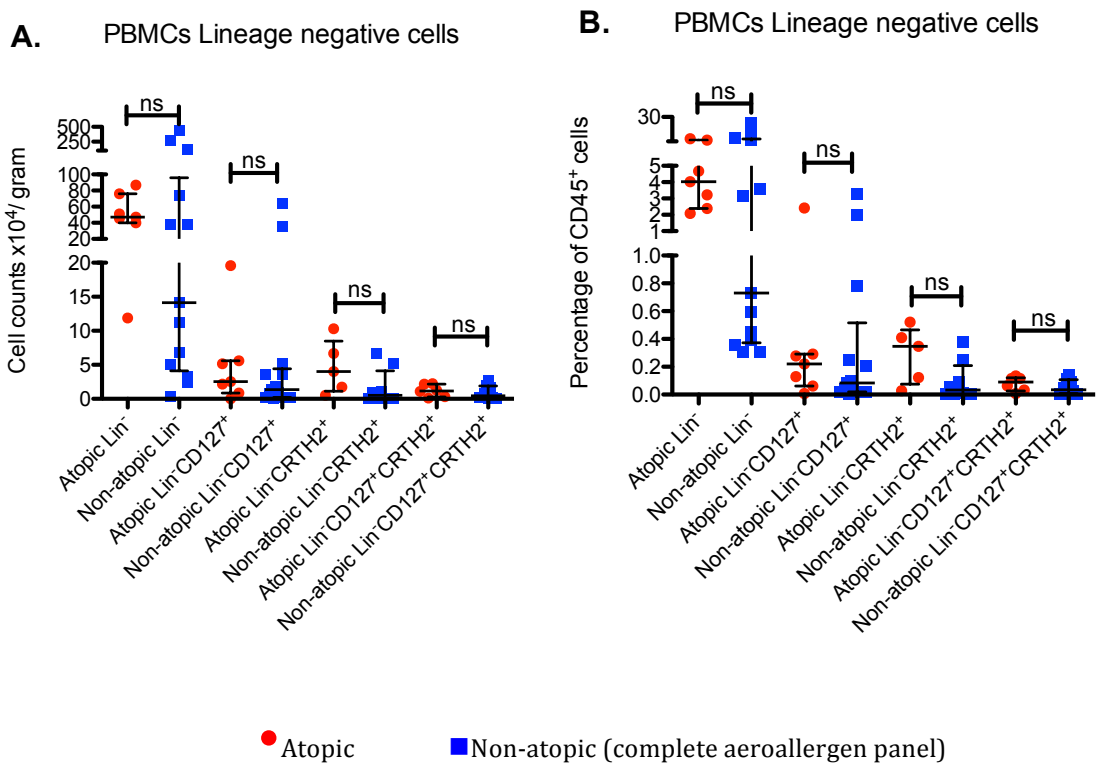


Figure 6.2.2. A comparison of peripheral blood (PBMCs) lineage negative cells identified by published extracellular markers as cell counts per ml of blood (A.) and as percentage of total lymphoid cells (CD45⁺) between atopic (Red) and non-atopic (complete aeroallergen panel)(Blue) subjects. All displayed values are medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

The samples were then analysed comparing lineage negative cells expressing the type 2 intra-cellular cytokines that define the ILC groups. No significant differences were observed comparing tonsils or PBMCs from atopic and non-atopic (complete aeroallergen panel) subjects either as cell counts or as percentage of total cells. (Figure 6.2.3.).

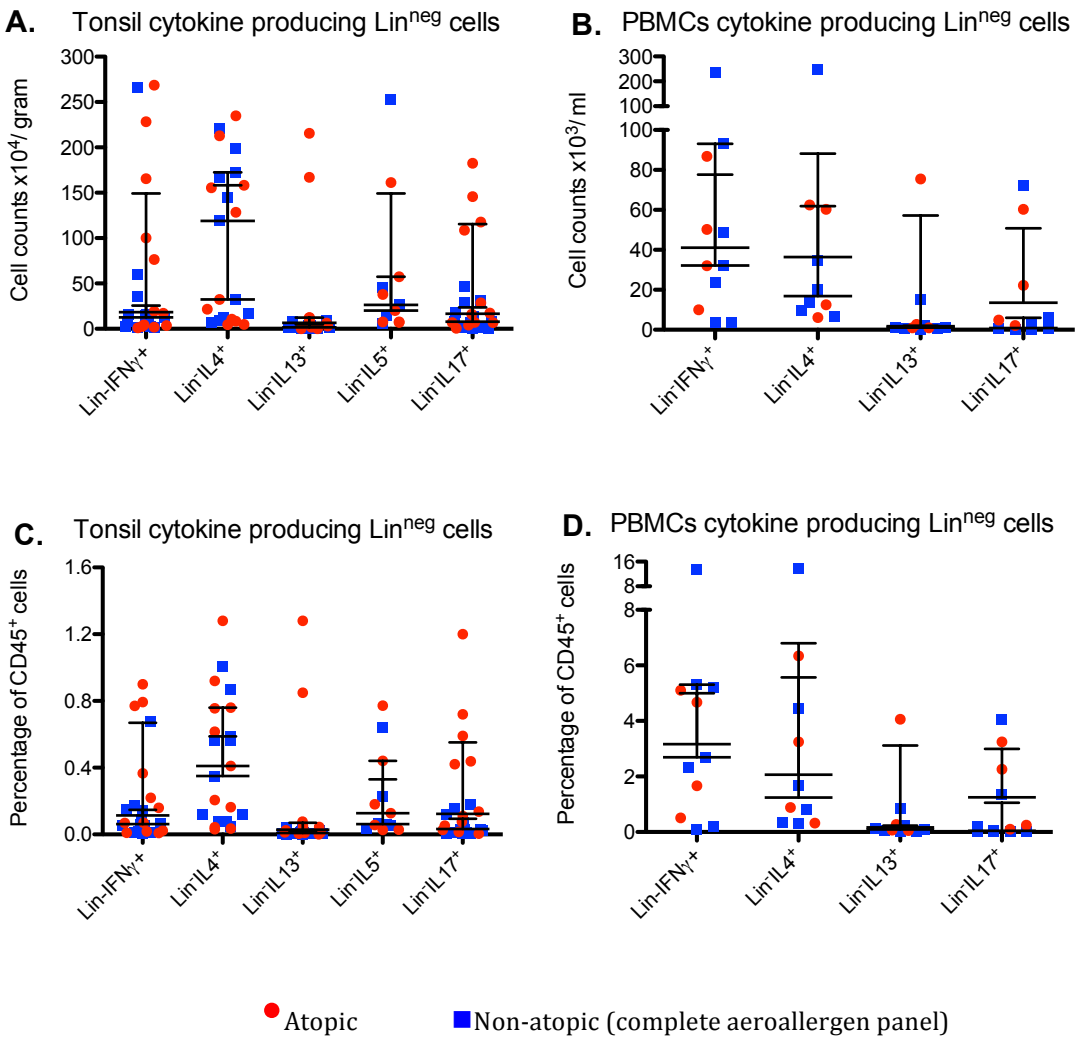


Figure 6.2.3. A comparison of tonsil lineage negative cytokine producing cells as cell counts per gram of tissue (A.) and percentage of total lymphoid cells ($CD45^+$) (C.) comparing atopic (Red) subjects with non-atopic (complete aeroallergen panel) (Blue). The same comparison this time with peripheral blood cells (PBMCs) as cells per ml of blood (B.) and as percentage of total lymphoid cells (D). All displayed values as medians with interquartile ranges.

Analysis was then focused on lineage negative cells possessing the extracellular marker CD127 and also capable of producing key cytokines. The results for cells producing IL-13 were extremely low making comparison impossible. The cell counts in the peripheral blood samples were also reduced to an undetectable level and therefore no further analysis could be made. For the tonsil samples, no significant difference was observed either as cells per gram of tissue or as percentage of $CD45^+$ cells (Figure 6.2.4.).

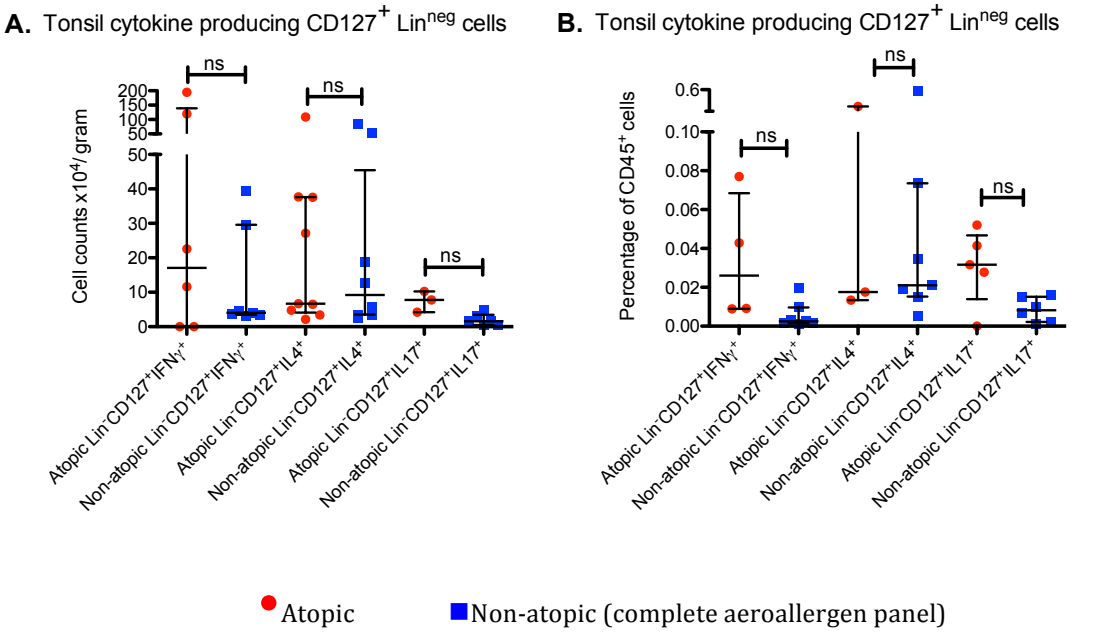


Figure 6.2.4. A comparison of tonsil lin^{neg} CD127^{+} and cytokine producing cells as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) (B.) between atopic (Red) and non-atopic (complete aeroallergen panel) (Blue). All displayed values as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

Finally, assessing Tonsil ILC group 2 cells defined using the type 2 transcription factor, as Lin^{neg} Gata3^{+} cells. These cells together with those with type 2 intracellular cytokines were analysed. Once again, the IL-13 producing cell numbers and the PBMCs results were so small, comparison was not possible and these results excluded. No significant difference was observed in the tonsil samples as cells per gram of tissue or as percentage of CD45^{+} cells (Figure 6.2.5.).

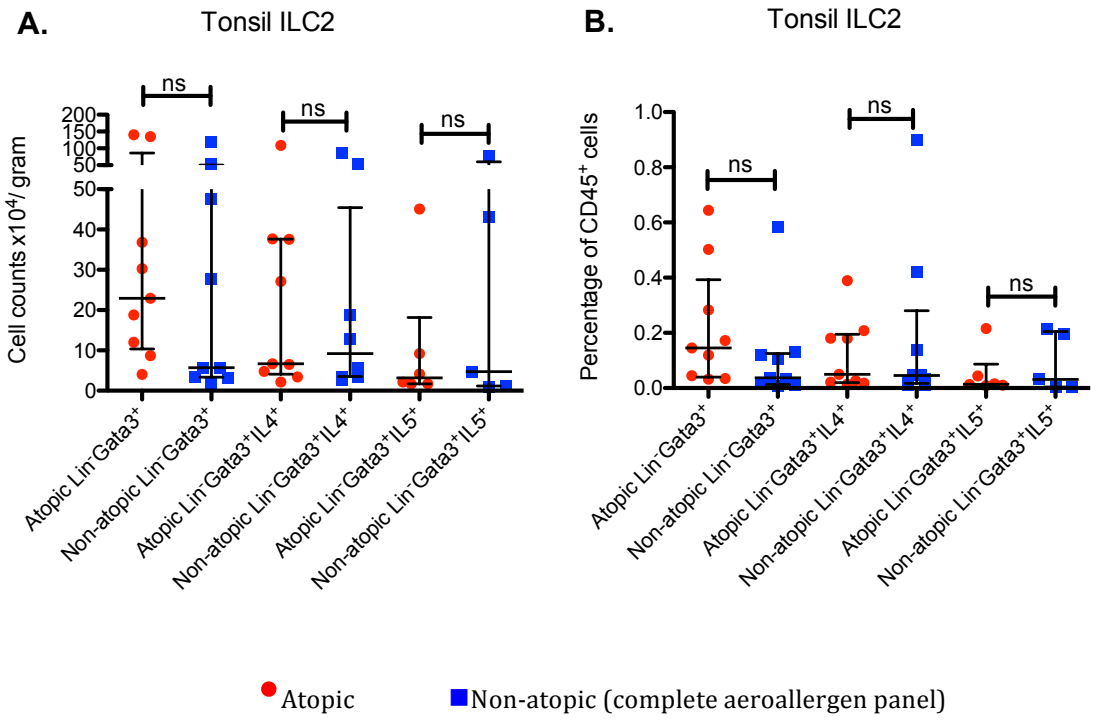


Figure 6.2.5. A comparison of tonsil IL2s (Lin⁻Gata3⁺) as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) between atopic (Red) and non-atopic (complete aeroallergen panel) (Blue) subjects. All displayed values as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

6.5.2.b. ILCs in tonsil tissue and PBMCs comparing atopic vs non-atopic (all results) children.

As with the T-cell analysis, comparisons were made between atopic and non-atopic children including those with an insufficient set of specific IgE results defined as non-atopic. Consistent with the previous findings, the CD127⁺ lineage negative cells were significantly raised in atopic subjects as cells per gram of tissue (Median values 52.7x10⁴ (IQR 37.2x10⁴-127.2x10⁴) for atopic samples vs 7.6x10⁴ (IQR 3.9x10⁴-16.3x10⁴) for non-atopic (all results), $p<0.0001$ (Figure 6.2.6.). The lineage negative CD127⁺CRTH2⁺ cells were also still higher in atopic subjects as percentage of CD45+ cells (Median values 0.008% (IQR 0.003%-0.02%) in atopic samples vs 0.003% (IQR 0.001%-0.004%) in non-atopic (all results), $p=0.02$) although this did not reach the desired significance level of $p<0.01$ stated in chapter 2 (Figure 6.2.6.). These differences were not present in PBMCs from atopic and non-atopic children. (Figure 6.2.7.)

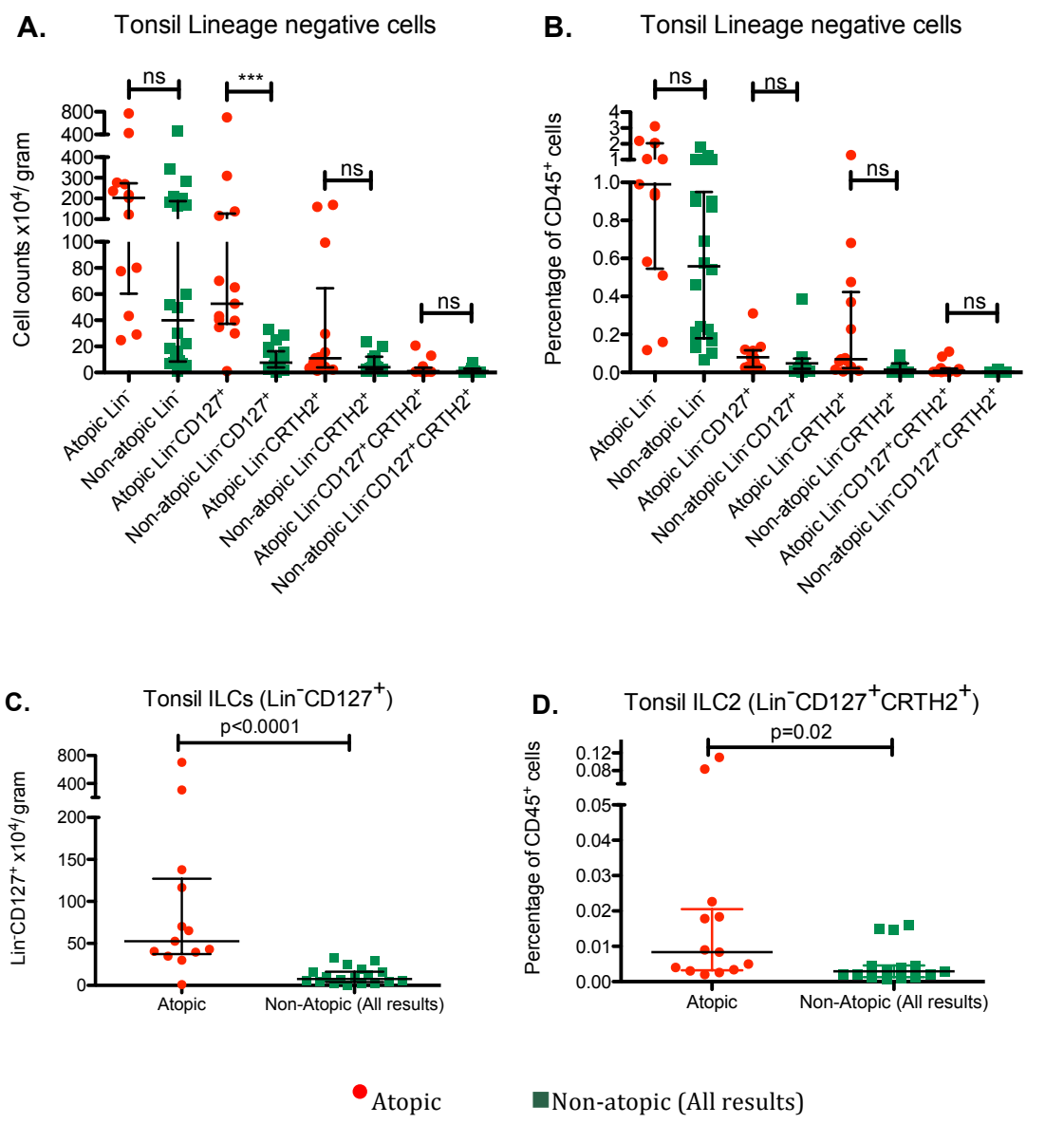


Figure 6.2.6. A comparison of tonsil lineage negative cells identified by published extracellular markers as cell counts per gram of tissue (A.) and as percentage of total lymphoid cells ($CD45^+$) (C.) between atopic (red) with non-atopic (all results) (Green). The $Lin^{neg}CD27^+$ cells are significantly higher in atopic subject as cells per gram of tissue compared to the non-atopic subjects (All results) (C.). The ILC2s ($Lin^{neg}CD127^+CRTH2^+$) are significantly higher in the atopic subjects compared to non-atopic (All results) (D.). All values displayed as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

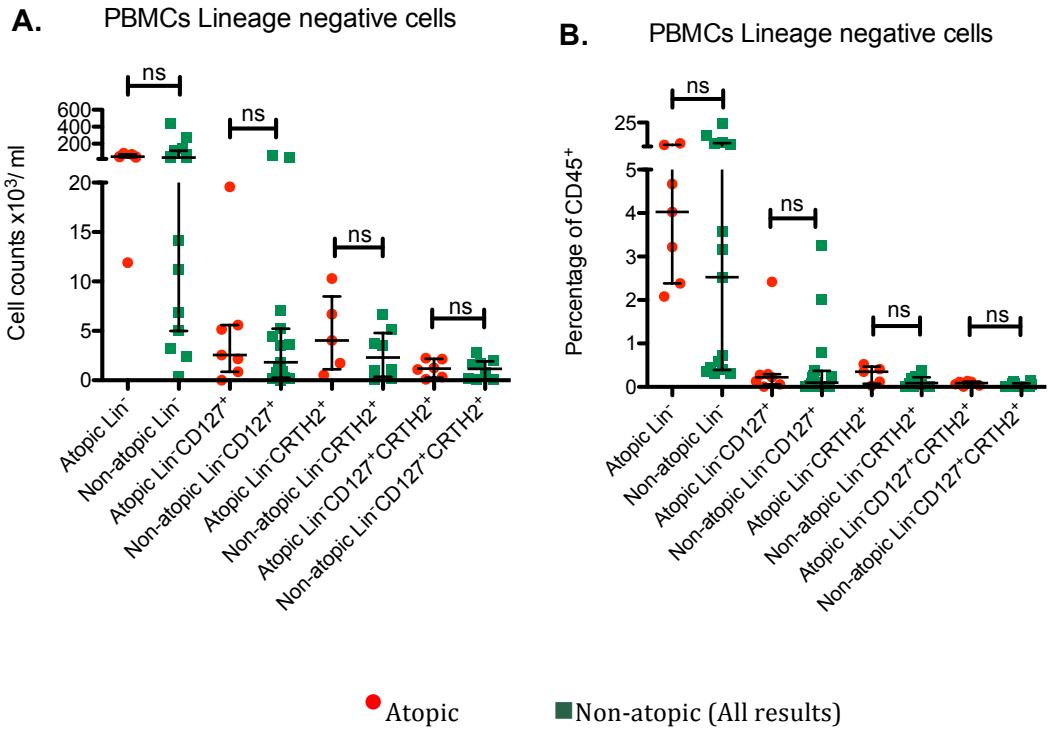


Figure 6.2.7. A comparison of peripheral blood (PBMCs) lineage negative cells identified by published extracellular markers as cell counts per ml of blood (A.) and as percentage of total lymphoid cells ($CD45^+$) between atopic (Red) and non-atopic (All results)(Green) subjects. All displayed values are medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

Cytokine producing lineage negative cells were analysed next. No significant difference was observed between samples from atopic and non-atopic (all results) subjects both as cells per gram of tissue and as percentage of $CD45^+$ cells for tonsil samples or PBMCs (Figure 6.2.8.).

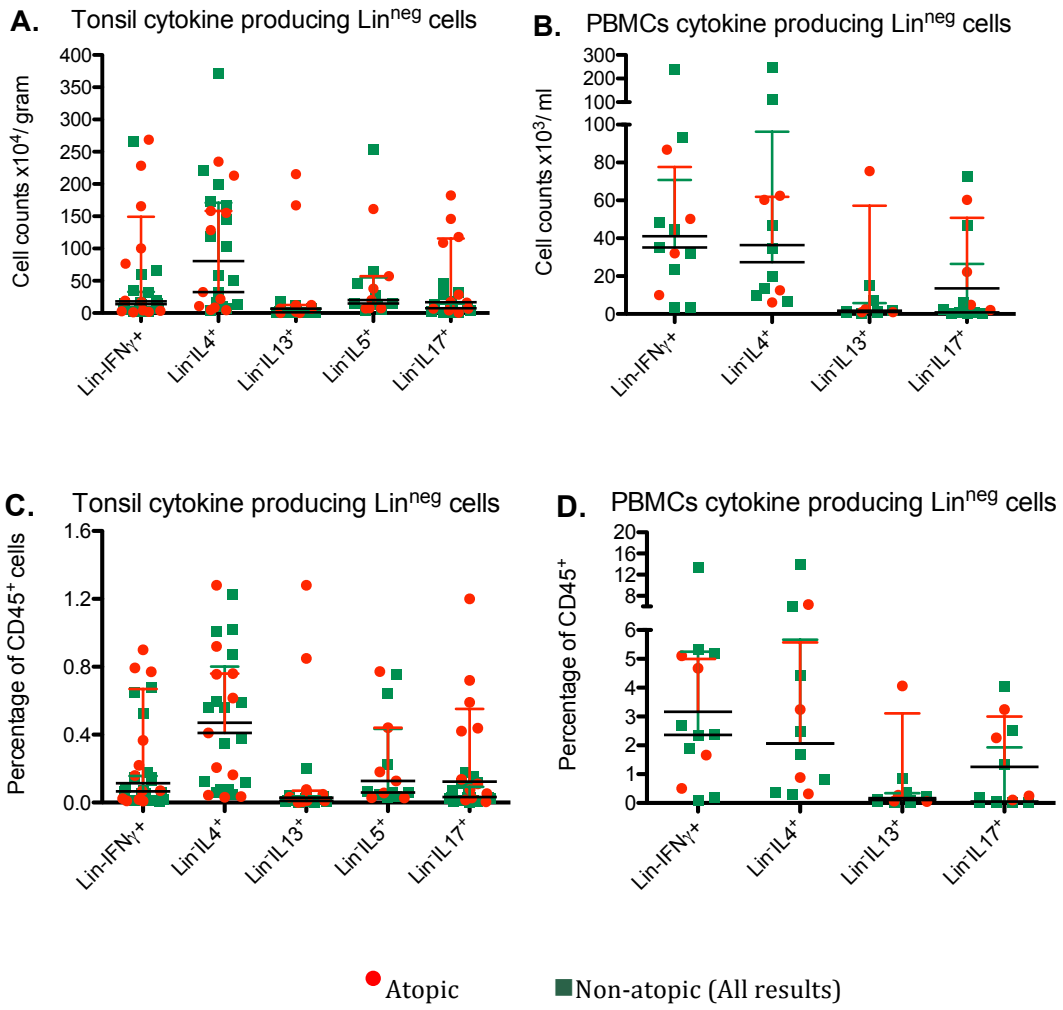


Figure 6.2.8. A comparison of tonsil lineage negative cytokine producing cells as cell counts per gram of tissue (A.) and percentage of total lymphoid cells ($CD45^+$) (C.) comparing atopic (Red) subjects with non-atopic (All results) (Green). The same comparison this time with peripheral blood cells (PBMCs) as cells per ml of blood (B.) and as percentage of total lymphoid cells (D). All displayed values as medians with interquartile ranges.

When including all samples from non-atopic subjects, there were no significant differences observed in $CD127^+$ lineage negative cells producing cytokines for the tonsil tissue samples comparing atopic subjects against non-atopic (all results) (Figure 6.2.9.).

