

## **Title**

Relationship between serum vitamin D, disease severity and airway remodeling in children with asthma

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## **Running head**

Vitamin D and pediatric severe asthma

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#### **Authorship credit**

AG recruited majority of the patients, collected patient samples, conducted majority of experiments, analyzed the data and took the lead on writing the manuscript.

AS performed some of the experimental work.

AG, AB, CH and SS conceptualized, delineated the hypotheses and designed the experiments

AB, CH, DR and SS contributed to the interpretation of the analyses, supervised the project and helped with revising the manuscript.

AB and SS also performed bronchoscopies and obtained samples.

WB performed some of the statistical analyses.

## **Scientific knowledge on the subject**

Children with mild to moderate asthma are more likely to have vitamin D insufficiency. Epidemiologic data suggest that low serum vitamin D in asthmatic children is associated with poor asthma control, reduced lung function and increased medication usage. However, little is known about the relationship between serum vitamin D levels and pathophysiology in children with severe therapy resistant asthma (STRA).

## **What this study adds to the field**

Children with STRA have lower serum vitamin D levels than moderate asthmatics and controls. Reduced vitamin D levels in STRA are associated with lower lung function, poor asthma control, increased medication use and asthma exacerbations. Serum vitamin D level is inversely associated with airway smooth muscle mass.

This article has an online data supplement, which is accessible from this issue's table of content online at [www.atsjournals.org](http://www.atsjournals.org)

## Abstract

### Rationale

Little is known about vitamin D status and its effect on asthma pathophysiology in children with severe, therapy resistant asthma (STRA). Relationships between serum vitamin D, lung function, and pathology were investigated in pediatric STRA.

### Methods

Serum 25-hydroxyvitamin D (25[OH]D<sub>3</sub>) was measured in 86 children (mean age 11.7 years), 36 STRA, 26 moderate asthmatics (MA) and 24 non-asthmatic controls. Relationships between 25[OH]D<sub>3</sub>, the asthma control test (ACT), spirometry, corticosteroid usage, and exacerbations were assessed. 22/36 children with STRA underwent fiberoptic bronchoscopy, bronchoalveolar lavage and endobronchial biopsy with assessment of airway inflammation and remodeling.

### Results

25[OH]D<sub>3</sub> levels (median [IQR]) were significantly lower in STRA (28[22-38])nmol/L than MA (42.5[29-63])nmol/L and controls (56.5[45-67])nmol/L ( $p<0.001$ ). There was a positive relationship between 25[OH]D<sub>3</sub> levels and %predicted forced expired volume (FEV<sub>1</sub>) ( $r=0.4$ ,  $p<0.001$ ) and forced vital capacity (FVC) ( $r=0.3$ ,  $p=0.002$ ) in all subjects. 25[OH]D<sub>3</sub> levels were positively associated with ACT ( $r=0.6$ ,  $p<0.001$ ), and inversely associated with exacerbations ( $r=-0.6$ ,  $p<0.001$ ) and inhaled steroid dose ( $r=-0.39$ ,  $p=0.001$ ) in MA & STRA. Airway smooth muscle (ASM) mass, but not epithelial shedding or reticular basement membrane thickness, was inversely related to 25[OH]D<sub>3</sub> levels ( $r=-0.6$ ,  $p=0.008$ ). There was a positive correlation between ASM

mass and bronchodilator reversibility ( $r=0.6$ ,  $p=0.009$ ) and an inverse correlation between ASM mass and ACT ( $r=-0.7$ ,  $p<0.001$ )

### Conclusions

Lower vitamin D levels in children with STRA were associated with increased ASM mass, worse asthma control and lung function. The link between vitamin D, airway structure and function suggests vitamin D supplementation may be useful in pediatric STRA.

Abstract word count - 250

## Introduction

For most children with ready access to healthcare, asthma can be controlled with low doses of inhaled steroids. There remains, however, a significant number of asthmatics who, despite apparently appropriate treatment, remain symptomatic and at risk of exacerbations. Most assessments have suggested that this difficult-to-treat population represents 5-10% of asthmatics (1-3). Even though this high risk group only represents a small portion of all asthmatics, they suffer the greatest morbidity and need for healthcare utilization.

Exposure to solar ultraviolet radiation within a wavelength band of 290-315nm leading to production of vitamin D in the skin is the primary source of vitamin D for humans (4). Over the last few decades, vitamin D deficiency and insufficiency are increasingly being recognized in the general population, and have been largely attributed to dietary, lifestyle and behavioral changes (4). The musculoskeletal consequences of vitamin D deficiency are well established, however, a number of pulmonary disorders, including asthma, have now been linked to vitamin D deficiency and insufficiency. Cross-sectional data suggest that low serum vitamin D in children with mild to moderate asthma is associated with poor asthma control, more exacerbations, reduced lung function and increased medication usage (5-7). However, little is known about vitamin D levels and their impact on disease control and airway pathology in children with severe, therapy-resistant asthma (STRA).

An increase in airway smooth muscle (ASM) mass is a key feature of airway remodeling in asthma (8-10). Importantly, increased ASM hypertrophy and hyperplasia has been demonstrated in endobronchial biopsies from children with

severe asthma and is significantly related to bronchodilator responsiveness (10). However, to date, there is little evidence that any asthma therapies affect airway remodeling. *In vitro* studies have shown that vitamin D may influence ASM remodeling by exerting an inhibitory effect on passively sensitized ASM growth and contractility (11-13). However, the relationship between airway pathology in bronchial tissue from asthmatics and serum vitamin D levels has not been reported.