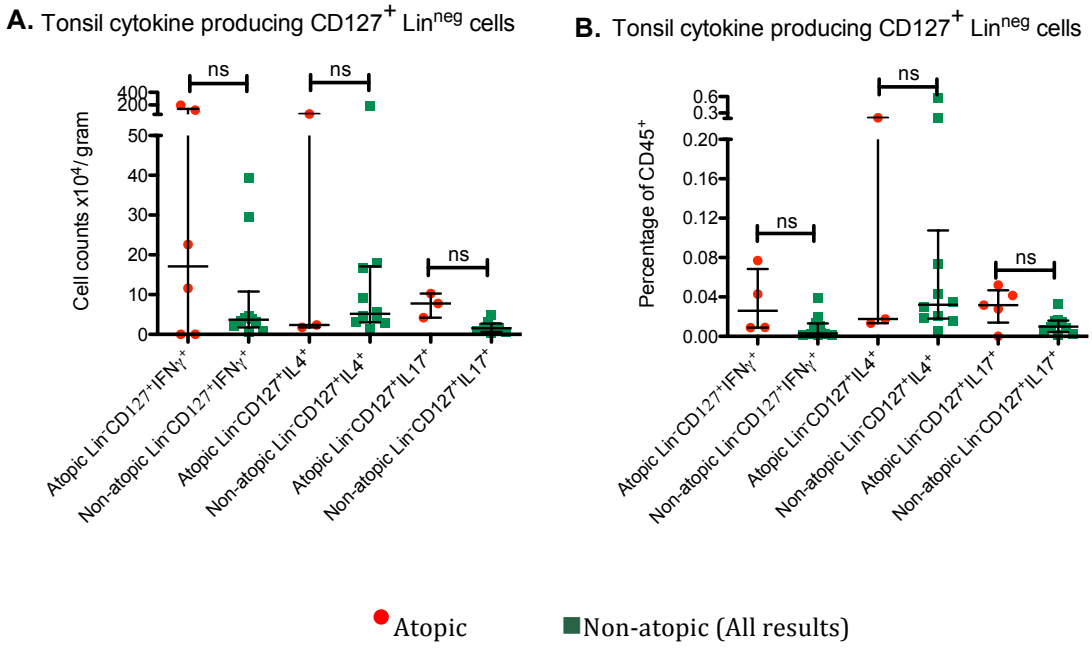


Figure 6.2.9. A comparison of tonsil lin^{neg} $CD127^{+}$ and cytokine producing cells as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) (B.) between atopic (Red) and non-atopic (All results) (Green). All displayed values as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

Finally, no differences between atopic and non-atopic children were seen when innate lymphoid 2 cells were defined using transcription factor ($Lin^{-}Gata3^{+}$). IL-13+ cells were in such small numbers, analysis was not possible (Figure 6.3.1.).

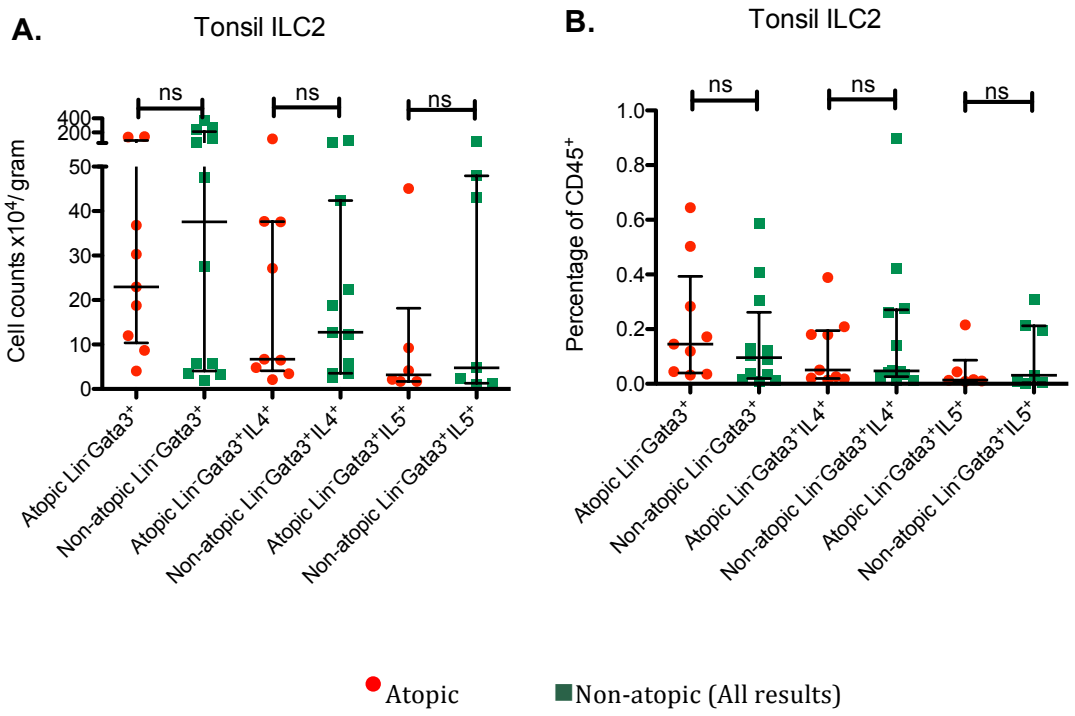


Figure 6.3.1. A comparison of ILC3s (Lin^-Gata3^+) as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) between atopic (Red) and non-atopic (All results) (Green) subjects. All displayed values as medians with interquartile ranges. Significance calculated using Mann-Whitney U-test.

6.5.2.c. ILCs in tonsil tissue and PBMCs comparing children with raised serum IgE vs normal serum IgE

The third phase of the ILC comparisons involved the use of total IgE as a definition of atopy with the cut off as <62IU for normal. Total lineage negative cells were found to be significantly higher in tonsil samples from subjects with a raised total IgE compared to those with a normal total IgE (median value $83.8x10^4$ (IQR $50x10^4$-$200.7x10^4$) in raised total IgE samples vs $21.5x10^4$ ($5.6x10^4$-$29.9x10^4$) in samples from subjects with normal total IgE, $p<0.0001$). This was also the case for the cells expressing extracellular markers CD127 and CRTH2 (median value $11x10^4$ (IQR $5.9x10^4$-$23x10^4$) in samples from subjects with raised total IgE vs $0.6x10^4$ (IQR $0.4x10^4$-$2.6x10^4$) in samples from subjects with normal total IgE, $p=0.0002$). The same was not observed when the cells were analysed as percentage of $CD45^+$ cells (Figure 6.3.2.). This data supported the previous findings of an emerging theme whereby atopic subjects have a higher proportion of tissue ILCs at least based on the extracellular markers. This was once again not the case with the results from

the PBMCs but it is important to note that many of the atopic results were extremely low (Figure 6.3.2).

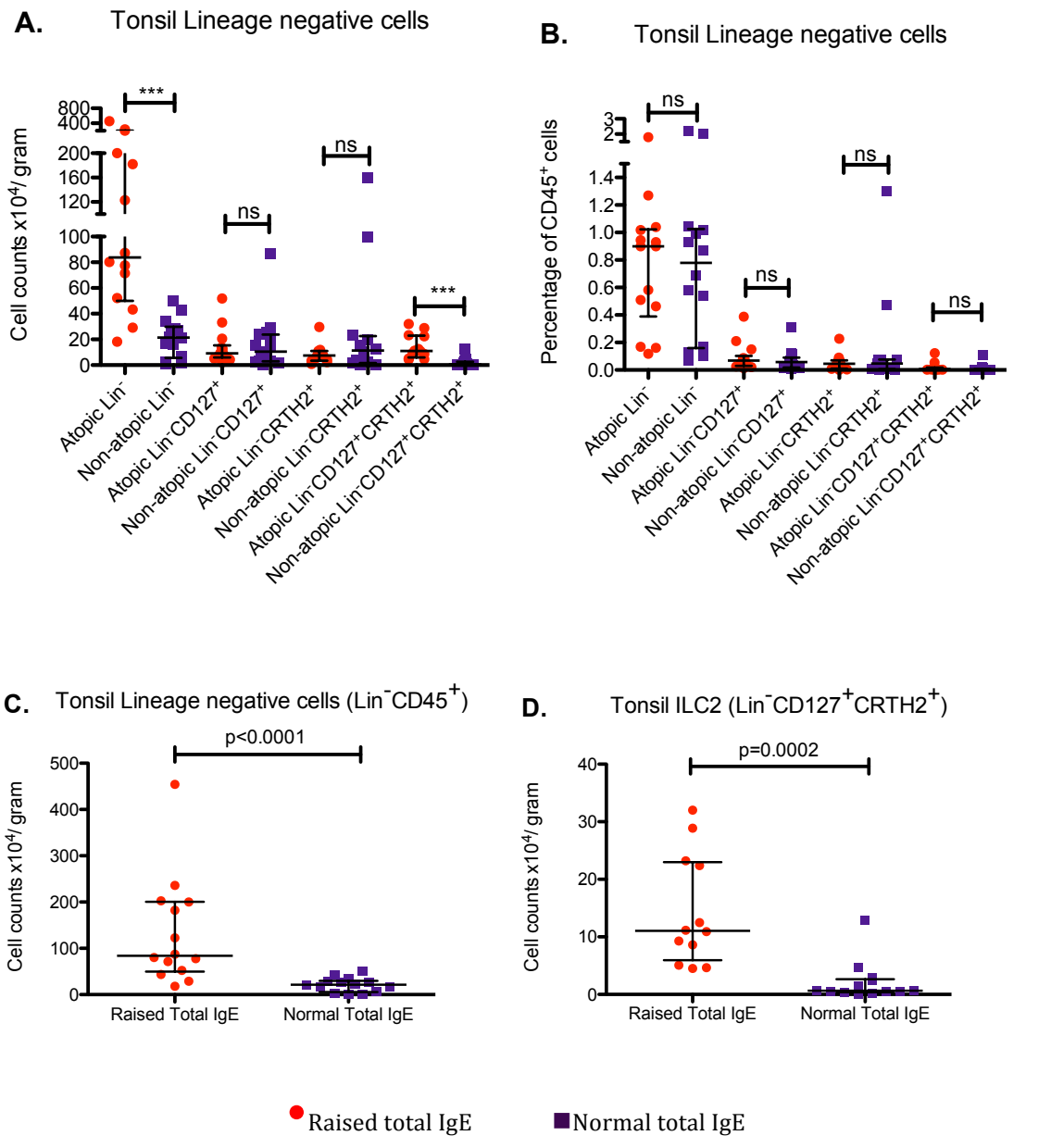


Figure 6.3.2. A comparison of tonsil lineage negative cells identified by published extracellular markers as cells per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) (B.) between subjects with raised total IgE (Red) and normal total IgE (Purple). The Lin^{neg} cells are significantly raised in the subjects with raised total IgE (C.). The ILC2s ($Lin^{neg}CD127^{+}CRTH2^{+}$) are significantly raised in the subjects with raised total IgE (D.). All displayed values as medians with interquartile ranges. Significance calculated by Mann-Whitney U-test

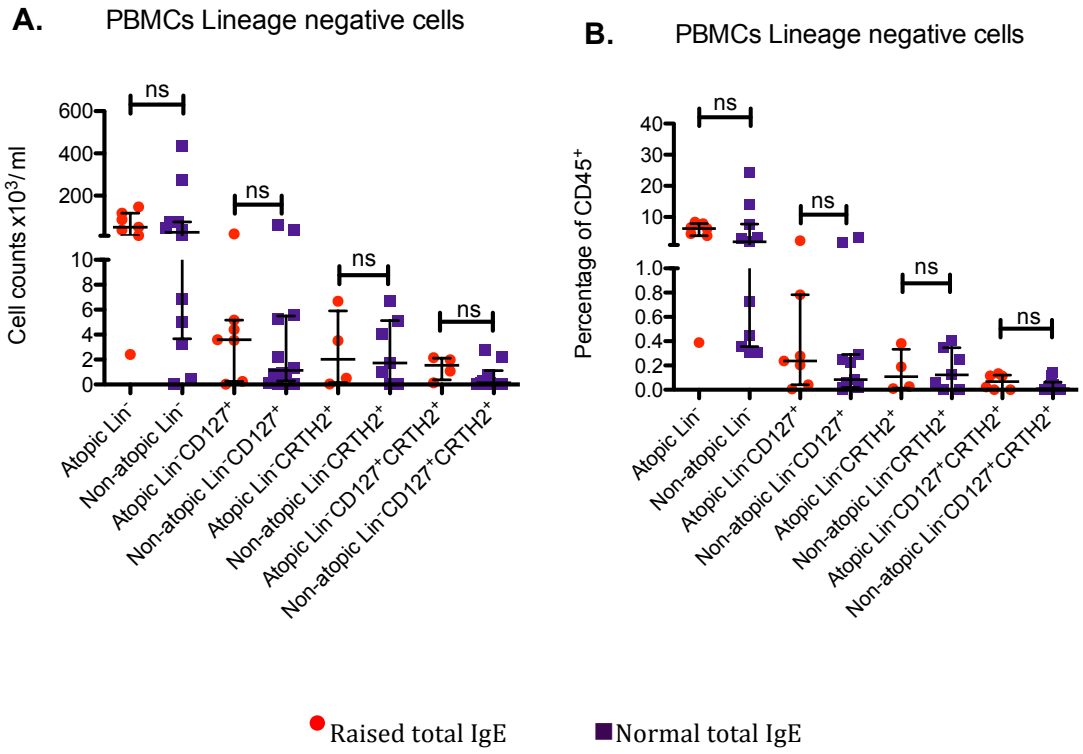


Figure 6.3.3. A comparison of PBMCs lineage negative cells identified by published extracellular markers as cell counts per ml of blood (A.) and percentage of total lymphoid cells (CD45+) (B.) between subjects with raised total IgE (Red) and subjects with normal total IgE (Purple). All displayed values as medians with interquartile ranges. Significance calculated by Mann-Whitney U-test

The tonsil samples were then compared according to lineage negative cells with the capacity to produce type 2 cytokines. No significant difference was observed as cell counts per gram of tissue or as percentage of $CD45^+$ cells between children with normal total IgE and those with a raised total IgE (Figure 6.3.4.).

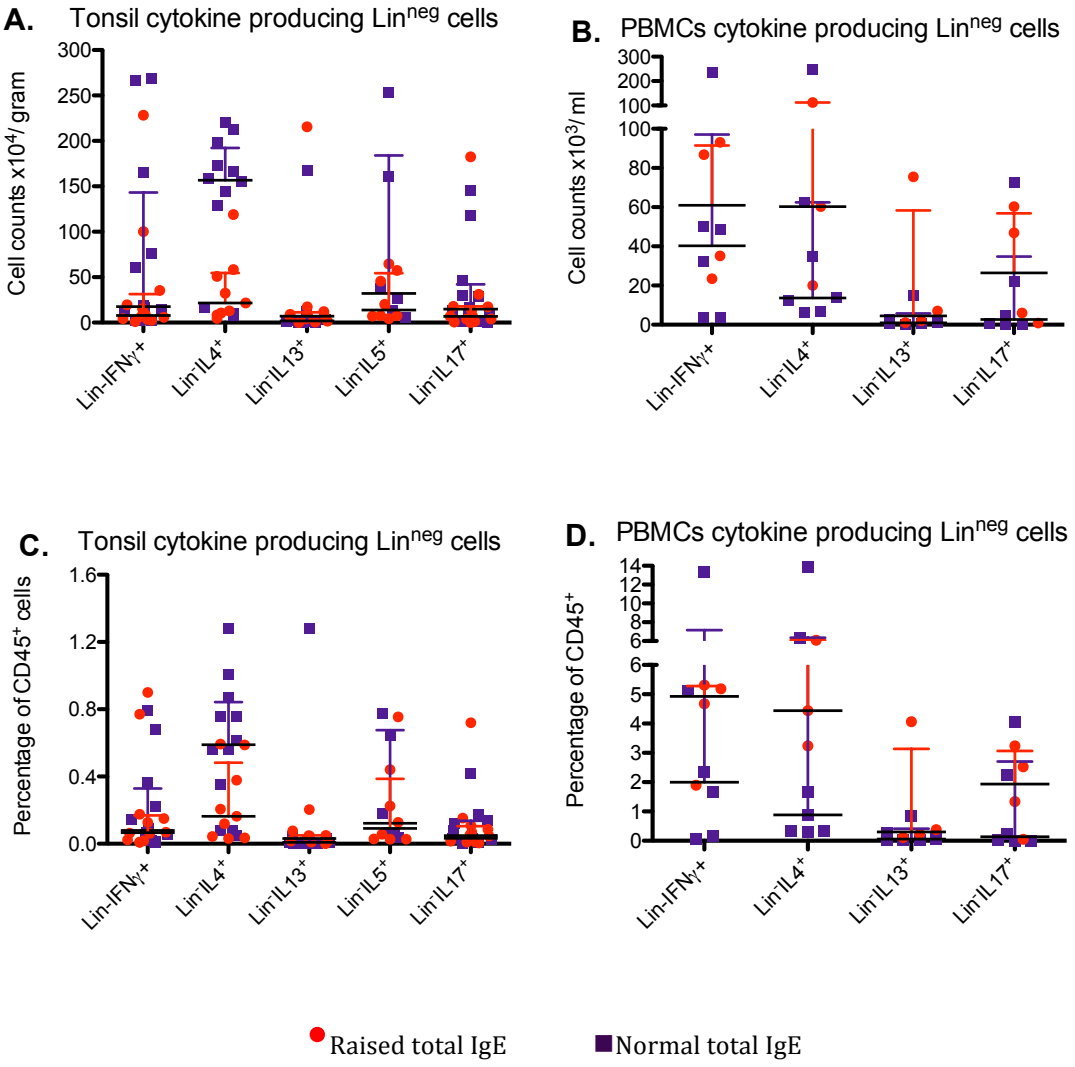


Figure 6.3.4. A comparison of tonsil lin^{neg} cytokine producing cells as cells per gram of tissue (A,) and percentage of total lymphoid cells (CD45+) (C.) between subjects with raised total IgE (Red) and subjects with normal total IgE (Purple). The same comparison this time with peripheral blood (PBMCs) comparing cells per ml of blood (B.) and percentage of total lymphoid cells (D.) All displayed values as medians with interquartile ranges.

The lineage negative cells expressing the receptor CD127 were then analysed for cytokine production. No significant difference was observed between samples from subjects with a raised total IgE and those with a normal total IgE (Figure 6.3.5.).

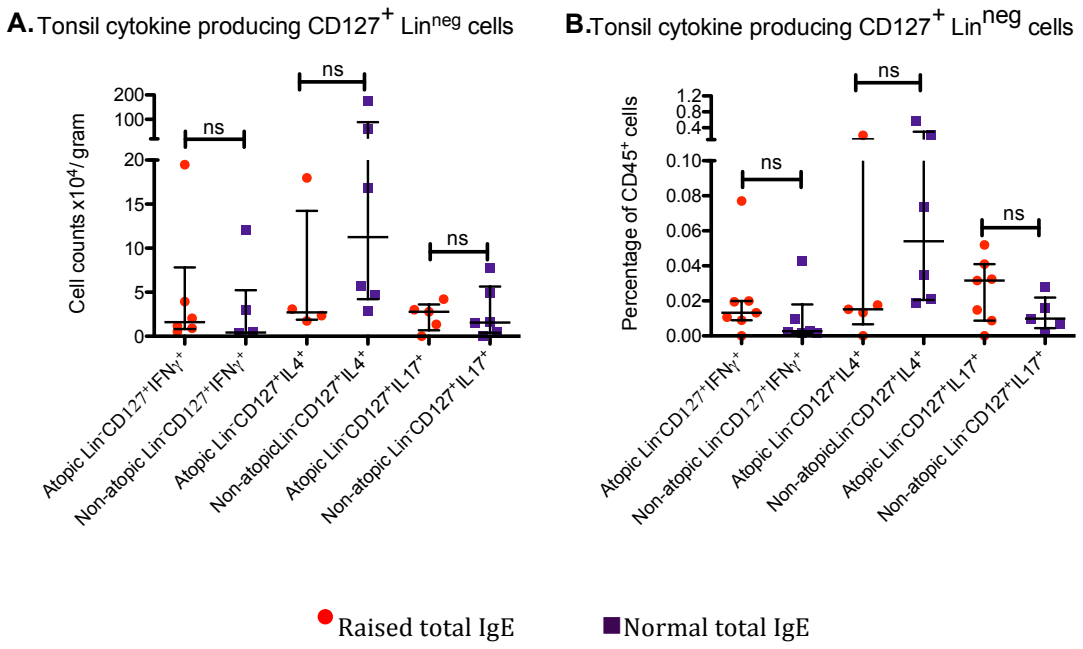


Figure 6.3.5. A comparison of tonsil lin^{neg}CD127^{+} and cytokine producing cells as cells per gram of tissue (A.) and percentage of total lymphoid cells (CD45+) (B.) between samples from subjects with raised total IgE (Red) against those with normal total IgE (Purple). All displayed values as medians with interquartile ranges. Significance calculated by Mann-Whitney U-test.

Finally, innate lymphoid group 2 cells defined by the presence of the Gata3 transcription factor were analysed. No difference was observed between the tonsil samples from subjects with raised total IgE and those with normal total IgE (Figure 6.3.6.).

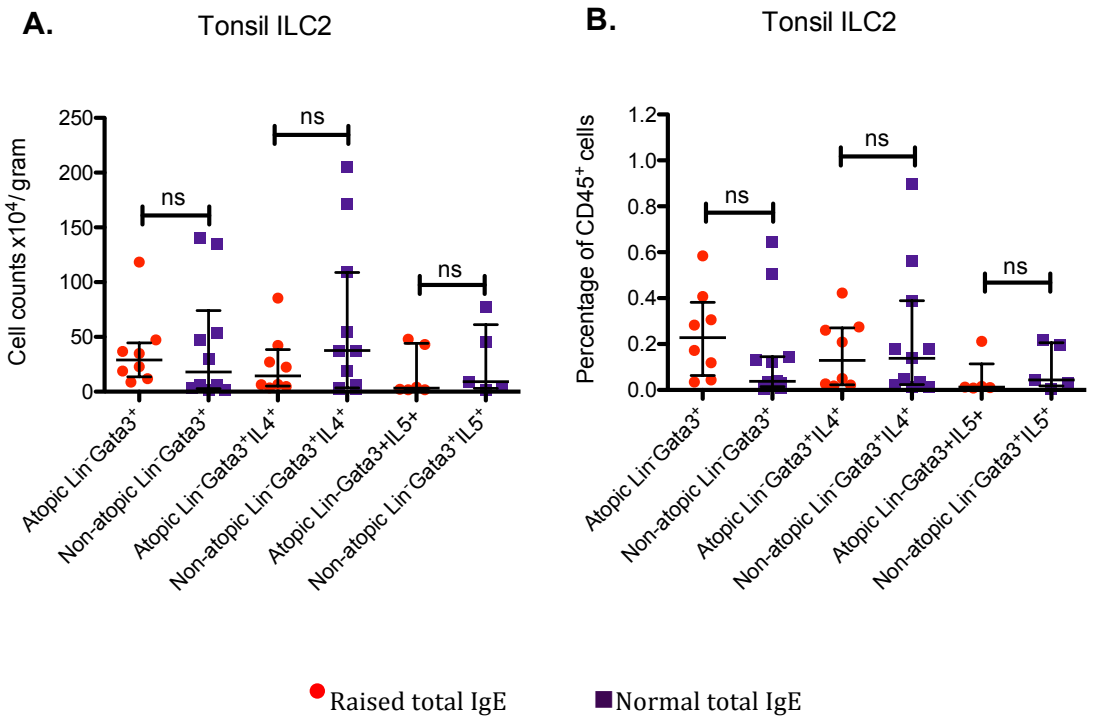


Figure 6.3.6. A comparison of tonsil ILC2s (Lin^-Gata3^+) as cell counts per gram of tissue (A.) and as percentage of total lymphoid cells (CD45+) (B.) between samples from subjects with raised total IgE (Red) against those with normal total IgE (Purple). All displayed values as medians with interquartile ranges. Significance calculated by Mann-Whitney U-test.

6.5.2.d. Tonsil Lineage negative cells vs total serum IgE.

Utilising the total IgE as a measure of atopy and not as a categorical value with a cut off, ILCs were plotted against the total IgE to see if a correlation existed. No relationship was apparent when comparing against cell counts per gram of tissue or as a percentage of total CD45+ cells.

6.6 Discussion

6.6.1. Principle findings

For both the tonsil tissue and peripheral blood samples, based on the comparisons I have performed, there were no differences in T-cells between atopic and non-atopic subjects. My

hypothesis that atopic children would demonstrate a stronger Th2 signature than non-atopic children was not true based on these analyses. The only significant finding was a higher proportion of IL-4 producing $CD4^+$T-cells amongst the non-atopic (all results) group. This result cannot be explained as the opposite finding would be physiologically logical. This finding was isolated and is not supported in any other results.

I have, however demonstrated that atopic children have increased tonsillar ILCs than the non-atopic children. When comparing the tonsil results from only those individuals with a complete set of specific IgE results to the aeroallergen panel, the $Lin^{neg}CD127^+$ cells were higher as cell counts per gram of tissue in the atopic individuals. This was also the case when the non-atopic group included subjects who had an incomplete set of specific IgE results and were non-atopic by default. When using the total IgE as the definition of atopy, this difference was not observed. However, total lineage negative cells, were higher in atopic individuals (those with raised total IgE) compared to the non-atopic.

Tonsil ILC2s defined using the extracellular markers $Lin^{neg}CD127^+CRTH2^+$ were higher in atopic individuals compared to non-atopic as percentage of total $CD45^+$ cells. This difference was true when using the non-atopic definition including and excluding insufficient IgE aeroallergen panel results. For the comparison using total IgE as the definition of atopy, the same cells were higher in tonsils from atopic subjects as cell counts per gram of tissue. To my knowledge, this is the first reported finding of this difference detected in paediatric tonsil tissue.

This finding was not apparent in peripheral blood. However, total lineage negative cells, in the atopic subjects were proportionally higher compared to the non-atopic suggesting the trend for higher numbers of ILCs in atopic subjects in the peripheral blood. It is however worth noting that none of these differences were observed when using cytokine production or the transcription factor Gata3 as a definition of the ILC groups. These differences were only observed using extracellular ILC markers.

6.6.2. Strengths and weaknesses of study

There is a possibility of selection bias with the analysis. Not all samples underwent the same level of analysis specifically the inclusion of the cytokines and transcription factors due to the development of the flow cytometry panel running concurrent with recruitment of

patients. The significant differences observed in the ILCs are based on extracellular marks alone however, the first 10 samples were excluded from analysis as the gating strategy for removing all lineage positive cells was not refined until the 11th recruited sample. I have no way of commenting on how those first 10 subjects samples may have impacted the results however, no active selection took place at recruitment. Those initial 10 recruits were no different to any other therefore this should have little impact in the data produced.

Despite standardizing and refining the sample collection, the processing of the specific IgE results on occasion was incomplete. The same group of employees following standard operating procedures processed all samples at the same laboratory. To maximize the data, I have grouped the subjects in three different ways: excluding those with incomplete specific IgE results; including those with an incomplete specific IgE results; using total IgE along as the definition. It is reassuring that there is evidence for higher ILCs in atopic subjects using all three definitions.

Since inception of this work, the research field regarding innate lymphoid cells has grown significantly. The roles transcription factors play in the development and definition of an ILC are widely described in the literature as well as the full range of cytokine a cell has the potential to produce in each group[146]. For example, ILC3s have been found to be a heterogeneous group comprising two sub-types based on the presence of the chemokine CCR6. The CCR6- ILC3s are sub-categorised again by the presence or absence of the natural cytotoxic receptor (NCR) and are both capable of producing IL-17 but also IL-22, TNF and GMC-SF[146].

One of the most significant discoveries in the field recently is the plastic nature of ILCs and their ability to switch subgroups and cytokine production in different environments. ILC3s expressing the RORγt transcription factor are capable of produce IFNγ after being exposed to IL-12 and IL-23 and down regulate the RORγt [275, 276]. IL-1β and IL-12 can induce ILC2s to express the ILC1 transcription factor T-bet and to produce IFN-γ while down-regulating ST2 and GATA3, and losing the ability to produce IL-5 and IL-13 [277] and enhanced GATA3 expression by ILC1s results in their conversion to ILC2s with the capacity to produce greater amounts of type 2 cytokines[278]. This feature of ILCs has expanded our appreciation of what the innate immune system is capable of.

Despite the advances in the field, the significant findings from my work involving total lineage negative cells as still credible as the definition of an ILC still includes a lack of conventional extracellular markers known to identify other immune cells and the IL-7 receptor CD127[146, 279]. My positive findings include one or both of these factors. With hindsight and taking into account the field as it stands, my flow cytometry panel would have needed to be more sophisticated to properly identify the ILC group 1, 2 and 3 subsets including their transcription factors as well as their full cytokine producing potential.

While the inclusion of the ILC2 transcription factor Gata3 and the ILC prototypical cytokines IL-4, IL-5 and IL-13 did not result in significant differences in the study populations, human ILC2 cells are recognized to possess the prostaglandin D-2 receptor named chemoattractant receptor expressed on Th2 cells (CRTH2)[144, 154]. My flow cytometry panel included this marker and showed significantly higher proportions of ILC2 cells in atopic subjects based on this definition.

6.6.3. Findings in relation to other studies

My finding of increased ILCs in the tonsils of atopic children has not been reported previously. I have identified higher proportions in the tonsil tissue compared to the peripheral blood, which is consistent with previous findings [153, 280]. Not only are ILCs now considered to be tissue-resident cells, they can be maintained and expand locally under different immunological conditions supporting the notion that these cells are sentinels and local keepers of tissue function [280].

The significant findings from my results are the higher proportion of lineage negative cells and specifically the ILC2s in the tonsils of atopic individuals. To recap, in response to IL-25 and IL-33 these cells secrete a typical Th2 pattern of cytokines namely IL-4, IL-5, IL-9 and IL-13 and require the transcription factor Gata3. While this has not been reported previously in children, all ILC subgroups have been identified in human tonsils [281] with ILC1s first being identified in this tissue along with human intestine [282]. Kouse and colleagues have looking at transcriptome profiling in human ILCs using paediatric tonsil samples as the source. No clinical information was published about the children however the number of ILC2s was found to be too small to analyse and compared to the ILC1 and ILC3 present [283]. Another publication has reported higher proportions of ILC3 cells in tonsils, with low frequencies of ILC1 cells and ILC2 cells[281]. This was not the finding from

my analysis however these publications have used more sophisticated definitions of an ILC3 including the NCR-/+ subtypes, IL-22 and the transcription factor RORγt. Modifications to my own flow cytometry panel may have found results with such modifications.

Poposki et al [284] looked at ILC2s in human tonsils extracted from adults but used the tissue as a disease control for the extracted polyps of subjects with chronic rhinosinositis. Some of the subjects from whom tonsils were extracted were atopic defined by plasma total and specific IgE however inter-group comparisons between the atopic and non-atopic subjects were not performed. Comparing peripheral blood, nasal polyp and tonsils, the predominant cell in the tonsil was once again NCR+ ILC3s (defined as $Lin^{neg}CD127^{+}$, $CRTH2^{-}CD1117^{+}NKp44^{-}$).

My finding of ILC2s in the context of atopy is consistent with the current understanding of their role in allergic pathology. ILC2s were first described in 2001 in mouse model as non-T/non-B cells capable of producing IL-5 and IL-13 leading to a type-2 response but were not defined till some 10 years later, initially as nuocytes [285]. In animal models of allergic airways disease ILC2-derived IL-13 is an essential inducer of airway hyper responsiveness, mucus hyper-secretion and inflammation[286, 287]. In mice with allergic lung inflammation, ILC2 populations expand in response to cytokines and immune activators, including IL-25, TSLP, IL-33 produced by type 2 pneumocytes, alveolar macrophages and granulocytes. The ILC2s then contribute to type 2 inflammation within the lung [288-292] even in the absence of $CD4^{+}$ T-cells.[147, 293-298]. Studies have also shown that the activation of ILC2 in these models occurs not only following intranasal IL-25 or IL-33, but also following the exposure to fungal aeroallergens such as *Alternaria alternata* [147, 148, 287, 293]. In all cases type2 cytokines released from ILC2 cells into the lungs is at least similar to that released from Th2 cells and may be greater than the Th2-dependent release [299].

As well as working independently, recent work has suggested that ILC2s coordinate with dendritic cells and $CD4^{+}$ T cells at mucosal sites to shape T-cell responses that contribute to allergic airway inflammation [300, 301]. The ILC2s and CD4+Th2 cells have a synergistic effect on the development of airway hyper-responsiveness in mouse models. The ILC2 produced IL-13 directly activated dendritic cells to express CCL17 enhancing CD4+Th2 cell activation. They also activate the Th2 cells by producing IL-4 [300, 302, 303]. In co-culture

with autologous B cells, ILC2 are also able to differentiate the cells and induce production of IgM, IgA and IgE in vitro [304, 305].

Research has also shown the ability of ILC2s to develop memory-like properties upon allergen exposure in a Th2 style. Again in the mouse model, ILC2s stimulated by inhalation of IL-33 or papain persisted long after the inflammatory response has ceased. These allergen-experienced ILC2s responded better to unrelated allergens than naïve ILC2s mediating a more allergic inflammation, producing higher amounts of cytokines. These memory-ILC2s are also able to be stimulated by specific allergens rather than cytokines like the naïve ILCs [306]. This may explain increased tonsil ILC2s in my atopic children without a Th2 phenotype to the tissue being that these cells are tissue bound and capable of propagating allergic disease without Th2 involvement.

In human adults ILC2s were found to be present in the peripheral blood of atopic individuals (defined by plasma total and specific IgE) in similar frequencies and numbers comparable with those found in non-atopic healthy subjects, but their ability to produce IL-4 in response to PMA/I appeared to be significantly higher [304]. When stimulated with IL-25 or IL-33, peripheral blood mononuclear cells from patients with allergic asthma produced significantly greater amounts of IL-5 and IL-13 than those from patients with allergic rhinitis and those from healthy donors[307]. Recently, ILC2s were found in the bronchoalvoelar lavage (BAL) and sputum of adult patients with asthma, and the proportion of IL-5+IL-13+ ILC2s in the sputum of patients with severe asthma was higher than that in corresponding samples from patients with mild asthma [152]. ILC2 levels are also increased in the sputum from severe asthmatic children [153].

Human ILC2s are robustly expanded in another type 2 inflammatory disease, chronic rhinosinusitis (CRS), which is characterized by pronounced eosinophilia and high IgE levels in serum. Elevated numbers of $CRTH2^+$ ILCs were observed in the nasal polyps of adult patients with CRS compared to control subjects. These cells responded to IL-2, IL-25 and IL-33 [154]. Recently, using the same definition, ILC2 enriched mucosa has also been found in the ethmoid sinus of adult patients with CRS and nasal polyps compared to controls with CRS without nasal polyps. This study also showed that the ILC2s from CRS patients responded to IL-33-mediated stimulation by producing IL-13 [172], further suggesting that ILC2s may contribute to the pathogenesis of allergic upper airway disease in humans.

Another prominent type-2 disease is atopic dermatitis, an inflammatory skin condition characterized by the presence of T_h2 cells and high levels of IL-5 and IL-13 in the skin. Recent work has suggested that ILC2s might be involved in the pathogenesis of atopic dermatitis, because the ILC2 population is expanded in the lesions of patients with atopic dermatitis [175, 176]. These cells are stimulated by TSLP and are also likely to be stimulated by IL-25 and IL-33, because increased transcripts of these cytokines have been detected in atopic dermatitis skin lesions [175-177]. Notably, the lectin inhibitory receptor KLRG1 may have a regulatory role in the control of ILC2 activation during atopic dermatitis, because the KLRG1 ligand epithelial cadherin (E-cadherin) diminishes ILC2 cytokine production, and E-cadherin is down-regulated in atopic dermatitis lesions [175].

6.7. Conclusion

In this chapter, the research question asked was whether the tonsils and peripheral blood from atopic children had higher proportions of Th2 cells and ILC2 cells compared to non-atopic children. The hypotheses proposed were that there would be more Th2 cells and ILC2 cells in the tissue obtained from atopic children. I did not find a higher proportion of Th2 cells and am therefore rejecting hypothesis no. 4. I did however identify an increased proportion of innate lymphoid cells, particularly the group 2 cells in tonsils from atopic children. This is a novel finding and supports our understanding of the role ILC2s play in the pathology of allergic disease. I am therefore accepting hypothesis 5.

Chapter 7: Final Discussion

The findings from the hypotheses tested for this work were as follows:

1. The incidence of OSA in children with Severe Therapy Resistant Asthma is not as high as the proposed incidence in the general non-asthmatic paediatric population. This hypothesis has been rejected.
2. There is no difference in rates of atopy based on clinical evaluation or laboratory confirmation between children undergoing surgical intervention for the treatment of OSA and those undergoing the same procedure for the treatment of recurrent tonsillitis. This hypothesis has been rejected.
3. The palatine tonsils from children with sleep study proven OSA do not have a higher proportion of Th17 cells, but do have fewer Treg cells than a control group without OSA. The same finding was present in the corresponding peripheral blood samples. In this case, the hypothesis has been accepted in part.
4. Tonsils from atopic subjects undergoing adenotonsillectomy do not contain a stronger Th2 cellular phenotype than children without evidence of atopy undergoing the same procedure. This hypothesis has been rejected.
5. There is an increased proportion of lineage negative cells, specifically type 2 innate lymphoid cells (ILC2s) in the tonsils from atopic children compared to children without evidence of atopy. This hypothesis has been accepted.

7.1. Principle findings in relation to other studies

7.1.1. The incidence of OSA in children with severe therapy resistant asthma is comparable with the suggested incidence in the general paediatric population.

The incidence of OSA in the STRA group was firstly not statistically significantly different compared to published data on the incidence in the general paediatric population and was also very mild compared to the severity reported in the Kheirandish-Gozal[241] trial of 'problematic asthmatics'.

The Kheirandish-Gozal study is not alone in identifying a link between OSA and asthma. Several other groups have employed sleep studies to confirm disease, used physician diagnosis of asthma and identified an association between the two (Redline et al 1999[25],

Goldstein 2015[239]). There are also numerous studies using a combination of parent reported sleep disordered breathing or parent reported diagnosis of asthma and wheeze with mixed outcomes as discussed in chapter 1. There are several possibilities as to why my data did not show a higher incidence and severity. Firstly there is the size of my study population being smaller than that of the published studies, Kheirandish-Gozal[241] by factor of 4.6, Redline[25] by factor of 19 and Goldstein[239] by a factor of 10. The severe therapy resistant asthmatic (STRA) children were used to answer this research question owing to the fact that they have severe disease therefore increasing the chances of detecting sleep disordered breathing, if an association existed. This is however a rare asthma phenotype resulting in a small study population and one in which sleep disordered breathing has not been addressed previously making a power calculation difficult to perform. However, as all children defined as STRA during the study period were captured and only mild OSA was found in 3 out of the 21, it is unlikely that a larger study population would result in a more significant finding.

Consideration needs to be made regarding the ethnicity of the children involved. All three of the STRA children in my study were non-Caucasian. While some large-scale studies do take into consideration ethnicity when performing multiple regression analysis, none of the studies named in the paragraph above took this into consideration including the Kheirandish-Gozal study, which contained a considerable amount of African American children with an ethnic predisposition to OSA.

The very nature of this group is also an explanation for the findings. STRA patients have severe disease and therefore are receiving a considerable amount of anti-inflammatory medication many of which are also indicated for the treatment of mild to moderate OSA and persistent disease after surgical intervention. Their medication burden is higher than that for subjects in the published studies above and may have already reduced the severity of disease before undergoing a sleep study. In comparison to my study population, the children in the studies named above have milder asthma were an association has been demonstrated.

A third reason for my conflicting finding also relates to the study group or more precisely the definition of severe asthma. As well as their severe nature, this group was chosen because they are a well-defined asthmatic population with a clear and logical rationale for their diagnosis based on multifactorial and multidisciplinary assessment. Much of the

published research into childhood asthma particularly concerning the link between asthma and OSA can be criticised for the diagnosis of the asthma used. Lack of measurements such as lung function and bronchodilator reversibility, lack of physician diagnosis and no consideration to compliance with therapy make the diagnosis of asthma less reliable. Severity based on patient or parent perceptions of disease can be open to interpretation without formalized measurements of severity and frequency of exacerbation. The Royal Brompton Problematic asthma protocol used to define STRA children is a gold standard use to diagnosis severe disease. Therefore, the children in my study are the best defined amongst the other named studies and a lack of association with OSA has been more reliably demonstrated.

7.1.2. Children undergoing tonsillectomy for proven OSA do not have a higher incidence of atopy than a disease control group.

Using children without evidence of OSA undergoing tonsillectomy for recurrent tonsillitis as a control group, I found no difference in rates or proportions of atopy or atopic disease compared to children with OSA (5/24 (20%) in the OSA group, 10/21 (48%) in the control group). This was based on clinical history as well as serum analysis for evidence of sensitization.

While there are several large-scale studies published suggesting a relationship between OSA and atopy (see Chapter 1), the majority of previously published work relies on questionnaire definitions for both OSA and atopy and in many cases uses snoring as a surrogate marker of OSA. Two studies of note however utilised a sleep study to diagnose OSA and serum analysis for evidence of sensitization (Carr et al 2007[242], McColly et al 1997[231]). While these studies lacked a control group, neither found a strong association between atopy and OSA. My interpretation of the relationship between OSA and atopy backed up by my own findings is that atopy, and more precisely, atopic disease predisposes a child to a congested and obstructed upper airway, which will impact sleep but not necessarily result in obstructive sleep apnoea. A study comparing the sleep study results of children with upper airway allergic disease such as rhinitis and a disease free control group would be a useful method of investigating this hypothesis.

The majority of the published studies looking at an association between OSA and atopy use reports of snoring as a surrogate marker of sleep disordered breathing and OSA. My data

has demonstrated that there is no association and that snoring does not imply OSA in these children.

7.1.3. Children with OSA have a lower proportion of T-regulatory cells in tonsils and peripheral blood compared to control subjects.

There is evidence from my data that children with OSA have fewer T-regulatory (Treg) cells in their tonsils and peripheral blood than children without OSA suggesting that a lack of inflammatory regulation may result in tissue hyper-proliferation and upper airway congestion leading in OSA. While my data did not show a difference in tonsil dimensions or weight between those with OSA and those without, this pathology may have more significance in other tonsillar compartments that make up the Waldeyer's Ring of the upper airway such as the adenoids.

While my data is the first to discuss Tregs in the palatine tonsils compared to a control group, they have been implicated in the pathology of OSA previously in other body compartments. Several authors have reported a reduction in Tregs in tonsil, adenoidal tissue and peripheral blood along with an increase in Th17 cells. My results did not show the same findings for Th17 cells however that may be due to the nature of the control group. These children had undergone recurrent infections that led to the tonsil removal. Their tonsils may already be at a heightened inflammatory state, hence no difference in Th17 cells between OSA and control subjects. This is unfortunately a hypothesis that is difficult to test being ethically not appropriate to extract normal tonsils tissue from well children. However, in a mouse model, group A Streptococcus infection has been shown to induce an IL-17 response, Th17 signature cytokine in upper airway tissue [308].

7.1.4. The tonsils of atopic children have a higher proportion of innate lymphoid cells particularly ILC group 2 cells compared to non-atopic.

In the tonsil tissue of children with confirmed atopy based on serum analysis of sensitization to specific IgE to an aeroallergen panel, there was a high proportion of lineage negative cells expressing the extracellular marker CD127 and therefore consistent with the description of innate lymphoid cells (ILCs). While not statistically significant, the lineage negative cells in peripheral blood were also higher amongst non-atopic individuals. In addition to these findings, The ILC group 2 cells (ILC2s) defined as $Lin^{neg}CD127^{+}CRTH2^{+}$ were also significantly raised in atopic individuals.

ILCs are tissue-resident cells with clear evidence of not only their interaction and synergistic function with the adaptive immune system but also their role in immune homeostasis and disease pathology independently. As with the ILC2s identified in the nasal polyps of subjects with chronic allergic rhinitis or the cells identified in allergic dermatitis, the tissue resident ILC2s in the mucosa of the upper airway could also be responsible for allergic disease pathology and airway compromise.

7.2. Strengths

For the study of OSA amongst STRA children, the strength of this work is in the strict criteria used to define the patient groups in OSA, asthma and atopy. As discussed previously, the field was and still is lacking large-scale studies addressing this research question with robust criteria of disease definition. The children in my study underwent either the gold standard for OSA diagnosis, polysomnography or the UK standard of cardiorespiratory polygraphy to diagnose their sleep disordered breathing. Coming through the problematic asthma protocol, they have also been rigorously investigated to confirm firstly whether they truly have asthma and secondly that their disease is severe enough to warrant tertiary level care and treatment.

The same principle of strict criteria for group definition applies to the work studying atopy amongst children with proven OSA. Unlike many of the published studies on the subject, my work not only utilised sleep studies to prove and in many cases exclude OSA in the subjects recruited, I also employed techniques to define and quantify the rates of atopy amongst them. The use of the home ambulatory sleep studies while employed mainly to boost recruitment, also strengthens the definitions of disease particularly as they were used exclusively in the control group ruling out OSA. The inclusion of a control group in itself strengthens my findings and has been lacking in many previous publications on the subject of OSA, asthma and atopy.

The nature of the control group needs to be considered. This was a diseased group with a different pathology, therefore some findings may have been masked by the fact that the control subjects tonsil immunology was not normal or altered. While acknowledging this, the state of our knowledge of the immunology of the normal paediatric airway is lacking and any information on numbers or phenotypes of cells is enormously valuable.

For the conclusions based on the cellular analysis of the tonsils and peripheral blood samples obtained, I have used the same cellular markers for Tregs as previously published on the subject strengthening my agreement with previous findings. This is in part true with regards to my ILC data. The definition of an ILC being lineage negative and possessing the IL-7 receptor CD127 is still recognized in the literature despite the field expanding significantly since the inception of this work. My work with the ILCs has also demonstrated that the commercially available lineage negative cocktail used for studies is not really strong enough in lymphoid tissue with a great deal of B-cells present. Further modification may be needed to ensure lineage negativity in the ILCs.

7.3. Limitations

Regarding the OSA amongst STRA, there is the question of the real world implications of this data. STRA children are a rare sub-group of paediatric asthma therefore any findings will only have relevance for a very small proportion of children. Without a control group of either non-asthmatic children or even children with asthma to a lesser severity, conclusions drawn from this data are limited. I am unable to comment on the relationship between OSA and asthma in general, as this was not studied.

When discussing the work exploring the rates of atopy amongst children with proven OSA, the lack of formalized questionnaires could be a criticism of the data collected. The children (parent or guardian) were asked a series of focused questions on signs and symptoms of sleep disordered breathing and their result compared and tabulated in Chapter 4. Formalised questionnaires are available and could have been used to strength this data however it was not of significant consequence particularly when sleep study data was available for the majority of the group. A formalized questionnaire for signs and symptoms of atopy may also have been useful to strengthen the findings of clinical atopic disease. Obviously, once again, larger numbers would result in more reliable findings.

With regards to my cellular work with the tonsil tissue and peripheral blood, the aim was to investigate inflammation in the upper airway that could be contributing to OSA pathology. I have in part found evidence of failure to control inflammation in the Treg results and the possible role for upper airway ILC2s in allergic inflammation from the tonsil samples but to truly explore the upper airway pathologies, I could have expanded my sample collection to include induce sputum, bronchoalveolar lavage, cells from the nasopharynx. This would

have address the question of upper airway inflammation more effectively.

The reduction in Tregs in children with OSA is a significant finding and suggests a potential failure of anti-inflammatory control as an underlying pathology to the disease. However, any conclusion made can only be speculative as my work and that of others has only addressed the naturally occurring Tregs and has not considered adaptive Tregs or the array of other immune cells capable of anti-inflammatory function. This must be kept in mind when drawing conclusions.

For both the findings regarding Tregs and ILC2s, only observed proportions of the cells can be commented upon. The functionality of these cells has not been studied in this work. Higher or lower proportions may have no implications on pathophysiology if functionally the cellular output in terms of cytokine production and activity is the same in each comparison. The functional suppressive capacity of Tregs or the cytokine output for effector ILCs needs to be addressed before clinical implications made based on these results.

7.4. Future work

This project consisted of several directions of research addressing the overall question from different angles. It therefore has the potential to spawn several different avenues for future work, which I will discuss now individually.

7.4.1. OSA in asthmatic children

To further address this question, I would like to expand the criteria for asthmatic children to include different severities. I have not found evidence of a relationship between OSA and STRA but as discussed previously, this is a rare group of patients with mitigating circumstances such as medication load. Assessment of milder asthmatics would be useful to address the question of a relationship between OSA and asthma using the strict criteria I employed and therefore providing a definitive answer to the question. If no relationship exists in milder asthmatics, children could be assessed for evidence of sleep disordered breathing without a co-existing asthma diagnosis automatically resulting in the need for an unnecessary sleep study referral.

The problematic asthmatic cohort contains children referred, investigated and eventually labeled as difficult asthmatics (those with modifiable factors which if addressed will

improve their condition) or STRA. Investigating all children going through the protocol would increase numbers but also allow for comparison between the groups exploring my theory that those with the higher medication burden may be masking OSA severity. The effect ethnicity would need to be taken into consideration when analyzing the results.

7.4.2. Atopy in children with OSA

The first alteration for future work I would employ would be to increase my numbers. A larger study population with a comparable sample size to previously published work and with the same findings would result in a very clear message that atopy is not associated with formally diagnosed OSA. While the diagnosis of OSA is already robust particularly if all children undergoing adenotonsillectomy had sleep study evidence to prove or deny disease, I propose exploring the effects of atopy further by focusing on the upper airway. Studies have suggested that the tonsils themselves display evidence of sensitization [261]. Homogenizing the tissue and processing it in a similar fashion to the peripheral blood or staining the tissue using immunohistochemical techniques looking for IgE may highlight tissue specific sensitization impacting on disease.

Further exploration of the impact of sensitization of the upper airway would include analysis of the adenoids. This tissue has more ability to expand and regrow compared to the palatine tonsils that I studied. Evidence of sensitization in this tissue associated with hyper-proliferation would have considerable impact on airflow through the upper airway and therefore disease. As modern extraction techniques for the adenoids result in tissue destruction, alterations in surgical procedure would be needed for recruited subjects and ethical approval to do so.

7.4.3. Anti-inflammatory immune function and OSA

Investigating the proportions of Tregs in the adenoidal tissue of children with OSA would be my desired next direction of work. A correlation between pharyngeal tonsil, palatine tonsil and peripheral blood all showing a reduction in Tregs would be a very interesting finding. I would also include the cytokine IL-10 widening the categories of Tregs analysed to include adaptive cells. The obtained tissue could also be utilized further not only to identify Tregs but also to locate them within structures. As mentioned in Chapter 2, solid paraffin preserved sections of the palatine tonsils have been obtained. Immunohistochemical techniques can be employed to locate and orientate the Tregs within the tissue and

comparisons made between OSA and non-OSA. Their location may have implications for pathology such as whether they are lacking at epithelial level of within germinal centers.

A limitation of my work thus far is that there is no functional data on the cells identified. This would be a direction I would endeavor to pursue with future work. Using flow cytometry cell sorting, a pure population of Tregs could be isolated and RNA sequencing techniques used to assess different Treg cells present. The separated cells could then be cultured and suppression assays performed to look at the effectiveness of Tregs in the context of OSA measuring cytokine production and proliferative markers. A lack of functionality to the cells may also have implications to the proposed pathophysiology.

Another direction to explore would be the function of the epithelium of the tonsils in the context of OSA. Immunological regulation of the mucosal epithelium has been shown to be very important in lung and intestine. The same may be true of the upper airway mucosal layer. For example the epithelial tight junctions in asthma has been proposed a weakness of the immune defense allowing for antigen presenting cells exposure to antigens resulting in an inflammatory response.

7.4.4. The role of ILC2s in allergic disease pathology

Future proposed work on this subject would firstly involve further development of the flow cytometry panel. To determine more precisely the ILC populations in a sample, a flow cytometry cell sort could be employed once again to isolate this rare cell group, using the lineage negative markers I employed, before RNA sequencing used to identify the transcription factors and expanding groups of cellular markers and cytokines attributed to the ILC subgroups. While my work is pointing towards an ILC2 directed pathology with atopy, it is important not to exclude the other cell types especially in light of ILC3s being found in higher proportions in tonsils compared to ILC2s.

As with the Tregs proposed work, the function of these cells needs to be explored within allergic disease. Once isolated, these cells could be cultured in vitro and by assessing cytokine production and proliferative markers studied in a variety of environments such as the presence of corticosteroids. Evidence of reduced inflammatory activity could point to targeted medical therapies against the ILC mediated disease. Linking this work with other similar research questions would also be of benefit and maximise potential outcomes. A colleague in my laboratory group has been looking at ILCs in the context of STRA children,

all of whom were atopic. By combining our data, we were able to demonstrate how ILCs were in a higher proportion amongst atopic children in a range of tissues including bronchoalveolar lavage, induced sputum, peripheral blood and tonsil tissue (figure 7.1). Further collaborative work could look at the tissue resident cells, their function and differences in different environments.

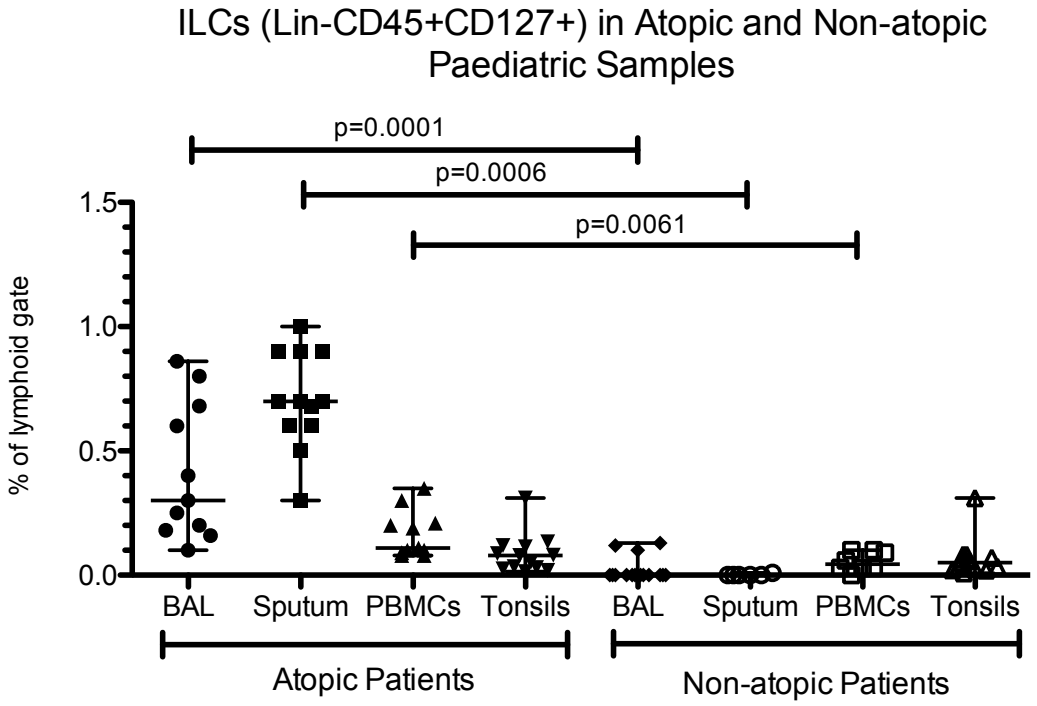


Figure 7.1. Collaborative work combining data on total ILCs in four different compartment in children (BAL, Sputum, PBMCs and tonsils) demonstrating a higher proportion in atopic individuals defined by the presence of positive specific serum IgE tests to an aeroallergen panel. Data demonstrated is percentages of the lymphoid gate (CD45+) with median valued and interquartile ranges. Significance tested by Mann-Whitney U-test.

7.5 Conclusion

This thesis presents the generation of an original research question, its study design including the successful application for ethical approval, it's implementation and recruitment, the processing of samples, data analysis and finally the presentation of the outcomes with proposals for future work.

The results have contributed to the proposed relationship between obstructive sleep apnoea, asthma and atopy in children. I have rejected my original hypothesis and demonstrated that there is no increased association between severe therapy resistant asthma and OSA. I have also demonstrated that there is no association between atopy and OSA and postulated that allergic disease has the potential to compromise the upper airway, disturbing sleep without actually reaching diagnostic criteria of OSA thus disproving my second hypothesis.

I have added to the research field suggesting a role for diminished T-regulatory cells in OSA pathology and while acknowledging the limitations of my study, proposed a direction for future work exploring the functionality of these cells. I have also contributed to the expanding field of work exploring the role innate lymphoid cells play in disease pathology by identifying higher proportions within the palatine tonsil of atopic individuals. This finding supports the emerging role ILCs play in allergic disease.

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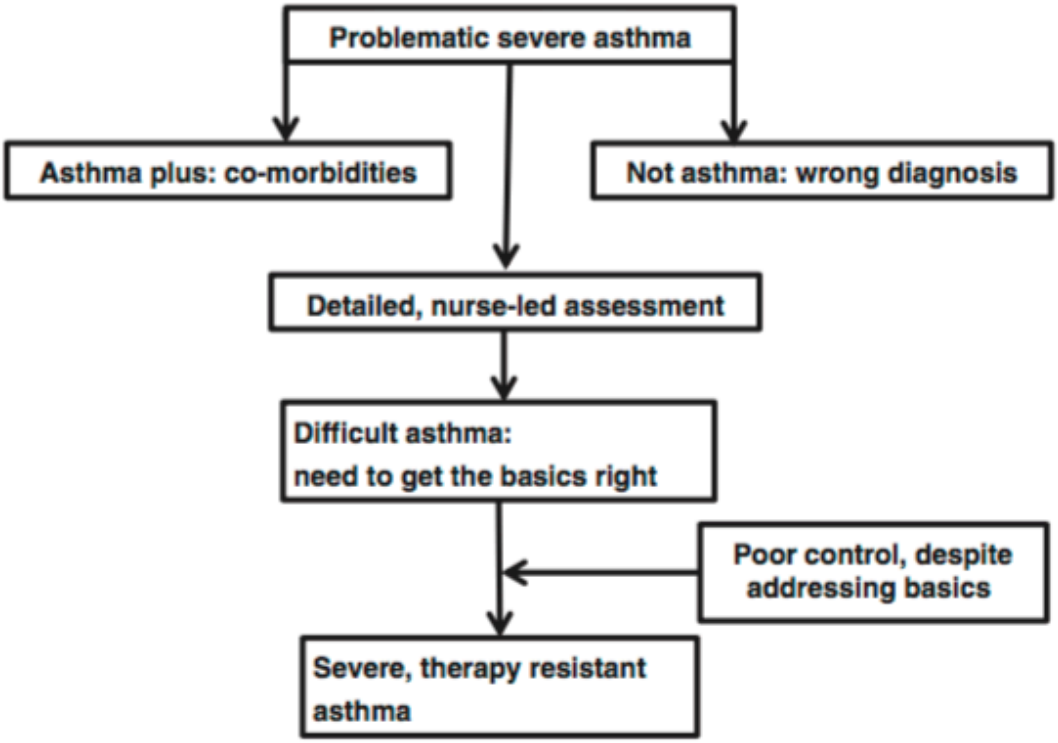
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Appendix 1. Figure 1. Problematic severe asthma protocol. Bush et al. Respirology 2017

Appendix 1: Problematic asthma protocol



Appendix 1. Figure 1. Problematic asthma protocol. Bush et al. Respirology 2017.

Appendix 2: The Asthma control tests

In the past 4 weeks how much of the time did your asthma keep you from getting as much done at school or home?				
All of the time **1**	Most of the time **2**	Some of the time **3**	A little of the time **4**	None of the time **5**
In the past 4 weeks how often have you had shortness of breath?				
More than once a day **1**	Once a day **2**	3-6 times a week **3**	Once or twice a week **4**	Not at all **5**
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?				
4 or more nights a week **1**	2 to 3 nights a week **2**	Once a week **3**	Once or twice **4**	Not at all **5**
In the past 4 weeks how often have you used your rescue inhaler or nebulizer solution?				
3 or more times per day **1**	1 to 2 times per day **2**	2 to 3 times per week **3**	Once a week or less **4**	Not at all **5**
How would you rate your asthma control over the past 4 weeks?				
Not controlled at all **1**	Poorly controlled **2**	Somewhat controlled **3**	Well controlled **4**	Completely controlled **5**

TOTAL SCORE /25

Appendix 3: Paediatric asthma quality of life questionnaire.

PAEDIATRIC ASTHMA QUALITY OF LIFE QUESTIONNAIRE WITH STANDARDISED ACTIVITIES (PAQLQ(S))

I want you to tell me how much you have been bothered by your asthma during the past week. I will tell you which card to use. Pick the number that best describes how much you were bothered by your asthma during the past week.

A	1.	How much have you been bothered by your asthma in **PHYSICAL ACTIVITIES** (such as running, swimming, sports, walking uphill/upstairs and bicycling) during the past week? [BLUE CARD]
A	2.	How much have you been bothered by your asthma in **BEING WITH ANIMALS** (such as playing with pets and looking after animals) during the past week? [BLUE CARD]
A	3.	How much have you been bothered by your asthma in **ACTIVITIES WITH FRIENDS AND FAMILY** (such as playing during school break and doing things with your friends and family) during the past week? [BLUE CARD]
S	4.	How much did **COUGHING** bother you in the past week? [BLUE CARD]
E	5.	How often did your asthma make you feel **FRUSTRATED** during the past week? [GREEN CARD]
S	6.	How often did your asthma make you feel **TIRED** during the past week? [GREEN CARD]
E	7.	How often did you feel **WORRIED, CONCERNED, OR TROUBLED** because of your asthma during the past week? [GREEN CARD]
S	8.	How much did **ASTHMA ATTACKS** bother you during the past week? [BLUE CARD]
E	9.	How often did your asthma make you feel **ANGRY** during the past week? [GREEN CARD]
S	10.	How much did **WHEEZING** bother you during the past week? [BLUE CARD]
E	11.	How often did your asthma make you feel **IRRITABLE (grumpy*)** during the past week? [GREEN CARD] (*use only if patient does not understand the word "irritable")
S	12.	How much did **TIGHTNESS IN YOUR CHEST** bother you during the past week? [BLUE CARD]
E	13.	How often did you feel **DIFFERENT OR LEFT OUT** because of your asthma during the past week? [GREEN CARD]

S 14. How much did **SHORTNESS OF BREATH** bother you during the past week? [BLUE CARD]

E 15. How often did you feel **FRUSTRATED BECAUSE YOU COULDN'T KEEP UP WITH OTHERS** during the past week? [GREEN CARD]

S 16. How often did your asthma **WAKE YOU UP DURING THE NIGHT** during the past week? [GREEN CARD]

E 17. How often did you feel **UNCOMFORTABLE** because of your asthma during the past week? [GREEN CARD]

S 18. How often did you feel **OUT OF BREATH** during the past week? [GREEN CARD]

A 19. How often did you feel **YOU COULDN'T KEEP UP WITH OTHERS** because of your asthma during the past week? [GREEN CARD]

S 20. How often did you have trouble **SLEEPING AT NIGHT**, because of your asthma, during the past week? [GREEN CARD]

E 21. How often did you feel **FRIGHTENED BY AN ASTHMA ATTACK** during the past week? [GREEN CARD]

A 22. Think about all the activities that you did in the past week. How much were you bothered by your asthma doing these activities? [BLUE CARD]

S 23. How often did you have difficulty taking a **DEEP BREATH** in the past week? [GREEN CARD]

DOMAIN CODE:

S = **Symptoms**
A = **Activity Limitation**
E = **Emotional Function**

Appendix 4: Sleep questionnaire

Sleep Symptoms		**Comment**
Snoring	**yes/no**	
Apnoea's	**Yes/no**	(duration, how many)
Arousals	**Yes/no**	
Mouth breathing	**Yes/no**	
Morning headache	**Yes/no**	
Excessive daytime sleepiness	**Yes/no**	
Difficulty concentrating	**Yes/no**	
Behavioural problems	**Yes/no**	
Usual bed time	**Yes/no**	
Usual wake time	**Yes/no**	

Appendix 5: Modified Epworth sleepiness score

Modified Epworth Sleepiness Scale - Ages 3-17yrs *(Parent to complete)*

Daytime Description (please circle)

When your child wakes in the morning, how do they feel?	Refreshed Tired/Sleepy Irritable
Does your child have headaches first thing in the morning?	Yes No
If yes, how many days a week?	1 2 3 4 5 6 7
Is your child sleepy during the day?	Never Rarely Occasionally Frequently Always
Does your child take naps during the day? If yes, how many naps per day?	Never Rarely Occasionally Frequently Always 1 2 3 4 5 6

In the following situations, what are the chances of your child falling asleep? (please tick)

Situation	No Chance	Slight Chance	Moderate Chance	High Chance
1. Sitting and Reading				
2. Watching TV				
3. Sitting inactive in a public place (eg Cinema)				
4. As a passenger in a car for 1 hour with no break				
5. Lying down in the afternoon				
6. Sitting and talking to someone				
7. Sitting quietly after lunch				
8. In a car, while stopped for a few minutes in traffic				

Has a teacher been concerned about your child being sleepy or inattentive at school?	Yes No
Has your child fallen asleep at school?	Never Rarely Occasionally Frequently Always
What time of day does your child seem most alert?	AM PM

Appendix 6: Parent information leaflets

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton Hospital
Sydney Street
London
SW3 6NP

PARENT INFORMATION SHEET
Children aged 0-5 years with Obstructive Sleep Apnoea
Obstructive Sleep Apnoea in children with atopic disease.



Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Invitation paragraph
We would like to invite your child to take part in a research study. Before you decide whether you want your child to take part, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your child to take part.
Thank you for reading this.

What is the purpose of the study?
Obstructive sleep apnoea (OSA, which is when breathing stops temporarily during sleep because the airway becomes blocked) is now recognised as a common health problem in children. As many as 3% of children are affected and it can lead to poor growth and intellectual development. It is an inflammatory disorder of the upper airways usually due to enlargement of the tonsils and/ or adenoids. Treatment may involve medical management (tablets and/or nose sprays), which has varying levels of success or surgical removal of the enlarged tissues.

There are links with inflammatory disorders of the lower airways such as asthma and other allergic diseases. There is already a significant amount of research suggesting links between these disorders.

We plan to explore this relationship further with more robust diagnoses of both OSA and allergies and would like to learn more about the cells in the enlarged tonsil and adenoids causing the obstruction.



Why has my child been invited?
Your child is being asked to participate in this research study because they have been diagnosed with OSA and an ear, nose and throat (ENT) specialist has decided that it would be best for their adenoids and/or tonsils to be removed.

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 be asked to sign a consent form to show you have agreed to take part. You may choose not to participate in this study or you may leave the study at any time without giving a reason. This would not affect the standard of care you receive.

What will happen to my child if they take part?
Your child will experience no change to their planned clinical care. If after discussion with the ENT surgeon you all decide that surgical removal of the obstruction is necessary, surgery will be scheduled at either Chelsea & Westminster Hospital or The Royal Brompton Hospital. The tissues removed are usually discarded. We aim to collect the tissues along with as much data as possible without inconveniencing you or your child. No additional meetings will be required other than those planned as part of your child's clinical course.

What will I have to do?
After the treatment options are explained and if you and your child decide to progress to surgery, your child will be suitable for this research and their involvement would be as follows:

1. Once you are fully informed and happy for your child to participate, you as the parent sign the consent form. You then complete a series of questionnaires about your child's health lasting approximately 30 minutes.
2. You will also be asked to fill out a questionnaire with supplementary video clips to see whether your child has ever suffered from wheeze.
3. You will also have the option for your child to have a skin prick test performed by the investigator. This involves placing a small amount of a substance called an allergen, which is a concentrated liquid containing parts of whatever is being tested for on your child's arm. Our common practice is to test against cat dander, dog dander, grass pollen, tree pollen, house dust mite and a fungus called aspergillus but we can add others in if there is something you suspect your child is allergic to or has had a reaction to in the past(depending on whether we have the allergen in our kit).
A small scratch is then made in the skin allowing the dot of liquid to react. If a small swelling develops, this is suggestive of an allergy to the allergen in that dot.



your child from the study. We will explain the reasons and arrange for your care to continue.

What if I do not want my child to carry on with the study?
If you do not want your child to take part in this study, you will receive standard care as determined by your doctor. Your participation in this study is voluntary and you may withdraw your child from the study at any time without prejudice to your future medical care. Should you decide to withdraw your child from the study for any reason, you are asked to contact Dr Christopher Grime (study coordinator) immediately. Should your child's participation in the study be stopped, regardless of the reason, you will not suffer any penalties or loss of benefits to which you are otherwise entitled.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure- PALS, The Royal Brompton Hospital - 02073497715, pals@rbht.nhs.uk.

In an emergency, please call the on-call paediatric respiratory registrar at The Royal Brompton Hospital who is contactable 24 hours a day via the switch board- 02073528121

Harm
In the event that something does go wrong and your child is harmed during the research and this is due to someone's negligence, you may have grounds for legal action for compensation against the Royal Brompton and Harefield NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. NHS indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm.

Will my child's involvement in the study be kept confidential?
All information collected about your child during the course of the research will be kept strictly confidential. Any information about your child that leaves the hospital will have their name, address and personal details removed so that your child cannot be recognised from it. We will request your consent before informing your child's GP about their participation in the study.

Will my child's General Practitioner / Family doctor (GP) be informed of my involvement?
Provided you consent to this, your child's GP will be informed that your child is participating in the study and kept informed of their medical progress.

What will happen to the results of the research study?
Your child's medical records will be made available for review by the study investigators and regulatory authorities (who periodically check that the studies are being carried out correctly). The information in these records will be kept confidential but on rare occasions

your child from the study. We will explain the reasons and arrange for your care to continue.

What if I do not want my child to carry on with the study?
If you do not want your child to take part in this study, you will receive standard care as determined by your doctor. Your participation in this study is voluntary and you may withdraw your child from the study at any time without prejudice to your future medical care. Should you decide to withdraw your child from the study for any reason, you are asked to contact Dr Christopher Grime (study coordinator) immediately. Should your child's participation in the study be stopped, regardless of the reason, you will not suffer any penalties or loss of benefits to which you are otherwise entitled.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure- PALS, The Royal Brompton Hospital - 02073497715, pals@rbht.nhs.uk.

In an emergency, please call the on-call paediatric respiratory registrar at The Royal Brompton Hospital who is contactable 24 hours a day via the switch board- 02073528121

Harm
In the event that something does go wrong and your child is harmed during the research and this is due to someone's negligence, you may have grounds for legal action for compensation against the Royal Brompton and Harefield NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. NHS indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm.

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All information collected about your child during the course of the research will be kept strictly confidential. Any information about your child that leaves the hospital will have their name, address and personal details removed so that your child cannot be recognised from it. We will request your consent before informing your child's GP about their participation in the study.

Will my child's General Practitioner / Family doctor (GP) be informed of my involvement?
Provided you consent to this, your child's GP will be informed that your child is participating in the study and kept informed of their medical progress.

What will happen to the results of the research study?
Your child's medical records will be made available for review by the study investigators and regulatory authorities (who periodically check that the studies are being carried out correctly). The information in these records will be kept confidential but on rare occasions

Parent Information Sheet ages 0-5 years OSA- SDB & Atopy version no. 3 date 297/01/2014 4

the law may require disclosure to third parties. At the end of the project all the research results are gathered together and analysed. The researchers have a professional responsibility to publish their findings, however your child's identity will not be revealed. Most research is published in the medical press – if you are interested in knowing the overall results of the study, ask the researchers about this.

Who is organising and funding the research?
The Paediatric Respiratory Department at The Royal Brompton Hospital and The Leukocyte Biology department at Imperial College London are paying for this research to be carried out. The doctor conducting the research is a member of the paediatric respiratory team.

Who has reviewed the study?
All research in the NHS is looked at by an Independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by NRES Committee London-Riverside. In addition approval has been gained from local R&D Offices and the Medicines and Health Regulatory Authority (MHRA) at The Royal Brompton and Harefield Hospital NHS Trust.

Contact for Further Information
If you would like any further information about the study, either now or at time during the course of the study, please ask a member of the Research Team:

Dr Christopher Grime
Pediatric Sleep Research Fellow
The Royal Brompton Hospital
Email: C.Grime@rbht.nhs.uk
Tel: 020 73528121 (ext. 2267)

Thank you for taking the time to consider this study. If you do choose to participate, you will be given a copy of this information sheet to keep and also a copy of the consent form that you will be asked to sign.



Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton Hospital
Sydney Street
London
SW3 6NP

PARENT INFORMATION SHEET
Children aged 0-5 years - Controls

Obstructive Sleep Apnoea in children with atopic disease.

Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital



Invitation paragraph
We would like to invite your child to take part in a research study. Before you decide whether you want your child to take part, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish your child to take part.
Thank you for reading this.

What is the purpose of the study?
Obstructive sleep apnoea (OSA, which is when breathing stops temporarily during sleep because the airway becomes blocked) is now recognised as a common health problem in children. As many as 5% of children are affected and it can lead to poor growth and intellectual development. It is an inflammatory disorder of the upper airways usually due to enlargement of the tonsils and/ or adenoids. Treatment may involve medical management (tablets and/or nose sprays), which has varying levels of success or surgical removal of the enlarged tissues.

There are links with inflammatory disorders of the lower airways such as asthma and other allergic diseases. There is already a significant amount of research suggesting links between these disorders.

We plan to explore this relationship further with more robust diagnoses of both OSA and allergies and would like to learn more about the cells in the enlarged tonsils and/ or adenoids causing the obstruction.



Why has my child been invited?
Your child is being asked to participate in this research study because their ear nose and throat specialist has decided that it would be best for their adenoids and/or tonsil to be removed. Your child has not been diagnosed with OSA but would be acting as what we call a 'control' patient who is free of the disease and can be used to compare results against those who do have OSA.

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 be asked to sign a consent form to show you have agreed to take part. You may choose not to participate in this study or you may leave the study at any time without giving a reason. This would not affect the standard of care you receive.

What will happen to my child if they take part?
Your child will experience no change to their planned clinical care. If after discussion with the ENT surgeon you all decide that surgical removal of your child's tonsils and/ or adenoids is necessary, surgery will be scheduled at either Chelsea & Westminster Hospital or The Royal Brompton Hospital. The tissues removed are usually discarded. We would like to collect the tissue along with as much data as possible without inconveniencing you or your child. No addition meetings will be required other than those planned as part of your child's clinical course.

What will I have to do?
If you and your child decide to progress to surgery, your child will be suitable for this research and their involvement would be as follows:

1. Once you are fully informed and happy for your child to participate, you as the parent sign the consent form. You then complete a series of questionnaires about your child's health lasting approximately 30 minutes.
2. You will also be asked to fill out a questionnaire with supplementary video clips to see whether your child has ever suffered from wheeze.
3. You will also have the option for your child to have a skin prick test performed by the investigator. This involves placing a small amount of a substance called an allergen, which is a concentrated liquid containing parts of whatever is being tested for on your child's arm. Our common practice is to test against cat dander, dog dander, grass pollen, tree pollen, house dust mite and a fungus called aspergillus but we can add others in if there is something you suspect your child is allergic to or has had a reaction to in the past (depending on whether we have the allergen in our kit). A small scratch is then made in the skin allowing the dot of liquid to react. If a small swelling develops, this is suggestive of an allergy to the allergen in that dot.



4. We may also ask permission for a home oxygen saturation monitor to be worn overnight before your child's surgery. This is a very simple piece of equipment, which if used will be shown to you and your child at the initial consultation. It is worn by your child over night and measures their oxygen levels, heart rate and effort of breathing. It will then be delivered to your home and collected the following morning. This is an optional element to our research, which you can decline if you wish.
5. On the day of surgery, if you are happy for your child to continue to participate, we will collect the tissues removed during surgery. We would also like to collect a sample of blood from your child, which will be taken while they are under the general anaesthetic.

Involvement in this study should not impose any restrictions to your child's current lifestyle. There are no specific dietary restrictions with the exception of those related to the surgery proposed in which case the ENT team will keep you full informed. Your child should continue taking any medication they are currently prescribed unless instructed otherwise. The skin prick tests performed and blood taken for analysis during the anaesthetic are all addition steps and would not necessarily be performed as part of your child's proposed care.

What are the possible disadvantages and risks of taking part?
There are no anticipated disadvantages to taking part in this work. As stated above, data and tissue collection will take place as part of your child's planned clinical care. There will be no addition interventions with the exception of optional skin prick testing and blood sampling both of which can take place during the anaesthetic if you and your child wish. The blood can be taken via the cannula (drip) that the anaesthetist will need to insert as part of the general anaesthetic process. There is also the possibility of a home saturation monitoring mentioned above, which is again optional.

What are the side effects when taking part?
The skin prick test is a commonly performed procedure. The results are small skin reactions to the droplet of allergens (cat, dog, grass etc.) that can be measured with a special ruler. Some children may experience slightly larger skin reactions that once measured may require a cream to the site or a dose of antihistamine orally to treat. The investigator collecting all the data and instigating all the tests will be a senior paediatric respiratory trainee and can administer any treatment required.

Are there any benefits to my child if they participate in the study?
Other than the allergy tests, there are no direct benefits of participation for your child. The results of the allergy tests will be posted to you during the course of the work. Anything we learn about these conditions may benefit other children in the future.

What happens when the research study stops?

At the end of the research your care will continue as usual. If there are any samples we obtained from your child left over after our planned investigations we would like to keep them for future research of a similar nature but only if you agree to this.

What if relevant new information becomes available?
Sometimes during the course of a research study, new information becomes available about the diseases that are being studied. If this happens, we will tell you about it and discuss with you whether you want your child to continue in the study. If you decide not to carry on we will make arrangements for your child's care to continue. Also, upon receiving new information we might consider it to be in your child's best interests to withdraw you from the study. We will explain the reasons and arrange for your child's care to continue.

What if I do not want my child to carry on with the study?
If you do not want your child to take part in this study, you will receive standard care as determined by your doctor. Their participation in this study is voluntary and you may withdraw them from the study at any time without prejudice to their future medical care. Should you decide to withdraw them from the study for any reason, you are asked to contact Dr Christopher Grime (study co-ordinator) immediately. Should your child's participation in the study be stopped, regardless of the reason, you will not suffer any penalties or loss of benefits to which you are otherwise entitled.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure- PALS, The Royal Brompton Hospital - 02073497715, pals@rbht.nhs.uk.

In an emergency, please call the on-call paediatric respiratory registrar at The Royal Brompton Hospital who is contactable 24 hours a day via the switch board- 02079528121

Harm
In the event that something does go wrong and your child is harmed during the research and this is due to someone's negligence, you may have grounds for legal action for compensation against the Royal Brompton and Harefield NHS Foundation Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you. NHS indemnity does not offer no-fault compensation i.e. for non-negligent harm, and NHS bodies are unable to agree in advance to pay compensation for non-negligent harm.

Will my child's involvement in the study be kept confidential?
All information collected about your child during the course of the research will be kept strictly confidential. Any information about your child that leaves the hospital will have their name, address and personal details removed so that they cannot be recognised from it. We will request your consent before informing your child's GP about your participation in the study.

Will my child's General Practitioner / Family doctor (GP) be informed of my child's involvement?
Provided you consent to this, your child's GP will be informed that they child is participating in the study and kept informed of their medical progress.

What will happen to the results of the research study?
Your child's medical records will be made available for review by the study investigators and regulatory authorities (who periodically check that the studies are being carried out correctly). The information in these records will be kept confidential but on rare occasions the law may require disclosure to third parties. At the end of the project all the research results are gathered together and analysed. The researchers have a professional responsibility to publish their findings, however your child's identity will not be revealed. Most research is published in the medical press – if you are interested in knowing the overall results of the study, ask the researchers about this.

Who is organising and funding the research?
The Paediatric Respiratory Department at The Royal Brompton Hospital and The Leukocyte Biology department at Imperial College London are paying for this research to be carried out. The doctor conducting the research is a member of the paediatric respiratory team.

Who has reviewed the study?
All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee to protect your safety, rights, wellbeing and dignity. This study has been reviewed and given favourable opinion by NRES Committee London-Riverside. In addition approval has been gained from local R&D Offices and the Medicines and Health Regulatory Authority (MHRA) at The Royal Brompton and Harefield Hospital NHS Trust.

Contact for Further Information
If you would like any further information about the study, either now or at time during the course of the study, please ask a member of the Research Team:

Dr Christopher Grime
Paediatric Sleep Research Fellow
The Royal Brompton Hospital
Email: C.Grime@rbht.nhs.uk
Tel: 020 73528121 (ext. 2287)

Thank you for taking the time to consider this study. If you do choose to participate, you will be given a copy of this information sheet to keep and also a copy of the consent form that you will be asked to sign.

Appendix 7: Child information leaflets

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS
NHS Foundation Trust

PATIENT INFORMATION SHEET
Children aged 0-5 years with OSA
Obstructive Sleep Apnoea in children with atopic disease.

Why did I have a sleep study:
We studied you last night to see if you snored or have noisy breathing when you sleep.



Why might this happen:
Maybe you have big tonsils which ring like bells when you sleep making that funny noise.

What are tonsils:
Round balls of pink tissue at the back of your throat.



What can I do about it:
You will be seen by an ear, nose and throat doctor who can take out your big tonsils.

If the doctor takes them out, we want to collect and test them so we can learn more about children like you.



Will it hurt:
If they are removed, you will be asleep and won't know anything about it. When you wake up, you might have a sore throat and will only be able to eat soft things like jelly or ice-cream for a little while.

Will I need a blood test:
We want to test your blood and skin for allergies too. We can do this when you are asleep having your tonsils taken out. This is just for us to learn more about you and not part of your treatment.

Do I have to do it:
No. And you can change your mind at any time if you want to.

Thank you.



Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS
NHS Foundation Trust

PATIENT INFORMATION SHEET
Children aged 0-5 years -Controls
Obstructive Sleep Apnoea in children with atopic disease.

Why am I being seen today:
You might be having lots of sore throats and are being seen by an ear, nose and throat doctor to see if they can help.



What can I do about it:
The doctor might suggest you have your tonsils taken out.

What are tonsils:
Round balls of pink tissue at the back of your throat.
If the doctor takes them out, we want to collect and test them so we can learn more about children like you.



Will it hurt:
If they are removed, you will be asleep and won't know anything about it. When you wake up, you might have a sore throat and will only be able to eat soft things like jelly or ice cream for a little while.

Will I need a blood test:
We want to test your blood and skin for allergies too. We can do this while you are asleep having your tonsils taken out. This is just for us to learn more about you and not part of your treatment.

We might also ask if we can check your breathing at night with a clever little machine you wear on your finger.

Do I have to do it:
No. And you can change your mind at any time if you want to.

Thank you.



Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton Hospital
Sydney Street
London
SW3 6NP

PATIENT INFORMATION SHEET
Children aged 5-10 years with OSA

Obstructive Sleep Apnoea in children with atopic disease.

Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital



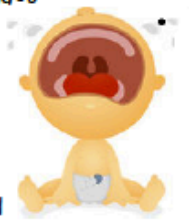
Why did I have a sleep study?

We studied you last night to see if you snored or have noisy breathing when you sleep. This can affect how well air moves in and out of your chest. When air doesn't move properly, we call is **obstructive sleep apnoea** which means pauses in breathing due to an obstruction.

Why might this happen?

Maybe you have big tonsils which make your throat narrow causing the obstruction. When you are asleep and relaxed, big tonsils can make you snore. We think they are big

because you might have allergies. That's when things like grass or dog hair can make you sneeze or have a runny nose.



What are tonsils?

Round balls of pink tissue at the back of your throat. They are either side of the bit that dangles down which is called the Uvula.

What can I do about it?



You will be seen by an ear, nose and throat doctor who can take out your big tonsils.
If the doctor takes them out, we want to collect them and test them for allergies. We also want to test you for allergies too so we can learn more about children like you and find better ways to treat you.

What are allergies?

When someone is allergic, they can become very snotty, sneeze a lot and develop a rash on their body when they come into contact with something they are allergic to like grass, cats, dogs etc. We are trying to learn more about allergies and your tonsils will help us.

Will it hurt?

If they are removed, you will be asleep and won't know anything about it. When you wake up, you might have a sore

throat and will only be able to eat soft things like jelly or ice-cream for a little while.

Will I need a blood test?



We want to check how big your lungs are by asking you to blow into our lung volume measuring machine call a spirometer.
We also want to test your blood and your skin for allergies too. We can do both these things while you are asleep having your tonsils taken out. This is just for us to learn more about you and not part of your treatment.

Do I have to do it?

No! And you can change your mind at any time if you want to.

Thank you!

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton Hospital
Sydney Street
London
SW3 6NP

PATIENT INFORMATION SHEET
Children aged 5-10 years - Controls

Obstructive Sleep Apnoea in children with atopic disease.

Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Why am I being seen today?



It might be because you have lots of sore throats or throat infections and are being seen by an ear, nose and throat doctor to see if they can help.

What can be done about it?

The doctor might suggest you have your tonsils taken out to stop all your throat infections.

What are tonsils?



Round balls of pink tissue at the back of your throat. They are either side of the bit that dangles down which is called the Uvula.
If the doctor takes them out, we want to collect them and test them for allergies. We also want to

test you for allergies too so we can learn more about children like you.

What are allergies?
When someone is allergic, they can become very snotty, sneeze a lot and develop a rash on their body when they come into contact with something they are allergic to like grass, cats, dogs etc. We are trying to learn more about allergies and your tonsils will help us.

Will it hurt?
If they are removed, you will be asleep and won't know anything about it. When you wake up, you might have a sore throat and will only be able to eat soft things like jelly or ice-cream for a little while.

Will I need a blood test?
We want to check how big your lungs are by asking you to blow into our lung volume measuring machine call a spirometer.



We want to test your blood and your skin for allergies too but we can do that while you are asleep having your tonsils taken out so you won't feel a thing. This is just for us to learn more about you and not part of your treatment.

We might also ask to check your breathing overnight with a clever little device you wear on your finger while you sleep.

Do I have to do it?
No! And you can change your mind at any time if you want to.

Thank you!



Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

Royal Brompton Hospital
Sydney Street
London
SW3 6NP

PATIENT INFORMATION SHEET
Children aged over 10 years with Obstructive Sleep Apnoea

Obstructive Sleep Apnoea in children with atopic disease.



Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Invitation paragraph
We would like to invite you to take part in a research study. A research study is when we investigate why things happen, to understand them and make better treatments in the future. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what it will involve for you. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.
Thank you for reading this.

What is the purpose of the study?
Obstructive sleep apnoea (OSA, which is when breathing stops temporarily during sleep because the airway becomes blocked) is a common health problem in children. It is usually due to enlargement of the tonsils and/ or adenoids, structures at the



back of the throat. Treatment may involve medical management (tablets and/or nose sprays), which doesn't always solve the problem or surgery to remove the enlarged tonsils.

There is already research linking OSA to conditions like asthma and other allergic diseases. We plan to look at this more closely to learn more about the cells in the enlarged tonsils and/or adenoids causing the obstruction.

Why have I been invited to take part?
You have been asked to participate in this research study because you have OSA and the ear nose and throat doctor has decided that it would be best to have your tonsils and/ or your adenoids removed.

Do I have to take part?
No, it is up to you. If you decide to take part you will have this information sheet to keep and be asked to sign a permission form to show you have agreed to take part. You may choose not to participate in this study or you may leave the study at any time without giving a reason. This will make no change to your care.

What will happen if I take part?
You will experience no change to your planned treatment. If you agree to have your tonsils and adenoids removed we would like to collect them. They would usually be thrown away. We will try to collect as much tissue as possible without inconveniencing you.

What will I have to do?
If you decide to have your tonsils and/ or adenoids taken out, you will be suitable for this research and your involvement would be as follows:

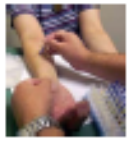
1. Your parents sign the permission form allowing you to take part and you sign a form showing you are willing to take part too. We will need you to then complete a series of questionnaires about your health lasting approximately 30 minutes.
2. We will then measure your lung function using a device called a spirometer. This involves blowing as hard and fast as you can into a machine, which records the force and calculates your lung capacity. We would like to do this twice, 20 minutes apart. Before the second attempt, we will give your an inhaler with a medicine to open up your airways called Salbutamol





(Tonsils).

3. You will also have the option for skin prick tests. This involves placing a small amount of a substance called an allergen, which is a concentrated liquid containing parts of something you might be allergic to. A small scratch is then made in the skin allowing the dot of liquid to react. If a small swelling develops, this might suggest an allergic reaction.



4. On the day of surgery, if you are still happy to take part, we will collect the tonsils and/ or adenoids removed. The doctors usually throw them away. We would also like to collect a sample of blood from you, which will be taken while you are asleep for surgery.

Involvement in this study should not impose any restrictions to your lifestyle. You should continue taking any medication you are currently prescribed unless instructed otherwise. The skin prick test and blood sample taken are just for our research and not necessarily part of your planned care.

What are the possible down sides of taking part?
There are no anticipated down sides to taking part in this work. Data and tonsil collection will take place as part of your hospital visits. The only difference is the optional skin prick testing and blood sampling both of which can take place while you are asleep.

What are the side effects of taking part?
The skin prick test can cause a rash which might need either a cream or a one off dose of a medicine call antihistamine. The investigator will be a senior doctor who can administer any treatment required.

Are there any benefits to me if I participate in the study?
You will learn more about any possible allergies you might have and if we learn more about OSA you might benefit from the results in the future.

What happens when the research study stops?
At the end of the research, your care will continue as usual. If there are any of the samples we collected from you left and you are in agreement, we might keep them for future research on the same subject.

What if I do not want to carry on with the study?

If you don't want to take part in this study, you will receive the same care as planned by your doctor. You may withdraw from the study at any time without any problems. If you decide to withdraw, tell your parents who can contact the research team.

What if there is a problem?
If you have a concern about any aspect of this study, you should speak to your parents about it who will have the contact details of the researchers on their information sheet. We will do our best to answer your questions.

Will my taking part in the study be kept confidential?
All information collected about you will be kept strictly confidential. Any information about you that leaves the hospital, will have your name, address and personal details removed so that you cannot be recognised from it.

Thank you for taking the time to consider this study. If you do choose to participate, you will be given a copy of this information sheet to keep and also a copy of the permission form that you will be asked to sign.



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PATIENT INFORMATION SHEET
Children aged over 10 years - Control

Obstructive Sleep Apnoea in children with atopic disease.

Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital



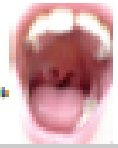
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Thank you for reading this.

What is the purpose of the study:

Obstructive sleep apnoea (OSA, which is when breathing stops temporarily during sleep because the airway becomes blocked) is a common health problem in children. It is usually due to enlargement of the tonsils and/ or adenoids, structures at the back of the throat. Treatments may involve medical management (steroid and/or nose sprays), which doesn't always solve the problem or surgery to remove the enlarged tonsils.



There is already research linking OSA to conditions like asthma and other allergic diseases. We plan to look at this more closely to learn more about the cells in the enlarged tonsils and/ or adenoids causing the obstruction.

Why have I been invited to take part:

You do not have OSA, but are being considered for removal of your tonsils and/ or adenoids for other reasons. We would like to recruit you as a 'control' subject. This is someone whose results we can compare with children who do have OSA.

Do I have to take part:

No, it is up to you. If you decide to take part you will have this information sheet to keep and be asked to sign a form showing you have agreed to take part. You may choose not to participate in this study or you may leave the study at any time without giving a reason. This will make no change to your care.

What will happen if I take part:

You will experience no change to your planned treatment. If you agree to have your tonsils and adenoids taken out we would like to collect them. They would usually be thrown away. We will try to collect as much data as possible without inconveniencing you.

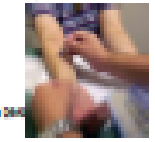
What will I have to do:

If you decide to have your tonsils and/ or adenoids taken out, you will be suitable for this research and your involvement would be as follows:

1. Your parents sign a permission form allowing you to take part and you sign a form showing you are willing to take part too. We will need you to complete a series of questionnaires about your health lasting approximately 30 minutes.
2. We will then measure your lung function using a device called a spirometer. This involves blowing as hard and fast as you can into a machine, which records the force and calculates your lung capacities. We would like to do this twice, 30 minutes apart. Before the second attempt, we will give you an inhaler with a medicine to open up your airways called Salbutamol (Ventolin).







a. You will also have the option for skin prick tests. This involves placing a small amount of a substance called an allergen, which is a concentrated liquid containing parts of something you might be allergic to. A small scratch is then made in the skin allowing the drop of liquid to mix. If a small swelling develops, this might suggest an allergic reaction.

4. We might ask you to have your oxygen saturations checked overnight at home. This will involve us delivering a small piece of equipment for you to wear on your finger overnight. We will then have it collected the next morning. It will show us what your oxygen levels and heart rate is over night.
5. On the day of surgery, if you are still happy to take part, we will collect the sample and or steroids removed. The doctors usually throw them away. We would also like to collect a sample of blood from you, which will be taken while you are asleep for your surgery.

Involvement in this study should not impose any restrictions to your lifestyle. You should continue taking any medication you are currently prescribed unless instructed otherwise. The skin prick tests and blood sample taken are just for our research and not necessarily parts of your planned care.

What are the possible disadvantages of taking part:
There are no anticipated disadvantages to taking part in this work. Nose and throat collection will take place as part of your planned hospital visit. The only difference is the optional skin prick testing and blood sampling both of which can take place while you are asleep having your steroids removed if you wish.

What are the side effects of taking part:
The skin prick tests can cause a rash which might need either a cream or a one off dose of a medicine call prednisolone. The investigator will be a senior doctor who can administer any treatments required.

Are there any benefits to me if I participate in the study:
You will learn more about any possible allergies you might have.

What happens when the research study stops:
At the end of the research, your care will continue as usual. If there are any of the samples we collected from you left and you are in agreement, we might keep them for future research on the same subjects.

What if I do not want to carry on with the study:
If you don't want to take part in the study, you will receive the same care as planned by your doctor. You may withdraw from the study at any time without any problems. If you decide to withdraw from the study, your personal care remains the research team.

What if there is a problem:
If you have a concern about any aspect of the study, you should speak to your personal doctor, who will have our contact details on their information sheet. We will do our best to answer your questions.

Will my taking part in the study be kept confidential:
All information collected about you will be kept strictly confidential. Any information about you that leaves the hospital will have your name, address and personal details removed so that you cannot be recognised from it.

Thank you for taking the time to consider this study. If you do choose to participate, you will be given a copy of this information sheet to keep and also a copy of the permission form that you will be asked to sign.



Appendix 8: Consent forms

Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

CONSENT FORM- Parents

Obstructive sleep apnoea in children with atopic diseases



Centre Number:
Patient Identification Number for this trial:

Chief / Principal Investigator
Dr Hui-Lang Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Please initial box if you agree

1. I confirm that I have read and understand the information sheet dated 29/01/2014 (version 3) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my child's participation is voluntary and that I am free to withdraw at anytime without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of any of my child's medical notes and data collected during the study, may be looked at by responsible individuals from the research team, from regulatory authorities or from the NHS Trust, where it is relevant to my child taking part in this research. I give permission for these individuals to have access to my child's records. ☐
4. I consent for the samples obtained from my child to be used for research purposes as outlined in the information sheet dated 29/01/2014 (version 3). ☐
5. I also consent for my child's samples to be stored for future research on the same subject. ☐
6. I agree to my child's GP being informed of my participation in the study. ☐
7. I agree for my child to take part in the above research ☐

Name of child	Relationship to parent/ guardian	
Name of Parent/ Guardian	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed: 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

ASSENT FORM- Patients

Obstructive sleep apnoea in children and atopic diseases

Centre Number:
Patient Identification Number for this trial:



Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Please initial box if you agree

1. I confirm that I have read and understand the information sheet dated 29/01/2014 (version 3) for this study. I have had time to think about the information, ask any questions I have had and am happy with the answers. ☐
2. I understand that I don't have to take part and can pull out at any time without . giving a reason without my planned treatment being affected. ☐
3. I understand that any information about me collected as part of this study will be look after very carefully and stored safely. I give permission for my medical notes to be looked at. ☐
4. I give permission for the samples collected from me to be used in this study as described in the information sheet dated 29/01/2014 (version 3). ☐
5. I also give permission for my samples to be stored for future research on the same subject. ☐
6. I agree to my GP being informed of my participation in the study. ☐
7. I agree to take part in this research ☐

Name	Date	Signature
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Chelsea and Westminster Hospital NHS
NHS Foundation Trust
Royal Brompton & Harefield NHS
NHS Foundation Trust

CONSENT WITHDRAWAL FORM

Sleep disordered breathing in children and atopic diseases

Centre Number:
Patient Identification Number for this trial:

Chief / Principal Investigator
Dr Hui-Leng Tan
Consultant in Paediatric Respiratory and Sleep Medicine
The Royal Brompton Hospital

Study Coordinator
Dr Christopher Grime
Paediatric Sleep Research Fellow
NHLI/ The Royal Brompton Hospital

Please initial box if you agree

1. I wish/ I wish for my child to withdraw from further participation in this trial ☐
   (Please delete as appropriate)
2. I wish to withdraw/ I wish for my child to be withdrawn from further trial-related follow-up ☐

Your participation in the obstructive sleep apnoea research study means that we have already gathered some data. We would like to use this information in the future for analysing this trial and for future research. However, if you DO NOT wish this information to be used, please complete the following section:

3. I wish to withdraw consent to using any tissue samples* ☐
4. I wish to withdraw consent to using blood samples I have given* ☐
5. I wish to withdraw consent to using any data gathered prior to the date of this form ☐

Name of child		Relationship to parent/ guardian
Name of parent/ guardian	Date	Signature child/ parent/ guardian
Name of Person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

When completed, 1 for patient; 1 for researcher site file; 1 (original) to be kept in medical notes

Appendix 9: Recruitment document

Background

Patient No._____

Name _____ Hospital number _____

DOB_____ Date of assessment_____

Age _____ Sex (circle) male / female

Ethnicity (circle) Caucasian / Asian / Black / Chinese / Mixed Caucasian and Asian / Mixed Caucasian and black / Other

Address _____

Birth and Early Life History

Gestational age:_____ weeks

Mode of delivery: (circle) vaginal / instrumental / elective LSCS/ emergency LSCS

Birth weight: _____ Kg IUGR: (circle) yes no

ventilated after birth: (circle) yes no Oxygen after birth: (circle) yes no

Infant feeding: breast / bottle / mixed Duration of breast feeding:

Age weaned (introduction of solids): _____ months

Sleep Symptoms		**Comment**
Snoring	**yes/no**	
Apnoea's	**Yes/no**	(duration, how many)
Arousals	**Yes/no**	
Mouth breathing	**Yes/no**	
Morning headache	**Yes/no**	
Excessive daytime sleepiness	**Yes/no**	
Difficulty concentrating	**Yes/no**	
Behavioural problems	**Yes/no**	
Usual bed time	**Yes/no**	
Usual wake time	**Yes/no**	

Modified Epworth Sleepiness Scale - Ages 3-17yrs *(Parent to complete)*

Daytime Description (please circle)

When your child wakes in the morning, how do they feel?	Refreshed Tired/Sleepy Irritable
Does your child have headaches first thing in the morning?	Yes No
If yes, how many days a week?	1 2 3 4 5 6 7
Is your child sleepy during the day?	Never Rarely Occasionally Frequently Always
Does your child take naps during the day?	Never Rarely Occasionally Frequently Always
If yes, how many naps per day?	1 2 3 4 5 6

In the following situations, what are the chances of your child falling asleep? (please tick)

Situation	No Chance	Slight Chance	Moderate Chance	High Chance
1. Sitting and Reading				
2. Watching TV				
3. Sitting inactive in a public place (eg Cinema)				
4. As a passenger in a car for 1 hour with no break				
5. Lying down in the afternoon				
6. Sitting and talking to someone				
7. Sitting quietly after lunch				
8. In a car, while stopped for a few minutes in traffic				

Has a teacher been concerned about your child being sleepy or inattentive at school?	Yes No
Has your child fallen asleep at school?	Never Rarely Occasionally Frequently Always
What time of day does your child seem most alert?	AM PM

Respiratory symptoms		**Comments**
Viral illness in past 2 weeks	**Yes/no**	
Persistent productive cough	**Yes/no**	
Any history of wheeze	**Yes/no**	**(If yes- wheeze questionnaire + video clips)**
Symptom pattern	Symptoms with colds/URTI	**Yes/no**
	Symptoms with or without colds/URTI	**Yes/no**
Main trigger	viral illness / exercise / anxiety / pets / pollen / dust / cigarette smoke / other allergy/other_____	
Main symptoms	wheeze / cough / SOB / other _____	

Allergy history		**Comments**
History of food allergy	**Yes/no**	
History of eczema	**Yes/no**	
History of hayfever	**Yes/no**	
History of rhinitis	**Yes/no**	
Rhinitis Score (See attached)		

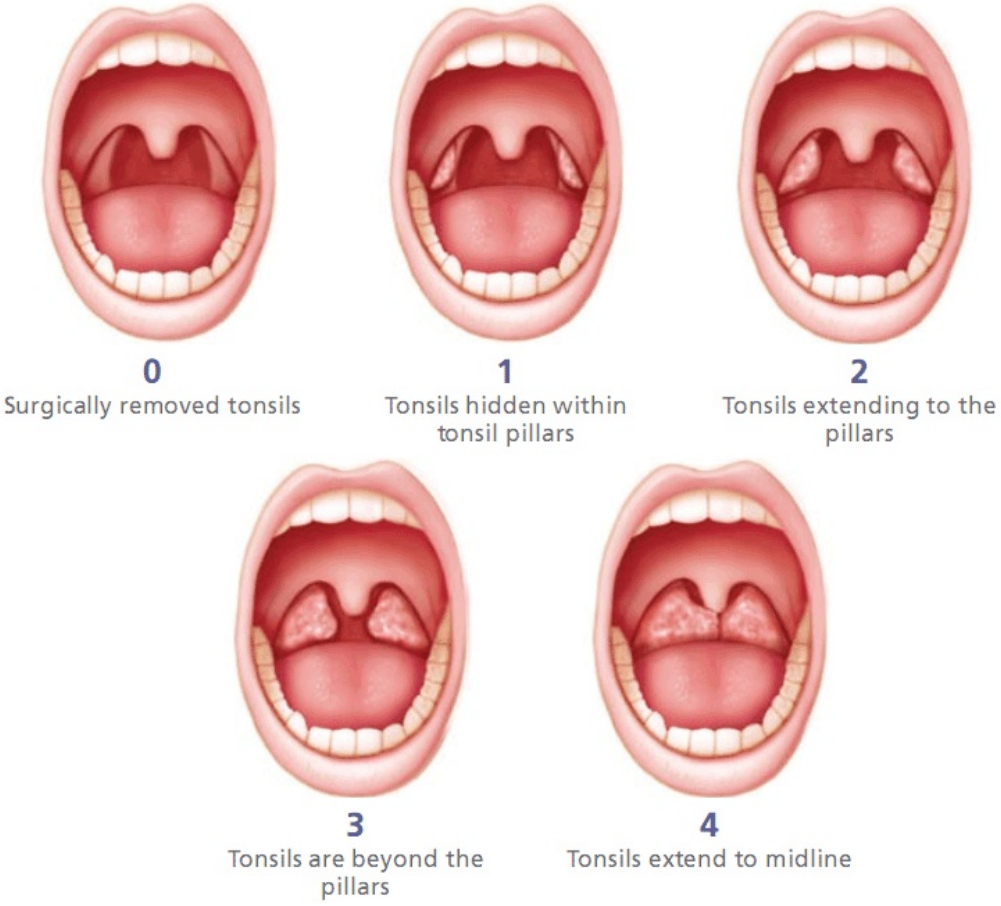
Family and Social History		**Comment**
Fhx asthma	**Yes/no**	Mother/father/siblings
Fhx eczema	**Yes/no**	Mother/father/siblings
Fhx hayfever	**Yes/no**	Mother/father/siblings
Exposure to pets at home	**Yes/no**	(Type & Number)
Smoking at home	**Yes/no**	mother / father / both / other household member / none

Other past medical history

Medications	**Dose**	**Route** (po, sc, IV, inh, neb)	**Frequency**
Allergy to medication	**Reaction**		

Mallampati Score (see attached) **1 2 3 4**

Tonsil Grade (see attached) **0 1 2 3 4**


0
Surgically removed tonsils
1
Tonsils hidden within tonsil pillars
2
Tonsils extending to the pillars
3
Tonsils are beyond the pillars
4
Tonsils extend to midline

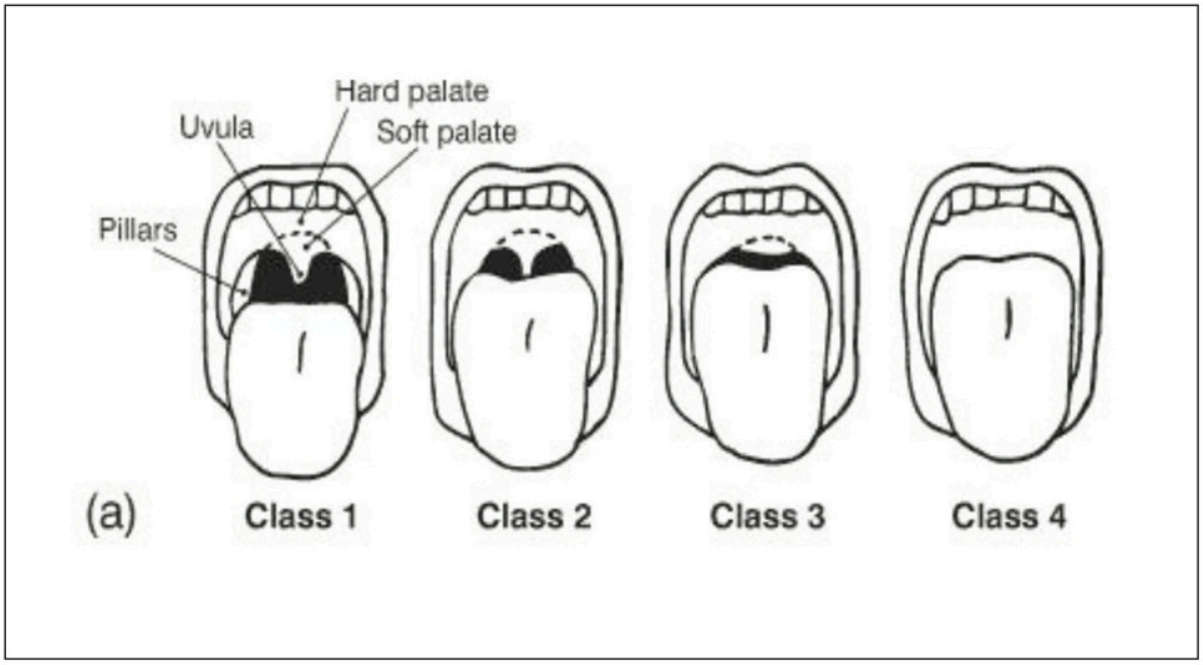


Figure 1. The Mallampati score:
Class 1. Complete visualization of the soft palate
Class 2. Complete visualization of the uvula
Class 3. Visualization of only the base of the uvula
Class 4. Soft palate is not visible at all

Pre-school Wheeze Questionnaire (under 5 years)

Has your child ever wheezed? Yes/ no

What is your child's main symptom? (*circle)* wheeze / cough / other (specify)

_____

Which of the video clips, if any, represents your child's symptoms?

0 1 2 3 4

How many episodes of wheeze has he / she ever had?	1	2	3	>3
How many episodes in the past 6 months?	1	2	3	>3
Has he / she ever been completely wheeze free?	Yes/ no			
Does he / she only wheeze with colds?	Yes/ no			
Does he /she wheeze apart from colds?	Yes/ no			

If yes:

Does the wheeze increase with a cold? Yes/ no

Does he / she wheeze most days? Yes/ no

Has he / she ever been completely wheeze free? Yes/ no

Has he / she ever been admitted to hospital with wheeze? Yes/ no

If Yes, number of admissions 1 2 3 >3

Appendix 10: Preliminary flow cytometry panel

Laser colour	Antibody name and wavelength	Cell stain	FMO1	FMO2	FMO3	FMO4	FMO5	FMO6	FMO7	FMO8	FMO9	FMO10	FMO11	Compensation
Ultra violet	Zombie UV													
Violet	BV421 (PB) 450/50	CD150		CD150	CD150	CD150	CD150	CD150	CD150	CD150	CD150	CD150	CD150	CD150
Violet	BV525 525/50	B220	B220		B220	B220	B220	B220	B220	B220	B220	B220	B220	B220
Violet	BV605 610/20	Thy1.2	Thy1.2	Thy1.2		Thy1.2	Thy1.2	Thy1.2	Thy1.2	Thy1.2	Thy1.2	Thy1.2	Thy1.2	Thy1.2
Blue	FITC 530/30	Foxp3	Foxp3	Foxp3	Foxp3		Foxp3	Foxp3	Foxp3	Foxp3	Foxp3	Foxp3	Foxp3	Foxp3
Blue	PerCP-Cy5.5 695/40	CD19	CD19	CD19	CD19	CD19		CD19	CD19	CD19	CD19	CD19	CD19	CD19
Yellow	PE 585/15	ICOS	ICOS	ICOS	ICOS	ICOS	ICOS		ICOS	ICOS	ICOS	ICOS	ICOS	ICOS
Yellow	PE Texas Red 610/20	CD44	CD44	CD44	CD44	CD44	CD44	CD44		CD44	CD44	CD44	CD44	CD44
Yellow	PE Cy7 780/60	PD1	PD1	PD1	PD1	PD1	PD1	PD1	PD1		PD1	PD1	PD1	PD1
Red	APC 670/14	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ	IFNγ		IFNγ	IFNγ	IFNγ
Red	Alexa700 730/45	CD62L	CD62L	CD62L	CD62L	CD62L	CD62L	CD62L	CD62L	CD62L	CD62L		CD62L	CD62L
Red	APC-Cy7 780/60	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4	CD4		CD4

Appendix 10. Figure 1. Staining panel for trial of 11 colour FACS used on mouse spleen lymphocytes. Three antibodies utilising the violet laser (BV421, BV525 and BV605), two utilising the blue laser (FITC and PerCP-Cy 5.5), three utilising the yellow laser (PE, PE Texas red and PE-Cy7) and three utilising the red laser (APC, Alexafluor 700 and APC-CY7). The cell surface markers and transcription factors chosen where those know to tbe present on the moue splenic cells and those for which antibodies were already available for use. A series of fluorescence minus 1 (FMO) where developed to use as measures when identifying the absence or presence of the markers on the cells. The compensations used for each antibody are listed on the right of the diagram.

Appendix 11. Chapter 3 results

	OSA (n=3)	Non-OSA (n=18)	p
Total Apnoea-hypopnea index (Inter quartile range)	3.9 (3.5-4.2)	0.9 (0.4-2.3)	0.04
Obstructive Apnoea-hypopnoea index (Inter quartile range)	**2 (2-2.6)**	**0.1 (0-0.3)**	**0.007**
Central Apnoea-hypopnoea index (Inter quartile range)	1.1 (0.4-1.8)	0.6 (0.2-1.4)	0.5
Median SpO_2 (%) (Inter quartile range)	96 (96-98)	96 (95-97)	0.64
Minimum SpO_2 (%) (Inter quartile range)	87 (85-92)	91 (86-94.5)	0.38
Desaturations per hour - 3% desaturation rule (Inter quartile range)	3.2 (2-5.6)	1.6 (0.6-5.7)	0.08
Mean TcPCO2 (kPa) (Inter quartile range)	5.7 (5.3-5.9)	5.5 (5.3-5.7)	0.56
Minimum TcPCO2 (kPa) (Inter quartile range)	5.3 (4-5.5)	4.8 (4.8-5.3)	0.89
Maximum TcPCO2 (kPa) (Inter quartile range)	6.5 (6.3-6.5)	6.4 (6.1-6.9)	0.68

Appendix 11.Table 1. Comparison of sleep study data between STRA children with confirmed OSA and those whos study did not demonstrate OSA. Median values plus interquartile ranges. Significance using Mann-Whitney U-test

REM AHI (Inter quartile range)	4.3 (3.5-4.0)
Non-REM AHI (Inter quartile range)	2 (0.3-3.8)

Appendix 11.Table 2. AHI values for the 8 STRA children to undergo full PSG sleep studies.

	OSA (n=3)	Non-OSA (n=5)	p
Percentage REM % (Inter quartile range)	16 (15.2-17.4)	20.9 (11.9-23.4)	0.39
Arousal Index (Inter quartile range)	13.7 (2.7-15.2)	7.3 (5.4-10.2)	0.57

Appendix 11. Table 3. Percentage REM and arousal index of the 8 STRA children to undergo full PSG sleep study. Significance using Mann-Whitney U-test.

	OSA (n=3)	Non-OSA (n=18)	p
Daily inhaled corticosteroid dose (Budesonide equivalent in micrograms)	1400 (800-2000)	1600 (950-2000)	0.9
No. receiving Montelukast	2 (66%)	12 (57%)	
No. receiving nasal steroids.	2 (66%)	3 (14%)	
No. receiving oral steroids (prednisolone)	1 (33%)	8 (38%)	
Median GP prescription collection (%)	100%	90%	

Appendix 11. Table 4. Medication comparison for both OSA and Non-OSA STRA subjetcs. Significance for corticosteroid dose calculated using Mann-Whitney U-test. Other comparison significance calculated using Fisher's Exact test.

	OSA	Non-OSA	p
Percentage predicted Mean Forced vital capacity (FVC) (Standard deviation)	101.7 (9)	96.2 (21.7)	0.1
Percentage predicted Mean Forced expiratory volume in 1 second (FEV1) (Standard deviation)	94.7 (10.1)	85.8 (26.9)	0.4

Appendix 11. Table 5. Lung function comparing STRA children with and without evidence of OSA on sleep study. Significance calculated using students t-test.

	OSA	Non-OSA	
Mean Paediatric Asthma Quality of Life Questionnaire (PAQLQ) (Standard deviation)	5.7 (1.1)	4.5 (1.4)	0.4
Mean Asthma Control Test (ACT) (Standard deviation)	22 (3.5)	13.9 (5.4)	0.2

Appendix 11. Table 6. Psychological data comparing STRA children with and without evidence of OSA on sleep study. Significance calculated using students t-test.

	OSA	Non-OSA	
Median Total IgE (IU) (interquartile ranges)	97.8 (123-1938)	414 (130-1560)	1.0
Median Sum of Specific IgE (IU) (interquartile ranges)	119.7 (16.9-114.8)	83.1 (24.6-262.8)	0.9
Median total IgE Z-scores (interquartile ranges)	2.7 (1.3-2.8)	2.9 (1.3-3.5)	0.7

Appendix 11. Table 7. Atopy result comparing STRA children with and without evidence of OSA on sleep study. Significance calculated using students t-test.

Appendix 12. Chapter 4 results

	OSA	Non-OSA	
Median tonsil tissue weight (Gram) (Interquartile range)	6.6 (4.7-8.7)	5.8 (4.2-8.7)	0.8
Modified Epworth Sleepiness score (Interquartile range)	5.5 (2-8.5)	4 (3-7)	0.5
Apnoea-hypopnoea index (AHI) (Interquartile range)	**5.4 (3.9-9.8)**	**0.5 (0.1-1)**	**<0.0001**
Obstructive AHI (Interquartile range)	**2.9 (2-5.9)**	**0 (0-0.05)**	**<0.0001**
Sum of specific IgE (IU) (Interquartile range)	0.07 (0.03-0.2)	0.2 (0.07-8.9)	0.04
Total IgE (IU) (Interquartile range)	44 (11.5-144.5)	39 (16-113)	0.7

Appendix 13. Chapter 5 results

Appendix 13.1. T-cells results

A.13.1.1. Tonsil tissue results

Lymphocyte	Median Cell count per gram of tissue ($x10^5$) (N=45)
B-cell (CD19+)	1092 (763.2-1783)
T-cell (CD3+)	632.5 (312.7-857.9)
Natural killer cell (CD3-CD56+)	8.74 (4.77-31.48)
Lineage negative cells	7.59 (2.28-21.63)

Table 1. Total cell counts per gram off tissue for B-cells, T-cells, NK-cells and lineage negative cells. Median values with inter-quartile ranges.

Lymphocyte	Median Cell count per gram of tissue ($x10^3$) (N=23)
B-cell (CD19+	1537 (896.7-2070)
T-cell (CD3+)	1049 (594.8-1362)
Natural killer cell (CD3-CD56+)	50.18 (23.75-147.8)
Lineage negative cells	42.74 (10.11-78.79)

Table 2. Total cell counts per ml off blood for B-cells, T-cells, NK-cells and lineage negative cells. Median values with inter-quartile ranges.

Lymphocytes	OSA (n=24)	Non-OSA (n=21)	P
Total T-cells (CD3+) (Median cell counts per gram $x10^6$)	52.81 (31.27-85.34)	69.86 (31.76-95.35)	0.78
Total B-cells (CD19+) (Median cell counts per gram $x10^6$)	94.45 (77.38-179.5)	122.6 (70.82-187.5)	0.60
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram $x10^6$)	1.69 (0.48-1.7)	1.11 (0.36-10.17)	0.61

Table 3. T-cells, B-cells and NK cell counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lymphocytes	OSA (n=24)	Non-OSA (n=21)	P
Total T-cells (CD3+) (% of total cell count)	19.8 (14.85-26.18)	19.23 (14.63-24.49)	0.77
Total B-cells (CD19+) (% of total cell count)	43 (34.7-50.2)	47.37 (26.58-53.27)	0.93
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.25 (0.17-0.5)	0.22 (0.15-1.7)	0.99

Table 4. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing OSA subjects to Non-OSA subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	OSA (n=24)	Non-OSA (N=21)	P
CD4+ (Median cell counts per gram x10^6)	43.5 (28.2-60.3)	44.7 (23.8-75.8)	0.85
CD8+ (Median cell counts per gram x10^6)	11.1 (5.4-15.4)	13.2 (5.7-19.8)	0.54
CD4+CD56+ (Median cell counts per gram x10^5)	4.6 (1.3-20.8)	8.7 (2.39-21.98)	0.52

Table 5. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

T-cell markers	OSA (n=24)	Non-OSA (N=21)	P
CD4+ (percentage of CD3+ cells)	74.1 (70.2-78.2)	74.4 (62.2-77)	0.54
CD8+ (percentage of CD3+ cells)	17.4 (16-19.2)	17.3 (14.5-19.8)	0.86
CD4+CD56+ (% of CD3+ cells)	0.38 (0.2-3.9)	1.33 (0.6-2.7)	0.46

Table 6. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

T-cell markers	OSA	Non-OSA	P
CD4+IFNγ+ (Median cell counts per gram x10^5)	22.2 (n=15) (11.59-60.33)	29.1 (N=15) (14.3-52.4)	0.74
CD4+IL4+ (Median cell counts per gram x10^5)	318 (n=13) (169-482.1)	255.5 (n=14) (124-390.7)	0.39
CD4+IL13+ (Median cell counts per gram x10^5)	3.3 (n=15) (0.5-8)	2.4 (n=15) (0.3-12.2)	0.89
CD4+IL5+ (Median cell counts per gram x10^5)	398.7 (n=7) (241.5-541.2)	238.3 (n=7) (113.1-445.2)	0.36
CD4+IL17+ (Median cell counts per gram x10^5)	16.5 (n=15) (11.3-82.3)	25 (n=15) (11.9-41)	0.61
CD4+CD25+FOXp3+ (Median cell counts per gram x10^5)	**5.6 (n=9) (3.2-7.1)**	**18.3 (n=12) (11.2-28.5)**	**0.006**

Table _7. Helper T-cell subsets as median total cell counts per gram of tonsil tissue comparing OSA and non-OSA subjects. Median cell counts and interquartile ranges and p value generates using the Mann-Whitney calculation.

T-cell markers	OSA	Non-OSA	P
CD4+IFNγ+ (% of CD4+ cells)	5.5 (n=15) (4-9.5)	5.9 (n=15) (4.4-7.7)	0.99
CD4+IL4+ (% of CD4+ cells)	81.9 (n=13) (29.8-91.4)	32.3 (n=14) (25.1-91.2)	0.09
CD4+IL13+ (% of CD4+ cells)	1.27 (n=15) (0.2-3.6)	1.34 (n=15) (0.2-8.4)	0.31
CD4+IL5+ (% of CD4+ cells)	70.9 (n=7) (65.9-80.8)	57 (n=7) (18.1-77.9)	0.23
CD4+IL17+ (% of CD4+ cells)	3.24 (n=15) (2.7-5.1)	3.85 (n=15) (1.92-5.34)	0.79
CD4+CD25+FOXp3+ (% CD4+ cells)	**2.28 (n=9) (0.9-1.4)**	**3.16 (n=12) (2.3-4.4)**	**0.003**

Table 8. Helper T-cell subsets as median percentage of total CD4+ cells comparing OS and Non-OSA subjects. Median values with interquartile ranges and p value calculated using Mann-Whitney testing.

A.13.1.2. Peripheral blood results

Lymphocyte	OSA (n=14)	Non-OSA (n=9)	P
Total T-cells (CD3+) (Median cell counts per gram x10^3)	951.1 (42.23-142.6)	1049 (549.6-1501)	0.52
Total B-cells (CD19+) (Median cell counts per gram x10^3)	121.1 (42.23-142.6)	317.1 (183.9-785.4)	0.007
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^3)	25.23 (6.52-48.81)	21.97 (3.26-48.52)	0.94

Table 9. T-cells, B-cells and NK cell counts per ml of blood comparing OSA subjects to Non-OSA subjects. Median cell counts with p value generated with Mann-Whitney calculation

Lymphocytes	OSA (n=14)	Non-OSA (n=9)	P
Total T-cells (CD3+) (% of total cell count)	61.8 (49.3-63.98)	65.8 (56.50-71)	0.29
Total B-cells (CD19+) (% of total cell count)	6.3 (4.6-8.46)	21.16 (10.18-31.45)	0.022
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	1.68 (1.04-3.11)	1.19 (0.18-4.25)	0.76

Table 10. T-cells, B-cells and NK cell median percentages of total cells comparing OSA subjects to Non-OSA subjects. Median cell counts with p value generated with Mann-Whitney calculation

T-cell markers	OSA (n=14)	Non-OSA (n=9)	P
CD4+ (Median cell counts per gram x10^3)	604 (262.6-810.2)	801.4 (547.5-1260)	0.17
CD8+ (Median cell counts per gram x10^3)	163.3 (72.7-292)	288.1 (33.47-414.8)	0.65
CD4+CD56+ (Median cell counts per gram x10^3)	2.9 (0.33-10.18)	0.75 (0-5.75)	0.35

Table 11. T-cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. Median cell counts with p value generated with Mann-Whitney calculation.

T-cell markers	OSA (n=14)	Non-OSA (n=9)	P
CD4+ (percentage of CD3+ cells)	62 (57.1-71.6)	66.3 (56.5-67.9)	0.78
CD8+ (percentage of CD3+ cells)	23.9 (8.3-30.7)	25.2 (22.8-31.9)	0.32
CD4+CD56+ (% of CD3+ cells)	0.7 (0.2-1.1)	0.7 (0.4-3.5)	0.65

Table 12. T-cells median percentages of total T-cells ($CD3^+$) comparing OSA subjects to Non-OSA subjects. Median cell counts with p value generated with Mann-Whitney calculation.

T-cell markers	OSA	Non-OSA	P
CD4+IFNγ+ (Median cell counts per ml x10^3)	145.1 (n=6) (74.2-270.1)	223.1 (n=6) (39.7-316.3)	0.82
CD4+IL4+ (Median cell counts per ml x10^3)	587.5 (n=6) (78.3-1282)	345.9 (n=6) (161.2-1834)	0.91
CD4+IL13+ (Median cell counts per ml x10^3)	14.7 (n=6) (4-101.8)	14.2 (n=6) (4-313.8)	1.00
CD4+IL17+ (Median cell counts per ml x10^3)	28.5 (n=6) (16.5-41.9)	28.6 (n=6) (17.7-70.2)	0.81
CD4+CD25+FOXp3+ (Median cell counts per ml x10^3)	**1.3 (n=5) (0.5-1.6)**	**6.3 (n=6) (2.5-13.7)**	**0.009**

Table 13. Helper T-cell subsets as median total cell counts per ml of blood comparing OSA to Non-OSA subjects. Median values with interquartile ranges and p values calculated using Mann-Whitney calculation.

T-cell markers	OSA	Non-OSA	P
CD4+IFNγ+ (% of CD4+ cells)	11.3 (n=6) (7.6-24.4)	10.6 (n=6) (6.5-24.2)	0.94
CD4+IL4+ (% of CD4+ cells)	73.5 (n=6) (17.6-96.1)	29.7 (n=6) (22.7-98.9)	0.91
CD4+IL13+ (% of CD4+ cells)	1.2 (n=6) (0.7-8.7)	0.6 (n=6) (0.5-1.1)	0.28
CD4+IL17+ (% of CD4+ cells)	3 (n=6) (2-3.7)	2 (n=6) (1.7-5)	0.48
CD4+CD25+FOXp3+ (% CD4+ cells)	0.3 (n=5) (0.1-0.7)	0.8 (n=6) (0.4-2.5)	0.19

Table 14. Helper T-cells subset median percentages of total Th cells (CD4+) comparing OSA subjects to Non-OSA subjects. Median cell counts with p value generated with Mann-Whitney calculation.

Appendix 13.2. Innate lymphoid cells

A.13.2.1. Tonsils tissue results

Lineage negative cell markers	OSA (n=15)	Non-OSA (n=15)	P
Lin- (Median cell counts per gram x10^5)	7.29 (2.7-21.9)	16.8 (5.2-27.7)	0.37
Lin-CD127+ (Median cell counts per gram x10^4)	6.3 (2.1-14.8)	10.5 (6.3-24.5)	0.11
Lin-CRTH2+ (Median cell counts per gram x10^4)	23 (0-52.1)	9.3 (3.4-24.4)	0.64
Lin-CD127+CRTH2+ (Median cell counts per gram x10^4)	0.5 (0.4-1.1)	1.1 (0.6-2.7)	0.07

Table 15. Lineage negative cells with extracellular markers as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA (n=15)	Non-OSA (n=15)	P
Lin- (% CD45+ cells)	0.3 (0.1-1.1)	0.6 (0.1-1)	0.21
Lin-CD127+ (% CD45+ cells)	0.03 (0.01-0.1)	0.06 (0.03-0.1)	0.30
Lin-CRTH2+ (% CD45+ cells)	0.02 (0.01-0.1)	0.1 (0.01-0.1)	0.93
Lin-CD127+CRTH2+ (% CD45+ cells)	0.003 (0.002-0.02)	0.004 (0.002-0.02)	0.67

Table 16. Lineage negative cells with extracellular markers as percentage of $CD45^+$ cells comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-IFNγ+ (Median cell counts per gram x10^4)	5.8 (n=13) (2.7-18.4)	17.7 (n=15) (3.4-82.4)	0.21
Lin-IL4+ (Median cell counts per gram x10^4)	21.7 (n=11) (10.7-58.4)	128.5 (n=14) (9-169.5)	0.3
Lin-IL13+ (Median cell counts per gram x10^4)	4.9 (n=13) (1.2-7.8)	2.0 (n=15) (0.5-7.3)	0.37
Lin-IL5+ (Median cell counts per gram x10^4)	10.3 (n=8) (6-14.9)	41.7 (n=8) (21.7-135.2)	0.02
Lin-IL17+ (Median cell counts per gram x10^4)	7 (n=13) (2.7-15.7)	14.7 (n=15) (3.5-29.7)	0.51

Table 17. Cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-IFNγ+ (% CD45+ cells)	0.1 (n=13) (0.01-0.1)	0.1(n=15) (0.01-0.4)	0.04
Lin-IL4+ (% CD45+ cells)	0.1 (n=11) (0.04-0.6)	0.6 (n=11) (0.1-0.7)	0.45
Lin-IL13+ (% CD45+ cells)	0.02 (n=13) (0.01-0.05)	0.01 (n=15) (0.004-0.03)	0.11
Lin-IL5+ (% CD45+ cells)	0.05 (n=8) (0.03-0.06)	0.2 (n=8) (0.1-0.6)	0.06
Lin-IL17+ (% CD45+ cells)	0.04 (n=13) (0.02-0.1)	0.1 (n=15) (0.01-0.1)	0.79

Table 18. Cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lineage negative cell markers	OSA	Non-OSA	P
Lin-CD127+IFNγ+ (Median cell counts per gram x10^4)	3.9 (n=13) (0-8.5)	20.7 (n=15) (3-59.6)	0.09
Lin-CD127+IL4+ (Median cell counts per gram x10^4)	3.8 (n=11) (2.1-11.2)	4.3 (n=11) (1.3-88.6)	0.81
Lin-CD127+IL5+ (Median cell counts per gram x10^4)	-	-	-
Lin-CD127+IL17+ (Median cell counts per gram x10^4)	2.7 (n=13) (0.5-4.9)	4 (n=15) (2-7.1)	0.41

Table _19 $CD127^+$ cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-CD127+IFNγ+ (% CD45+ cells)	0.002 (n=13) (0.001-0.01)	0.01 (n=15) (0.005-0.03)	0.23
Lin-CD127+IL4+ (% CD45+ cells)	0.02 (n=11) (0.005-0.03)	0.03 (n-14) (0.01-0.4)	0.34
Lin-CD127+IL5+ (% CD45+ cells)	-	-	-
Lin-CD127+IL17+ (% CD45+ cells)	0.02 (n=13) (0.002-0.04)	0.01 (n=15) (0.004-0.02)	0.54

Table 20. $CD127^+$ cytokine producing lineage negative cells as percentages of $CD45^+$ cells comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-Gata3+ (Median cell counts per gram x10^4)	22.9 (n=9) (4.8-31.3)	36.8 (n=11) (12-118.4)	0.12
Lin-Gata3+IL4+ (Median cell counts per gram x10^4)	6.7 (n=9) (3-17.6)	37.6 (n=11) (5.7-85.5)	0.05
Lin-Gata3+IL5+ (Median cell counts per gram x10^4)	2.3 (n=9) (1.5-15.5)	9.2 (n=11) (1.7-77.2)	0.44

Table 21. $Gata3^+$ lineage negative cells and cytokine producing $Gata3^+$ lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-Gata3+ (% CD45+ cells)	0.1 (n=9) (0.03-0.2)	0.1 (n=11) (0.1-0.6)	0.08
Lin-Gata3+IL4+ (% CD45+ cells)	0.04 (n=9) (0.02-0.2)	0.1 (n=11) (0.03-0.4)	0.17
Lin-Gata3+IL5+ (% CD45+ cells)	0.01 (n=9) (0.01-0.1)	0.04 (n=11) (0.01-0.2)	0.44

Table 22. $Gata3^+$ lineage negative cells and cytokine producing $Gata3^+$ lineage negative cells as percentage of $CD45^+$ cells comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

A.13.2.2. Peripheral bloods results

Lineage negative cell markers	OSA	Non-OSA	P
Lin- (Median cell counts per gram $x10^4$)	3.9 (n=14) (0.6-6.8)	6 (n=7) (1.2-8.4)	0.56
Lin-CD127+ (Median cell counts per gram $x10^3$)	3 (n=14) (0.6-6)	1.5 (n=7) (0.2-5.2)	0.56
Lin-CRTH2+ (Median cell counts per gram $x10^3$)	3.7 (n=14) (1-6.7)	1.4 (n=7) (0.4-4.3)	0.23
Lin-CD127+CRTH2+ (Median cell counts per gram $x10^3$)	1.3 (n=14) (0.1-2)	0.1 (n=7) (0-0.8)	0.12

Table 23. Lineage negative cells with extracellular markers as median cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin- (% CD45+ cells)	2.4 (n=14) (0.4-7)	3.8 (n=7) (1.3-7)	0.51
Lin-CD127+ (% CD45+ cells)	0.2 (n=14) (0.04-0.5)	0.1 (n=7) (0.01-0.3)	0.43
Lin-CRTH2+ (% CD45+ cells)	0.2 (n=14) (0.03-0.3)	0.1 (n=7) (0.02-0.3)	0.60
Lin-CD127+CRTH2+ (% CD45+ cells)	0.1 (n=14) (0.02-0.1)	0.02 (n=7) (0.01-0.1)	0.27

Table 24. Lineage negative cells with extracellular markers as percentage of $CD45^+$ cells comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lineage negative cell markers	OSA	Non-OSA	P
Lin-IFNγ+ (Median cell counts per gram $x10^3$)	32 (n=8) (13.3-42.4)	50.2 (n=6) (26-162)	0.22
Lin-IL4+ (Median cell counts per gram $x10^3$)	14.9 (n=8) (6.5-63.3)	47.5 (n=6) (13.4-108.9)	0.24
Lin-IL13+ (Median cell counts per gram $x10^3$)	1.1 (n=8) (0.8-3.3)	1.9 (n=6) (0.9-30.2)	0.81
Lin-IL17+ (Median cell counts per gram $x10^3$)	2.2 (n=8) (0.3-5.7)	22.2 (n=6) (0.4-66.3)	0.35

Table 25. Cytokine producing lineage negative cells as median cells counts per ml of blood comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Lineage negative cell markers	OSA	Non-OSA	P
Lin-IFNγ+ (% CD45+ cells)	2.1 (n=8) (0.8-4.6)	4.7 (n=6) (1.2-9.2)	0.62
Lin-IL4+ (% CD45+ cells)	1.6 (n=7) (0.3-4.9)	2.4 (n=5) (0.7-8.2)	0.59
Lin-IL13+ (% CD45+ cells)	0.1 (n=8) (0.04-0.3)	0.2 (n=6) (0.4-1.6)	0.70
Lin-IL17+ (% CD45+ cells)	0.1 (n=8) (0.01-1.1)	2.2 (n=6) (0.02-3.6)	0.35

Table 26. Cytokine producing lineage negative cells as median cells counts per gram of tissue comparing OSA subjects to Non-OSA subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

Appendix 14. Chapter 6 results

Appendix 14.1. T-cells

A14.1.1. Tonsil tissue results

Lymphocytes	Atopic (n=15)	Non-atopic (Comp. aero Panel) (n=20)	P
Total T-cells (CD3+) (Median cell counts per gram x10^6)	69.9 (44-104.6)	72.9 (26.5-98.3)	0.58
Total B-cells (CD19+) (Median cell counts per gram x10^6)	147.4 (81.7-163.7)	115.9 (77.9-192.3)	0.98
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^6)	0.6 (0.4-1.7)	1.6 (0.6-9)	0.16

Table 1. T-cells, B-cells and NK cell counts per gram of tissue comparing atopic and non-atopic (Suff IgE) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lymphocytes	Atopic (n=15)	Non-atopic (Comp. aero Panel) (n=20)	P
Total T-cells (CD3+) (% of total cell count)	24.4 (16.9-28.5)	19.4 (14.2-24)	0.1
Total B-cells (CD19+) (% of total cell count)	43 (34-55.5)	41.4 (31.4-52.2)	0.8
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.2 (0.1-0.5)	0.4 (0.2-2.4)	0.2

Table 2. T-cells, B-cells and NK cell counts as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic (n=15)	Non-atopic (Comp. aero Panel) (n=20)	P
CD4+ (median cell counts per gram x10^6)	51.8 (31.2-84.7)	45.9 (23.2-71.5)	0.75
CD8+ (median cell counts per gram x10^6)	12.6 (5.8-19.8)	12.06 (4.6-18)	0.71
CD4+CD56+ (median cell counts per gram x10^5)	7 (3.3-34.8)	10.1 (2.3-15.6)	0.6

Table 3. T-cells per gram of tissue comparing atopic and non-atopic (Suff IgE) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic (n=15)	Non-atopic (Comp. aero Panel) (n=20)	P
CD4+ (percentage of CD3+ cells)	74.1 (71.4-76)	75.2 (66.4-79.8)	0.37
CD8+ (percentage of CD3+ cells)	18 (16.2-19.6)	16.55 (14.3-17.8)	0.13
CD4+CD56+ (% of CD3+ cells)	1.11 (0.4-4.5)	1.14 (0.4-2.6)	0.67

Table 4. T-cells as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
CD4+IFNγ+ (median cell counts per gram x10^5)	23.83 (n=13) (16.9-76.7)	43.52 (n=14) (11-58.1)	0.8
CD4+IL4+ (median cell counts per gram x10^5)	167.9 (n=11) (18.6-339.3)	425.4 (n=11) (283-508.3)	0.04
CD4+IL13+ (median cell counts per gram x10^5)	3 (n=13) (0.6-17.1)	3 (n=14) (0.3-8.5)	0.6
CD4+IL5+ (median cell counts per gram x10^5)	260.7 (n=6) (178.9-902.5)	407.5 (n=6) (216.9-534.4)	0.9
CD4+IL17+ (median cell counts per gram x10^5)	33 (n=13) (8.93-123.5)	18.5 (n=14) (0.3-11.3)	0.5
CD4+CD25+FOXp3+ (median cell counts per gram x10^5)	12.47 (n=10) (8.1-18)	11.59 (n=10) (5.7-27.1)	0.8

Table 5. Helper T-cell subsets per gram of tissue comparing atopic and non-atopic (Suff IgE) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

T-cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
CD4+IFNγ+ (% of CD4+ cells)	5.62 (n=13) (4.9-8.8)	5.42 (n=14) (3.2-10.1)	0.97
CD4+IL4+ (% of CD4+ cells)	34.14 (n=11) (17.6-44.2)	72.9 (n=11) (35.8-95.1)	0.015
CD4+IL13+ (% of CD4+ cells)	0.7 (n=13) (0.19-3.1)	0.3 (n=14) (0.1-1.3)	0.3
CD4+IL5+ (% of CD4+ cells)	74.1 (n=6) (43.9-79.4)	57.6 (n=6) (38.8-74.5)	0.48
CD4+IL17+ (% of CD4+ cells)	9.34 (n=13) (3.95-14.3)	3.86 (n=14) (3-5.8)	0.08
CD4+CD25+FOXp3+ (% CD4+ cells)	2.26 (n=10) (1.1-4.2)	2.64 (n=10) (1.3-5.8)	0.78

Graph 6. Helper T-cells as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Values demonstrated are median percentage with interquartile ranges.

Lymphocytes	Atopic (n=15)	Non-atopic (all results) (n=30)	P
Total T-cells (CD3+) (Median cell counts per gram x10^6)	51.8 (31.2-84.7)	55.5 (26.4-85.2)	0.8
Total B-cells (CD19+) (Median cell counts per gram x10^6)	147.4 (81.7-163.7)	107.1 (75.7-185.7)	0.7
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^6)	0.6 (0.4-1.7)	1.2 (0.4-7)	0.6

Table 7. T-cells, B-cells and NK cell counts per gram of tissue comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lymphocytes	Atopic (n=15)	Non-atopic (all results) (n=30)	P
Total T-cells (CD3+) (% of total cell count)	24.4 (16.9-28.5)	18.8 (14.5-23.3)	0.05
Total B-cells (CD19+) (% of total cell count)	43 (34-55.5)	44 (35.3-50.8)	0.9
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.2 (0.1-0.5)	0.3 (0.2-0.8)	0.4

Table 8. T-cells, B-cells and NK cell counts as percentage of total cell counts atopic and non-atopic (all results) subjects. Values demonstrated are median percentage with interquartile ranges.

T-cell markers	Atopic (n=15)	Non-atopic (all results) (n=30)	P
CD4+ (median cell counts per gram $x10^6$)	51.8 (31.2-84.7)	41.3 (25.1-60.1)	0.4
CD8+ (median cell counts per gram $x10^6$)	12.6 (5.8-19.8)	9.19 (5-15.6)	0.37
CD4+CD56+ (median cell counts per gram $x10^5$)	7 (3.3-34.8)	6.3 (1.2-14.6)	0.34

Table 9. T-cells per gram of tissue comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic (n=15)	Non-atopic (all results) (n=30)	P
CD4+ (percentage of CD3+ cells)	74.1 (71.4-76)	74.4 (68-80.4)	0.54
CD8+ (percentage of CD3+ cells)	18 (16.2-19.6)	16.8 (15.2-18.9)	0.08
CD4+CD56+ (% of CD3+ cells)	1.1 (0.4-4.5)	0.9 (0.4-2.5)	0.5

Table 10. T-cells as percentage of total cell counts atopic and non-atopic (all results) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic	Non-atopic (all results)	P
CD4+IFNγ+ (median cell counts per gram x10^5)	23.83 (n=13) (16.9-76.7)	37.4 (n=17) (9-57)	0.88
CD4+IL4+ (median cell counts per gram x10^5)	167.9 (n=11) (18.6-339.3)	313.5 (n=16) (258.6-505.5)	0.03
CD4+IL13+ (median cell counts per gram x10^5)	3 (n=13) (0.6-17.1)	3.01 (n=17) (0.3-7.6)	0.47
CD4+IL5+ (median cell counts per gram x10^5)	260.7 (n=6) (178.9-902.5)	416.3 (n=8) (241.5-532.1)	0.83
CD4+IL17+ (median cell counts per gram x10^5)	33 (n=13) (8.93-123.5)	17.7 (n=17) (10.5-30.3)	0.28
CD4+CD25+FOXp3+ (median cell counts per gram x10^5)	12.47 (n=10) (8.1-18)	11.3 (n=11) (4.2-24.8)	0.23

Table 11. Helper T-cell subsets per gram of tissue comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic	Non-atopic (all results)	P
CD4+IFNγ+ (% of CD4+ cells)	5.6 (n=13) (4.9-8.8)	5 (n=19) (3.4-10.3)	0.88
CD4+IL4+ (% of CD4+ cells)	**34.1 (n=11) (17.6-44.2)**	**86.3 (n=16) (64.1-95.2)**	**0.002**
CD4+IL13+ (% of CD4+ cells)	0.7 (n=13) (0.2-3.1)	0.31 (n=19) (0.1-1.2)	0.15
CD4+IL5+ (% of CD4+ cells)	74.1 (n=6) (43.9-79.4)	59.7 (n=7) (47-71.4)	0.53
CD4+IL17+ (% of CD4+ cells)	9.34 (n=13) (3.9-14.3)	3.77 (n=19) (3.1-5.2)	0.04
CD4+CD25+FOXp3+ (% CD4+ cells)	2.26 (n=10) (1.1-4.2)	1.62 (n=14) (1.1-3.4)	0.7

Table 12. Helper T-cells as percentage of total cell counts atopic and non-atopic (all results) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

Lymphocytes	Raised IgE (n=15)	Normal IgE (n=22)	P
Total T-cells (CD3+) (Median cell counts per gram x10^6)	52.8 (40-89.3)	70.8 (42.8-89.3)	0.89
Total B-cells (CD19+) (Median cell counts per gram x10^6)	108.5 (81.6-163.7)	113 (72.9-188)	0.89
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^6)	0.8 (0.4-1.8)	1.6 (0.5-11.2)	0.4

Table 13. T-cells, B-cells and NK cell counts per gram of tissue comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lymphocytes	Raised total IgE (n=15)	Normal total IgE (n=22)	P
Total T-cells (CD3+) (% of total cell count)	25.7 (14.2-28.5)	20.6 (17.2-23.3)	0.3
Total B-cells (CD19+) (% of total cell count)	41.3 (34-53.3)	46 (33-53)	1.0
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.2 (0.1-0.5)	0.3 (0.2-4.2)	0.37

Table 14. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Raised total IgE (n=15)	Normal total IgE (n=22)	P
CD4+ (median cell counts per gram x10^6)	39.1 (31.2-74.6)	45.9 (35.5-63.3)	0.93
CD8+ (median cell counts per gram x10^6)	11.2 (4.6-20.8)	12.2 (6-17.5)	0.99
CD4+CD56+ (median cell counts per gram x10^5)	10.7 (2.2-29.4)	5.6 (1.8-11.2)	0.3

Table 15. T-cells counts per gram of tissue comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Raised Total IgE (n=15)	Normal total IgE (n=22)	P
CD4+ (% of CD3+ cells)	76 (71.4-80.4)	73.8 (68-78.7)	0.39
CD8+ (% of CD3+ cells)	16.2 (14-18)	17.5 (15.6- 20)	0.19
CD4+CD56+ (% of CD3+ cells)	1.8 (0.4-4.3)	1 (0.5-1.7)	0.41

Table 16. T-cells counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Raised Total IgE	Normal total IgE	P
CD4+IFNγ+ (median cell counts per gram x10^5)	19.2 (n=14) (10.2-81.1)	36.6 (n=12) (15.3-50.7)	0.66
CD4+IL4+ (cell counts per gram x10^5)	318.5 (n=12) (92-454.8)	284.7 (n=12) (203.2-456.7)	0.97
CD4+IL13+ (median cell counts per gram x10^5)	3 (n=14) (0.5-20)	3.8 (n=12) (0.3-10.7)	0.82
CD4+IL5+ (median cell counts per gram x10^5)	416.3 (n=5) (260.7-1086)	236.1 (n=6) (113.1-534.4)	0.12
CD4+IL17+ (median cell counts per gram x10^5)	22.8 (n=14) (10.5-144.3)	18.5 (n=12) (9.6-36.2)	0.62
CD4+CD25+FOXp3+ (median cell counts per gram x10^5)	11.2 (n=11) (5.1-13.8)	18.7 (n=11) (7.2-5.9)	0.22

Table 17. Helper T-cells counts per gram of tissue comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Raised Total IgE	Normal total IgE	P
CD4+IFNγ+ (% of CD4+ cells)	5.4 (n=14) (4.3-6.3)	5.2 (n=12) (3-9.8)	0.98
CD4+IL4+ (% of CD4+ cells)	43.4 (n=12) (35-81.9)	55 (n=12) (29.1-93.2)	0.97
CD4+IL13+ (% of CD4+ cells)	0.6 (n=14) (0.2-3.7)	0.5 (n=12) (0.1-1.8)	0.34
CD4+IL5+ (% of CD4+ cells)	77.8 (n=5) (65-80.9)	51.2 (n=6) (14.7-65.2)	0.08
CD4+IL17+ (% of CD4+ cells)	4.8 (n=14) (3.7-11.2)	3.8 (n=12) (1.5-8.4)	0.16
CD4+CD25+FOXp3+ (% CD4+ cells)	1.9 (n=15) (1.2-2.5)	3.2 (n=15) (1.6-5.5)	0.11

Table 18. Helper T-cells counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

A14.1.2. Peripheral blood results

Lymphocytes	Atopic (n=8)	Non-atopic (Comp. aero Panel) (n=13)	P
Total T-cells (CD3+) (Median cell counts per ml x10^5)	11.3 (6-13.5)	10 (3.2-13.6)	0.67
Total B-cells (CD19+) (Median cell counts per ml x10^5)	3.9 (1.8-5)	2.1 (0.8-5.5)	0.63
Total Natural Killer cells (CD3-CD56+) (Median cell counts per ml x10^5)	0.8 (0.03-0.4)	0.06 (0-0.1)	0.55

Table 19. T-cells, B-cells and NK cell counts per ml of blood comparing atopic and non-atopic (Suff IgE) subjects. Values demonstrated are median cell counts with interquartile ranges

Lymphocytes	Atopic (n=8)	Non-atopic (Comp. aero Panel) (n=13)	P
Total T-cells (CD3+) (% of total cell count)	63.8 (50.3-67.5)	63.9 (57.9-71)	0.72
Total B-cells (CD19+) (% of total cell count)	20 (11.1-39.5)	17.1 (11.8-28.8)	0.43
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.47 (0.2-3.9)	1.2 (0.2-2.3)	0.93

Table 20. T-cells, B-cells and NK cell counts as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic (n=8)	Non-atopic (Comp. aero Panel) (n=13)	P
CD4+ (median cell counts per gram x10^5)	7.2 (5.1-8.2)	6 (2.2-9.3)	0.72
CD8+ (median cell counts per gram x10^5)	2.5 (0.8-4.8)	1.2 (0.1-3.4)	0.4
CD4+CD56+ (median cell counts per gram x10^5)	3.6 (1-9.8)	0.3 (0.1-0.9)	0.03

Table 21. T-cells per ml of blood comparing atopic and non-atopic (Suff IgE) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic (n=8)	Non-atopic (Comp. aero Panel) (n=13)	P
CD4+ (percentage of CD3+ cells)	67.3 (60-77.9)	62 (53.4-68)	0.25
CD8+ (percentage of CD3+ cells)	22.1 (14.8-35.5)	25 (21.6-30.7)	0.65
CD4+CD56+ (% of CD3+ cells)	0.3 (0.2-0.7)	0.9 (0.3-1.5)	0.38

Table 22. T-cells as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
CD4+IFNγ+ (median cell counts per ml x10^5)	19.4 (n=5) (10.2-25.7)	9.7 (n=6) (2.6-34.7)	0.88
CD4+IL4+ (median cell counts per ml x10^5)	22.2 (n=5) (7.8-107.2)	42 (n=5) (15.1-234.5)	0.41
CD4+IL13+ (median cell counts per ml x10^5)	1 (n=5) (0.3-2.1)	0.8 (n=6) (0.3-1.9)	0.78
CD4+IL17+ (median cell counts per ml x10^5)	3.7 (n=5) (2.2-5.7)	3 (n=6) (1.4-5.5)	0.66
CD4+CD25+FOXp3+ (median cell counts per ml x10^5)	2.4 (n=4) (1.4-2.8)	0.7 (n=6) (0.4-2.6)	0.38

Table 23. Helper T-cell subsets per ml of blood comparing atopic and non-atopic (Suff IgE) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
CD4+IFNγ+ (% of CD4+ cells)	14 (n=5) (9.9-23.1)	8.9 (n=6) (6-27.6)	0.54
CD4+IL4+ (% of CD4+ cells)	28.8 (n=5) (9.4-82.7)	59 (n=5) (20-98.7)	0.73
CD4+IL13+ (% of CD4+ cells)	1.3 (n=5) (0.7-67)	0.6 (n=6) (0.5-1)	0.28
CD4+IL17+ (% of CD4+ cells)	3 (n=5) (1.8-7.3)	2.5 (n=6) (1.9-3.3)	0.66
CD4+CD25+FOXp3+ (% CD4+ cells)	1.5 (n=4) (0.4-2.3)	0.6 (n=6) (0.4-1.2)	0.38

Table 24. Helper T-cells as percentage of total cell counts atopic and non-atopic (Suff IgE) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

Lymphocytes	Atopic (n=8)	Non-atopic (all results) (n=15)	P
Total T-cells (CD3+) (Median cell counts per gram x10^5)	11.3 (6-13.5)	10.4 (3-12.6)	0.5
Total B-cells (CD19+) (Median cell counts per gram x10^5)	3.9 (1.8-5)	2.2 (0.9-5.6)	0.7
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^5)	0.1 (0.03-0.4)	0.1 (0-0.3)	0.8

Table 25. T-cells, B-cells and NK cell counts per ml of blood comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lymphocytes	Atopic (n=8)	Non-atopic (all results) (n=15)	P
Total T-cells (CD3+) (% of total cell count)	63.8 (50.3-67.5)	62.4 (57.5-69.6)	1.0
Total B-cells (CD19+) (% of total cell count)	20 (11.1-39.5)	20.2 (12.1-29.7)	0.7
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	0.47 (0.2-3.9)	2 (0.2-2.9)	0.8

Table 26. T-cells, B-cells and NK cell counts as percentage of total cell counts atopic and non-atopic (all results) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic (n=8)	Non-atopic (all results) (n=15)	P
CD4+ (median cell counts per gram x10^5)	7.2 (5-8.2)	6.2 (2.6-9.3)	0.89
CD8+ (median cell counts per gram x10^5)	2.5 (0.8-4.8)	1.4 (0.1-3.4)	0.48
CD4+CD56+ (median cell counts per gram x10^4)	3.6 (1-9.8)	0.5 (0.2-1)	0.05

Table 27. T-cells per ml of blood comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic (n=8)	Non-atopic (all results) (n=15)	P
CD4+ (percentage of CD3+ cells)	67.3 (60-77.9)	62.1 (54.1-90)	0.32
CD8+ (percentage of CD3+ cells)	22.1 (14.8-35.5)	24.9 (22.1-30.1)	0.60
CD4+CD56+ (% of CD3+ cells)	0.3 (0.2-0.7)	0.9 (0.3-1.5)	0.28

Table 28 T-cells as percentage of total cell counts atopic and non-atopic (all results) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Atopic	Non-atopic (all results)	P
CD4+IFNγ+ (median cell counts per gram x10^4)	19.4 (n=5) (10.2-25.7)	9.3 (n=7) (2.7-33.37)	0.72
CD4+IL4+ (median cell counts per gram x10^4)	22.2 (n=5) (7.8-107.2)	17.5 (n=7) (0-113.5)	0.93
CD4+IL13+ (median cell counts per gram x10^4)	1 (n=5) (0.3-2.1)	1.3 (n=7) (0.4-4.8)	1.00
CD4+IL17+ (median cell counts per gram x10^4)	3.7 (n=5) (2.2-5.7)	2.9 (n=7) (1.6-3.7)	0.53
CD4+CD25+FOXp3+ (median cell counts per gram x10^4)	0.9 (n=4) (0.4-1.4)	0.8 (n=7) (04-2.4)	0.6

Table29. Helper T-cell subsets per ml of blood comparing atopic and non-atopic (all results) subjects. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Atopic	Non-atopic (all results)	P
CD4+IFNγ+ (% of CD4+ cells)	14 (9.9-23.1)	8.7 (7-23.1)	0.43
CD4+IL4+ (% of CD4+ cells)	28.8 (9.4-82.7)	73.5 (24-98.6)	0.61
CD4+IL13+ (% of CD4+ cells)	1.3 (0.7-86)	0.7 (0.5-3.7)	0.48
CD4+IL17+ (% of CD4+ cells)	3 (1.8-7.3)	2.1 (1.6-3.2)	0.43
CD4+CD25+FOXp3+ (% CD4+ cells)	1.5 (0.4-2.6)	0.4 (0.2-0.8)	0.8

Table 30. Helper T-cells as percentage of total cell counts atopic and non-atopic (all results) subjects. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

Lymphocytes	Raised IgE (n=15)	Normal IgE (n=22)	P
Total T-cells (CD3+) (Median cell counts per gram x10^6)	8.9 (3.8-12.8)	10.4 (8.5-14.1)	0.4
Total B-cells (CD19+) (Median cell counts per gram x10^6)	1.7 (0.9-5.6)	4.9 (2.3-6.7)	0.1
Total Natural Killer cells (CD3-CD56+) (Median cell counts per gram x10^6)	0.2 (0.1-0.3)	0.04 (0.01-0.3)	0.2

Table 31. T-cells, B-cells and NK cell counts ml of blood comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

Lymphocytes	Raised IgE (n=15)	Normal IgE (n=22)	P
Total T-cells (CD3+) (% of total cell count)	63.5 (57.3-71.4)	61.9 (56.5-69.2)	0.7
Total B-cells (CD19+) (% of total cell count)	44.7 (33-84.7)	71.8 (59.6-95.7)	0.3
Total Natural Killer cells (CD3-CD56+) (% of total cell count)	5.6 (1.7-9.7)	1 (0.5-6.4)	0.2

Table 32. T-cells, B-cells and NK cell counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Raised Total IgE (n=15)	Normal total IgE (n=22)	P
CD4+ (median cell counts per gram x10^5)	6.8 (2.1-9)	6.7 (5.2-9.3)	0.49
CD8+ (median cell counts per gram x10^5)	1.3 (0.6-3.1)	2.8 (1.2-3.5)	0.37
CD4+CD56+ (median cell counts per gram x10^4)	0.1 (0-0.7)	0.6 (0.3-1.2)	0.15

Table 33. T-cells counts per ml of blood comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation

T-cell markers	Raised Total IgE (n=15)	Normal total IgE (n=22)	P
CD4+ (% of CD3+ cells)	69.2 (59.8-75.5)	62.3 (56.5-68)	0.34
CD8+ (% of CD3+ cells)	24.2 (6.7-33.8)	24.9 (21.4-28.5)	0.95
CD4+CD56+ (% of CD3+ cells)	0.5 (0.1-1)	0.7 (0.3-2.3)	0.48

Table 34. T-cells counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

T-cell markers	Raised Total IgE	Normal total IgE	P
CD4+IFNγ+ (median cell counts per gram x10^4)	12.6 (n=4) (4.2-43.1)	23 (n=7) (4.2-30.6)	0.79
CD4+IL4+ (cell counts per gram x10^4)	102 (n=3) (17.3-133.9)	27.1(n=7) (12.7-137)	0.67
CD4+IL13+ (median cell counts per gram x10^4)	6 (n=4) (0.2-12.9)	0.9 (n=7) (0.4-1.9)	0.54
CD4+IL17+ (median cell counts per gram x10^4)	2.2 (n=4) (1.2-3.4)	3.1 (n=7) (1.8-5.8)	0.41
CD4+CD25+FOXp3+ (median cell counts per gram x10^4)	0.1 (n=3) (0-0.2)	1.1 (n=6) (0.4-2)	0.02

Table 35. Helper T-cells counts per ml of blood comparing subjects with raised IgE to those with normal IgE. Median cell counts with interquartile ranges and p value generated with Mann-Whitney calculation.

T-cell markers	Raised Total IgE	Normal total IgE	P
CD4+IFNγ+ (% of CD4+ cells)	10.4 (n=4) (8-33.9)	9 (n=7) (7-23.1)	0.65
CD4+IL4+ (% of CD4+ cells)	88 (n=3) (59-99.8)	28 (n=7) (12.1-98.9)	0.18
CD4+IL13+ (% of CD4+ cells)	6 (n=4) (0.8-11.1)	0.6 (n=7) (0.5-1)	-
CD4+IL17+ (% of CD4+ cells)	2.4 (n=4) (1.7-3.3)	2.1 (n=7) (1.8-3.2)	0.92
CD4+CD25+FOXp3+ (% CD4+ cells)	0.5 (n=3) (0.1-0.8)	0.6 (n=6) (0.4-2.5)	-

Table 36. Helper T-cells counts as percentage of total cell counts comparing subjects with raised IgE to those with normal IgE.. Median cell counts and interquartile ranges with p value generated with Mann-Whitney calculation.

Appendix 14.2. Innate lymphoid cells

A14.2.1. Tonsils tissue results

Lineage negative cell markers	Atopic (n=15)	Non-atopic (Comp. aero Panel) (n=20)	P
Lin- (Median cell counts per gram x10^5)	20.3 (n=13) (2.5-27.4)	16.3 (n=15) (2.2-21.2)	0.27
Lin-CD127+ (Median cell counts per gram x10^4)	**52.7 (n=13) (37.2-127.2)**	**7.6 (n=15) (4.6-18)**	**0.0004**
Lin-CRTH2+ (Median cell counts per gram x10^4)	10.9 (n=12) (4-64.5)	4.2 (n=9) (2.6-16.3)	0.25
Lin-CD127+CRTH2+ (Median cell counts per gram x10^3)	1.1 (n=12) (0.7-3.8)	0.72 (n=9) (0.3-1.7)	0.20

Table 37. Lineage negative cells identified by published extracellular markers as cell counts per gram of tissue comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin- (% CD45+ cells)	1 (n=13) (0.5-2)	0.7 (n=15) (0.1-0.9)	0.05
Lin-CD127+ (% CD45+ cells)	0.1 (n=13) (0.03-0.1)	0.05 (n=15) (0.02-0.1)	0.20
Lin-CRTH2+ (% CD45+ cells)	0.07 (n=12) (0.02-0.4)	0.01 (n=9) (0-0.05)	0.2
Lin-CD127+CRTH2+ (% CD45+ cells)	**0.008 (n=12) (0.003-0.02)**	**0.001 (n=9) (0.0005-0.004)**	**0.005**

Table 38. Lineage negative cells identified by published extracellular markers as percentage of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin-IFNγ+ (Median cell counts per gram x10^4)	18.3 (n=10) (2.9-149.2)	12.8 (n=13) (1.8-25.7)	0.40
Lin-IL4+ (Median cell counts per gram x10^4)	32.5 (n=9) (8.5-158.2)	119 (n=11) (13-172.6)	0.64
Lin-IL13+ (Median cell counts per gram x10^3)	6.7 (n=10) (2-12.5)	1.5 (n=13) (0.6-5)	0.09
Lin-IL5+ (Median cell counts per gram x10^4)	20.1 (n=7) (7.5-57.3)	20.7 (n=6) (11.2-97.4)	0.94
Lin-IL17+ (Median cell counts per gram x10^4)	16.7 (n=10) (6.9-115.5)	7.8 (n=13) (1.3-23.6)	0.20

Table 39. Lineage negative cytokine producing cells as cell counts per gram of tissue comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin-IFNγ+ (% CD45+ cells)	0.1 (n=10) (0.02-0.7)	0.1 (n=13) (0.02-0.1)	0.24
Lin-IL4+ (% CD45+ cells)	0.4 (n=9) (0.04-0.8)	0.3 (n=11) (0.1-0.6)	0.84
Lin-IL13+ (% CD45+ cells)	0.03 (n=10) (0.01-0.1)	0.01 (n=13) (0.005-0.02)	0.05
Lin-IL5+ (% CD45+ cells)	0.1 (n=7) (0.03-0.4)	0.06 (n=6) (0.05-0.3)	0.94
Lin-IL17+ (% CD45+ cells)	0.12 (n=10) (0.03-0.5)	0.03 (n=13) (0.01-0.09)	0.04

Table 40. Lineage negative cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin-CD127+IFNγ+ (Median cell counts per gram x10^4)	17.1 (n=10) (0-138.8)	4 (n=13) (3.4-29.6)	0.72
Lin-CD127+IL4+ (Median cell counts per gram x10^4)	2.3 (n=9) (1.7-59.4)	4.6 (n=11) (2.9-16.8)	0.67
Lin-CD127+IL17+ (Median cell counts per gram x10^4)	7.8 (n=10) (4.2-10.3)	1.6 (n=13) (0.5-3.5)	0.05

Table 41. Lineage negative, $CD127^+$ and cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	OSA	Non-atopic (Comp. aero Panel)	P
Lin-CD127+IFNγ+ (% CD45+ cells)	0.02 (n=10) (0.01-0.1)	0.002 (n=13) (0.001-0.01)	0.07
Lin-CD127+IL4+ (% CD45+ cells)	0.02 (n=9) (0.01-0.2)	0.02 (n=11) (0.01-0.1)	0.83
Lin-CD127+IL17+ (% CD45+ cells)	0.03 (n=10) (0.01-0.04)	0.01 (n=13) (0.002-0.1)	0.12

Graph 42. Lineage negative, $CD127^+$ and cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation

Lineage negative cell markers	Atopic (n=9)	Non-atopic (Comp. aero Panel) (n=9)	P
Lin-Gata3+ (Median cell counts per gram x10^4)	22.9 (10.3-85.8)	5.7 (3.4-50.6)	0.29
Lin-Gata3+IL4+ (Median cell counts per gram x10^4)	6.7 (4.1-37.6)	9.2 (3.5-45.5)	0.89
Lin-Gata3+IL5+ (Median cell counts per gram x10^4)	3.2 (1.7-18.2)	4.7 (1.2-60.1)	1.00

Table 43. Innate lymphoid cell group 2 (Lin^-Gata3^+) as cell counts per gram of tissue comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation

Lineage negative cell markers	Atopic (n=9)	Non-atopic (Comp. aero Panel) (n=9)	P
Lin-Gata3+ (% CD45+ cells)	0.1 (0.04-0.4)	0.04 (0.01-0.1)	0.11
Lin-Gata3+IL4+ (% CD45+ cells)	0.05 (0.02-0.4)	0.04 (0.02-0.3)	0.86
Lin-Gata3+IL5+ (% CD45+ cells)	0.01 (0.01-0.1)	0.03 (0.003-0.2)	0.93

Table 44. Innate lymphoid cell group 2 ($Lin^{-}Gata3^{+}$) as percentages of $CD45^{+}$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin- (Median cell counts per gram $x10^5$)	20.3 (n=13) (2.5-27.4)	7.3 (n=22) (2.8-20.3)	0.17
Lin-CD127+ (Median cell counts per gram $x10^4$)	**52.7 (n=13) (37.2-127.2)**	**7.6 (n=22) (3.9-16.3)**	**<0.0001**
Lin-CRTH2+ (Median cell counts per gram $x10^4$)	10.9 (n=12) (3.9-64.5)	4 (n=14) (2.4-12)	0.09
Lin-CD127+CRTH2+ (Median cell counts per gram $x10^3$)	1.1 (n=12) (0.7-3.8)	1.2 (n=14) (0.4-2.9)	0.78

Table 45. Lineage negative cells identified by published extracellular markers as cell counts per gram of tissue comparing atopic subjects with non-atopic (all results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin- (% CD45+ cells)	1 (n=13) (0.5-2)	0.5 (n=22) (0.2-0.9)	0.03
Lin-CD127+ (% CD45+ cells)	0.1 (n=13) (0.03-0.1)	0.05 (n=22) (0.02-0.1)	0.15
Lin-CRTH2+ (% CD45+ cells)	0.1 (n=12) (0.02-0.4)	0.01 (n=14) (0.006-0.05)	0.02
Lin-CD127+CRTH2+ (% CD45+ cells)	**0.01 (n=12) (0.003-0.02)**	**0.003 (n=14) (0.001-0.004)**	**0.01**

Graph 46. Lineage negative cells identified by published extracellular markers as percentage of $CD45^{+}$ cells comparing atopic subjects with non-atopic (all results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin-IFNγ+ (Median cell counts per gram x10^4)	18.3 (n=10) (2.9-149.2)	13.8 (n=18) (3.7-32.9)	0.51
Lin-IL4+ (Median cell counts per gram x10^4)	32.5 (n=9) (8.5-158.2)	80.6 (n=16) (14-171.1)	0.67
Lin-IL13+ (Median cell counts per gram x10^3)	6.7 (n=10) (2-12.4)	2 (n=18) (0.6-7.8)	0.20
Lin-IL5+ (Median cell counts per gram x10^4)	20 (n=7) (7.5-57.3)	15 (n=9) (9.3-55.1)	1.00
Lin-IL17+ (Median cell counts per gram x10^4)	16.7 (n=10) (6.9-115.5)	7.6 (n=18) (1.5-14.5)	0.90

Table 47. Lineage negative cytokine producing cells as cell counts per gram of tissue comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin-IFNγ+ (% CD45+ cells)	0.1 (n=10) (0.02-0.7)	0.06 (n=18) (0.02-0.1)	0.36
Lin-IL4+ (% CD45+ cells)	0.4 (n=9) (0.04-0.8)	0.5 (n=16) (0.09-0.8)	0.90
Lin-IL13+ (% CD45+ cells)	0.03 (n=10) (0.01-0.07)	0.01 (n=18) (0.005-0.04)	0.15
Lin-IL5+ (% CD45+ cells)	0.13 (n=7) (0.03-0.4)	0.06 (n=9) (0.04-0.43)	1.00
Lin-IL17+ (% CD45+ cells)	0.12 (n=10) (0.03-0.5)	0.03 (n=18) (0.01-0.09)	0.03

Table 48. Lineage negative cytokine producing cells as cell counts per gram of tissue comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin-CD127+IFNγ+ (Median cell counts per gram x10^4)	17.1 (n=10) (0-138.8)	3.7 (n=18) (2-4.5)	0.51
Lin-CD127+IL4+ (Median cell counts per gram x10^4)	2.3 (n=9) (1.7-59.4)	5.1 (n=16) (3-17.1)	0.57
Lin-CD127+IL17+ (Median cell counts per gram x10^4)	7.8 (n=10) (4.2-10.3)	1.6 (n=18) (0.6-2.7)	0.02

Table 49. Lineage negative, $CD127^+$ and cytokine producing cells as cell counts per gram of tissue comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (all results)	P
Lin-CD127+IFNγ+ (% CD45+ cells)	0.03 (n=10) (0.009-0.07)	0.003 (n=18) (0.002-0.01)	0.13
Lin-CD127+IL4+ (% CD45+ cells)	0.02 (n=9) (0.01-0.2)	0.03 (n=16) (0.02-0.1)	0.69
Lin-CD127+IL17+ (% CD45+ cells)	0.03 (n=10) (0.01-0.05)	0.01 (n=18) (0.004-0.02)	0.15

Graph 50. Lineage negative, $CD127^+$ and cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic (n=9)	Non-atopic (all results) (n=11)	P
Lin-Gata3+ (Median cell counts per gram $x10^4$)	22.9 (10.3-85.8)	37.6 (4-213.2)	0.97
Lin-Gata3+IL4+ (Median cell counts per gram $x10^4$)	6.7 (4.1-37.6)	12.8 (3.5-42.4)	0.87
Lin-Gata3+IL5+ (Median cell counts per gram $x10^4$)	3.2 (1.7-18.2)	4.7 (1.3-47.9)	0.73

Table 51. Innate lymphoid cell group 2 (Lin^-Gata3^+) as cell counts per gram of tissue comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic (n=9)	Non-atopic (all results) (n=11)	P
Lin-Gata3+ (% CD45+ cells)	0.14 (0.04-0.4)	0.1 (0.02-0.3)	0.30
Lin-Gata3+IL4+ (% CD45+ cells)	0.05 (0.02-0.2)	0.05 (0.03-0.3)	0.80
Lin-Gata3+IL5+ (% CD45+ cells)	0.01 (0.01-0.1)	0.03 (0.004-0.2)	0.94

Table 52. Innate lymphoid cell group 2 (Lin^-Gata3^+) as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin- (Median cell counts per gram $x10^5$)	**83.8 (n=14) (50-200.7)**	**21.5 (n=15) (5.6-29.9)**	**<000.1**
Lin-CD127+ (Median cell counts per gram $x10^4$)	9.1 (n=14) (6-15.4)	10.5 (n=15) (3-23.7)	0.84
Lin-CRTH2+ (Median cell counts per gram $x10^4$)	7.5 (n=11) (3.4-10.9)	11.43 (n=13) (1.6-159.2)	0.60
Lin-CD127+CRTH2+ (Median cell counts per gram $x10^3$)	**10.9** (n=11) **(4.8-22.8)**	**0.5** (n=13) **(0.2-2.4)**	**0.008**

Table 53. Lineage negative cells identified by published extracellular markers as cells per gram of tissue comparing subjects with raised total IgE against subjects with normal total IgE. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin- (% CD45+ cells)	0.9 (n=14) (0.4-1)	0.8 (n=15) (0.1-1)	0.90
Lin-CD127+ (% CD45+ cells)	0.07 (n=14) (0.03-0.1)	0.06 (n=15) (0.02-0.09)	0.53
Lin-CRTH2+ (% CD45+ cells)	0.04 (n=11) (0.01-0.1)	0.05 (n=13) (0.01-0.1)	0.88
Lin-CD127+CRTH2+ (% CD45+ cells)	0.01 (n=11) (0.002-0.02)	0.002 (n=13) (0.001-0.006)	0.07

Table 54. Lineage negative cells identified by published extracellular markers as percentage of $CD45^+$ cells comparing subjects with raised total IgE against subjects with normal total IgE. Values demonstrated are median percentags with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin-IFNγ+ (Median cell counts per gram x10^4)	7.8 (n=11) (3.8-31.5)	17.7 (n=12) (5.4-143.3)	0.34
Lin-IL4+ (Median cell counts per gram x10^4)	21.7 (n=9) (9.6-54.7)	156.8 (n=12) (44.8-192.2)	0.02
Lin-IL13+ (Median cell counts per gram x10^3)	7.2 (n=11) (2.2-11.3)	2 (n=12) (0.7-6.7)	0.15
Lin-IL5+ (Median cell counts per gram x10^4)	13.8 (n=8) (7.2-54.4)	32.1 (n=6) (11.2-184.1)	0.41
Lin-IL17+ (Median cell counts per gram x10^4)	6.9 (n=11) (2-17.8)	14.8 (n=12) (93-42.2)	0.37

Table 55. Lineage negative cytokine producing cells as cell counts per gram of tissue comparing samples from subjects with raised total IgE against those with normal total IgE. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin-IFNγ+ (% CD45+ cells)	0.1 (n=11) (0.02-0.2)	0.1 (n=12) (0.03-0.3)	0.83
Lin-IL4+ (% CD45+ cells)	0.2 (n=9) (0.04-0.5)	0.6 (n=12) (0.1-0.8)	0.04
Lin-IL13+ (% CD45+ cells)	0.03 (n=11) (0.01-0.05)	0.005 (n=12) (0.005-0.03)	0.04
Lin-IL5+ (% CD45+ cells)	0.1 (n=8) (0.03-0.4)	0.1 (n=6) (0.05-0.7)	0.41
Lin-IL17+ (% CD45+ cells)	0.05 (n=11) (0.01-0.1)	0.03 (n=12) (0.01-0.1)	0.97

Table 56. Lineage negative cytokine producing cells as cell counts per gram of tissue comparing samples from subjects with raised total IgE against those with normal total IgE. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin-CD127+IFNγ+ (Median cell counts per gram x10^4)	1.6 (n=11) (0.8-7.8)	0.4 (n=12) (0.4-5.2)	0.18
Lin-CD127+IL4+ (Median cell counts per gram x10^4)	2.7 (n=9) (1.9-14.2)	11.2 (n=12) (4.2-88.6)	0.17
Lin-CD127+IL17+ (Median cell counts per gram x10^4)	2.7 (n=11) (0.7-3.6)	1.6 (n=12) (0.4-5.6)	1.00

Table 57. Lineage negative, $CD127^+$ and cytokine producing cells as cell counts per gram of tissue comparing samples from subjects with raised total IgE against those with normal total IgE. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal Total IgE	P
Lin-CD127+IFNγ+ (% CD45+ cells)	0.01 (n=11) (0.01-0.02)	0.003 (n=12) (0.002-0.02)	0.23
Lin-CD127+IL4+ (% CD45+ cells)	0.01 (n=9) (0.01-0.1)	0.05 (n=12) (0.02-0.3)	0.05
Lin-CD127+IL17+ (% CD45+ cells)	0.03 (n=11) (0.01-0.4)	0.01 (n=12) (0.004-0.02)	0.27

Table 58. Lineage negative, $CD127^+$ and cytokine producing cells as percentages of $CD45^+$ cells comparing samples from subjects with raised total IgE against those with normal total IgE. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE (n=8)	Normal Total IgE (n=11)	P
Lin-Gata3+ (Median cell counts per gram $x10^4$)	28.9 (13.7-44.6)	5.7 (3.2-53.8)	0.43
Lin-Gata3+IL4+ (Median cell counts per gram $x10^4$)	14.5 (5.2-38.6)	37.6 (3.5-108.9)	0.64
Lin-Gata3+IL5+ (Median cell counts per gram $x10^4$)	3.1 (1.7-44.2)	9.2 (3-61.2)	0.53

Table 59. Innate lymphoid cell group 2 (Lin^-Gata3^+) as cell counts per gram of tissue comparing atopic subjects with non-atopic (All results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE (n=8)	Normal Total IgE (n=11)	P
Lin-Gata3+ (% CD45+ cells)	0.2 (0.06-0.4)	0.04 (0.01-0.1)	0.13
Lin-Gata3+IL4+ (% CD45+ cells)	0.1 (0.02-0.3)	0.1 (0.02-0.4)	0.96
Lin-Gata3+IL5+ (% CD45+ cells)	0.01 (0.01-0.2)	0.04 (0.02-0.2)	0.79

Table 60. Innate lymphoid cell group 2 (Lin^-Gata3^+) as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

A14.2.2. Peripheral blood results

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin- (Median cell counts per gram $x10^4$)	4.7 (n=7) (4-7.6)	1.4 (n=13) (0.4-9.6)	0.20
Lin-CD127+ (Median cell counts per gram $x10^3$)	2.5 (n=7) (0.9-5.6)	1.4 (n=13) (0.2-4.4)	0.53
Lin-CRTH2+ (Median cell counts per gram $x10^3$)	4 (n=5) (1.1-8.5)	0.5 (n=7) (0-4.1)	0.12
Lin-CD127+CRTH2+ (Median cell counts per gram $x10^3$)	1.2 (n=5) (0.3-2.1)	0.1 (n=7) (0-1.2)	0.07

Table 61. Lineage negative cells identified by published extracellular markers as cell counts per ml of blood comparing samples from atopic subjects with samples from non-atopic subjects (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin- (% CD45+ cells)	4 (n=7) (2.4-6.3)	0.7 (n=13) (0.3-7.5)	0.30
Lin-CD127+ (% CD45+ cells)	0.2 (n=7) (0.06-0.3)	0.1 (n=13) (0.02-0.5)	0.43
Lin-CRTH2+ (% CD45+ cells)	0.34 (n=5) (0.1-0.5)	0.03 (n=7) (0-0.2)	0.07
Lin-CD127+CRTH2+ (% CD45+ cells)	0.09 (n=5) (0.03-0.1)	0.01 (n=7) (0-0.07)	0.05

Table 62. Lineage negative cells identified by published extracellular markers as percentage of $CD45^+$ cells comparing samples from atopic subjects with samples from non-atopic subjects (those with sufficient specific IgE test results only). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin-IFNγ+ (Median cell counts per gram $x10^3$)	41.1 (n=7) (15.5-77.7)	32.1 (n=7) (3.5-93.1)	0.92
Lin-IL4+ (Median cell counts per gram $x10^3$)	36.4 (n=4) (7.7-61.9)	16.9 (n=6) (8.9-88.2)	1.00
Lin-IL13+ (Median cell counts per gram $x10^3$)	1.8 (n=7) (1-57.3)	1 (n=7) (0.8-2)	0.40
Lin-IL17+ (Median cell counts per gram $x10^3$)	13.5 (n=7) (2.7-50.7)	0.9 (n=7) (0.1-6)	0.23

Table 63. Lineage negative cytokine producing cells as cell counts per ml of blood comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (Comp. aero Panel)	P
Lin-IFNγ+ (% CD45+ cells)	3.2 (n=7) (0.8-5)	2.7 (n=7) (0.2-5.3)	0.79
Lin-IL4+ (% CD45+ cells)	2 (n=4) (0.5-5.6)	1.2 (n=6) (0.3-6.8)	0.91
Lin-IL13+ (% CD45+ cells)	0.2 (n=7) (0.05-3.1)	0.09 (n=7) (0.02-0.2)	0.41
Lin-IL17+ (% CD45+ cells)	1.2 (n=7) (0.1-3)	0.04 (n=7) (0.001-1)	0.15

Table 64. Lineage negative cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (those with sufficient specific IgE test results only). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (All results)	P
Lin- (Median cell counts per gram $x10^4$)	4.7 (n=7) (4-7.6)	3.7 (n=15) (0.5-11.8)	0.40
Lin-CD127+ (Median cell counts per gram $x10^3$)	2.5 (n=7) (0.9-5.6)	1.8 (n=15) (0.2-5.2)	0.72
Lin-CRTH2+ (Median cell counts per gram $x10^3$)	4 (n=5) (1.1-8.5)	3.5 (n=7) (1-5.1)	0.53
Lin-CD127+CRTH2+ (Median cell counts per gram $x10^3$)	1.2 (n=5) (0.3-2.2)	0.1 (n=7) (0-1.6)	0.10

Table 65. Lineage negative cells identified by published extracellular markers as cell counts per ml of blood comparing samples from atopic subjects with samples from non-atopic subjects (all results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (All results)	P
Lin- (% CD45+ cells)	4 (n=7) (2.3-6.3)	2.5 (n=15) (0.4-7.9)	0.40
Lin-CD127+ (% CD45+ cells)	0.2 (n=7) (0.1-0.3)	0.1 (n=15) (0.02-0.4)	0.62
Lin-CRTH2+ (% CD45+ cells)	0.3 (n=5) (0.1-0.5)	0.1 (n=7) (0.005-0.2)	0.14
Lin-CD127+CRTH2+ (% CD45+ cells)	0.1 (n=5) (0.03-0.12)	0.01 (n=7) (0-0.08)	0.06

Table 66. Lineage negative cells identified by published extracellular markers as percentage of $CD45^+$ cells comparing samples from atopic subjects with samples from non-atopic subjects (all results). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (All results)	P
Lin-IFNγ+ (Median cell counts per gram $x10^3$)	41.1 (n=7) (15.5-77.7)	35.1 (n=7) (13.4-70.8)	0.94
Lin-IL4+ (Median cell counts per gram $x10^3$)	36.4 (n=5) (7.7-61.9)	27.4 (10.7-96.3)	0.80
Lin-IL13+ (Median cell counts per gram $x10^3$)	1.8 (n=7) (1-57.2)	1 (n=7) (0.8-5.8)	0.56
Lin-IL17+ (Median cell counts per gram $x10^3$)	13.5 (n=7) (2.7-50.7)	0.9 (n=7) (0.09-26.4)	0.26

Table 67. Lineage negative cytokine producing cells as cell counts per ml of blood comparing atopic subjects with non-atopic (all results). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Atopic	Non-atopic (All results)	P
Lin-IFNγ+ (% CD45+ cells)	3.2 (n=7) (0.8-5)	2.4 (n=7) (1-5.2)	0.82
Lin-IL4+ (% CD45+ cells)	2 (n=5) (0.5-5.6)	2 (n=7) (0.5-5.7)	1.00
Lin-IL13+ (% CD45+ cells)	0.2 (n=7) (0.5-3.1)	0.1 (n=7) (0.02-0.3)	0.57
Lin-IL17+ (% CD45+ cells)	1.2 (n=7) (0.1-3)	0.05 (0.003-2) (n=7)	0.3

Table 68. Lineage negative cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (all results). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal total IgE	P
Lin- (Median cell counts per gram x10^4)	5.1 (n=7) (1.2-11.7)	0.4 (0-2.7)	0.47
Lin-CD127+ (Median cell counts per gram x10^3)	3.6 (n=7) (0.2-5.2)	1.1 (0.3-5.5)	0.70
Lin-CRTH2+ (Median cell counts per gram x10^3)	2 (0.2-5.9)	1.7 (0-5.1)	0.90
Lin-CD127+CRTH2+ (Median cell counts per gram x10^3)	1.5 (0.4-2.1)	0.1 (0-1.1)	0.22

Table 69. Lineage negative cells identified by published extracellular markers as cell counts per ml of blood comparing samples from subjects with raised total IgE against subjects with normal total IgE. Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal total IgE	P
Lin- (% CD45+ cells)	6.3 (n=7) (4-7.9)	2 (n=11) (0.3-7.7)	0.20
Lin-CD127+ (% CD45+ cells)	0.2 (n=7) (0.4-0.8)	0.08 (n=11) (0.02-0.3)	0.59
Lin-CRTH2+ (% CD45+ cells)	0.1 (n=4) (0.01-0.3)	0.1 (n=7) (0-0.3)	0.92
Lin-CD127+CRTH2+ (% CD45+ cells)	0.1 (n=4) (0-0.1)	0.008 (n=7) (0-0.06)	0.70

Graph 70. Lineage negative cells identified by published extracellular markers as percentage of $CD45^+$ cells comparing samples from subjects with raised total IgE against subjects with normal total IgE. Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal total IgE	P
Lin-IFNγ+ (Median cell counts per gram x10^3)	60.1 (n=4) (26.3-91.5)	40.2 (n=7) (3.4-97)	0.61
Lin-IL4+ (Median cell counts per gram x10^3)	60.3 (20-112.8)	13.7 (n=7) (6.7-62.4)	0.38
Lin-IL13+ (Median cell counts per gram x10^3)	4.5 (n=4) (1.3-58.3)	0.9 (n=7) (0.7-5.8)	0.17
Lin-IL17+ (Median cell counts per gram x10^3)	26.5 (n=4) (2.2-56.9)	2.7 (n=7) (0.1-34.8)	0.35

Graph 71. Lineage negative cytokine producing cells as cell counts per ml of blood comparing comparing samples from subjects with raised total IgE against subjects with normal total IgE). Values demonstrated are median cell counts with interquartile ranges. Significance calculated using Mann Whitney calculation.

Lineage negative cell markers	Raised Total IgE	Normal total IgE	P
Lin-IFNγ+ (% CD45+ cells)	4.9 (n=4) (2.6-5.3)	2 (n=7) (0.1-7.1)	0.35
Lin-IL4+ (% CD45+ cells)	4.4 (n=3) (3.2-6)	0.9 (n=7) (0.3-6.3)	0.38
Lin-IL13+ (% CD45+ cells)	0.3 (n=4) (0.1-3.1)	0.05 (n=7) (0.01-0.4)	0.17
Lin-IL17+ (% CD45+ cells)	2 (n=4) (0.4-3)	0.1 (n=7) (0.003-2.7)	0.35

Graph 72. Lineage negative cytokine producing cells as percentages of $CD45^+$ cells comparing atopic subjects with non-atopic (all results). Values demonstrated are median percentages with interquartile ranges. Significance calculated using Mann Whitney calculation.