We hypothesised that our cohort of children with STRA would have lower serum vitamin D levels than moderate asthmatics (MA) and non-asthmatic controls, and that lower serum vitamin D levels would be associated with worse lung function, airway inflammation and remodeling.

Some of the results of these studies have been previously reported in the form of abstract (14).

## **Methods**

(Detailed methods are described in the online supplement (OLS))

### **Subjects**

Children aged 6-16 years with STRA (n=36), MA (n=26) and non-asthmatic controls (n=24) were recruited prospectively (Table1). The Royal Brompton and Harefield Research Ethics Committee approved the study. Informed consent was obtained from parents and age-appropriate assent from children.

A detailed definition of STRA is given in the OLS. Briefly, these were all children on  $\geq 800$ mcg/day beclomethasone equivalent inhaled steroids and additional controller

medications, and had undergone detailed assessments to exclude a wrong diagnosis, asthma with important co-morbidities and difficult asthma (underlying modifiable factors identified) (15, 16). Children with MA were well controlled on lower dose (<800mcg/day beclomethasone equivalent) inhaled corticosteroids. Non-asthmatic controls comprised either children with no respiratory disease whose parents had consented for blood tests during an elective surgical procedure (n=18) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (n=6).

### **Asthma Control Test (ACT)**

Symptom control was assessed using the childhood ACT(17)(OLS).

### **Exacerbations and medication usage**

Regular medications were recorded in MA and STRA. Acute exacerbations were defined as episodes necessitating high dose oral steroids for at least 3 days, in the previous six months.

### **Lung Function**

Spirometry was performed in accordance with American Thoracic Society guidelines (18)(OLS).

### **Serum 25-hydroxyvitamin D (25[OH]D<sub>3</sub>)**

Serum levels of 25[OH]D<sub>3</sub> were measured in all subjects using a 2 dimensional high performance liquid chromatography system - tandem mass spectrometry (2D LC-MS-MS) (19). Vitamin D deficiency was defined as serum 25[OH]D<sub>3</sub> <50nmol/L



(20ng per milliliter) (4, 20) and serum levels  $<75\text{nmol/L}$  were defined as insufficient (4, 20)(OLS).

### **Sputum eosinophils and neutrophils**

Sputum induction, processing and cell counts were performed in a subgroup of STRA (n=22) as previously described (21)(OLS).

### **Bronchoscopy, bronchoalveolar lavage (BAL) and endobronchial biopsy**

Bronchoscopy was performed under general anesthetic(22) in children with STRA (n=22) as part of clinical investigations(15)(OLS). Details of BAL and endobronchial biopsy processing are in OLS. Eosinophils and neutrophils were quantified in BAL and biopsy, and mast cells were quantified in biopsy alone. Smooth muscle proliferation was quantified by immunohistochemistry for proliferating cell nuclear antigen (PCNA). Reticular basement membrane (RBM) thickness (23), epithelial shedding (24) and airway smooth muscle (ASM) mass (10) were quantified in sections stained with haematoxylin and eosin(OLS).

### **Statistical analysis**

Differences between 3 groups were assessed using one way analysis of variance (normal data) or Kruskal-Wallis test (non-normal distribution). To assess the association between serum vitamin D levels and severity of disease, linear regression was used for continuous variables and logistic regression was used for categorical variables. When a non-normal variable remained skewed even after log transformation, this variable was dichotomized and used in the logistic regression. For both types of regression analyses, 2 models (an unadjusted and a multivariable

model were constructed). Follow-up tests performed after ANOVA were determined *a priori* unless specified otherwise. Correlations were assessed using the Pearson correlation (normal data) or the Spearman's rank correlation (skewed data). Statistical significance was reported at  $p < 0.05$ . Stata version 10.1 (Statacorp Texas, USA) and GraphPad Prism version 5.02 were used (OLS).

## Results

### Subjects

Demographic data of the children studied are presented in Table 1. There were no significant differences between children with MA, STRA, and non-asthmatic controls in age, ethnicity, gender distribution or BMI.

### Serum 25[OH]D<sub>3</sub> levels in asthmatics and controls

Serum 25[OH]D<sub>3</sub> levels (median [IQR] nmol/L) were significantly lower in children with STRA (28[22-38])nmol/L than those with MA (42.5[29-63])nmol/L, and non-asthmatic controls (56.5[45-67])nmol/L ( $p < 0.001$  for both) (Figure 1A). The prevalence of vitamin D deficiency (25[OH]D<sub>3</sub> level  $< 50$ nmol/L) was 94%, 54% & 33% in STRA, MA and controls respectively ( $p < 0.001$ ) (Figure 1B) and 97% of children with STRA, 92% of MA and 83% of controls had insufficient serum 25[OH]D<sub>3</sub> levels (25[OH]D<sub>3</sub> level  $< 75$ nmol/L).

There was no significant impact of season of sample collection on serum vitamin D level in the three groups (Figure E1 OLS).

### Vitamin D and atopic status

Serum 25[OH]D<sub>3</sub> was inversely related to serum total IgE ( $r=-0.3$ ,  $p=0.01$ ), specific IgE to cat ( $r=-0.27$ ,  $p=0.01$ ), dog ( $r=-0.29$ ,  $p=0.01$ ), tree pollen ( $r=-0.28$ ,  $p=0.02$ ), *Dermatophagoides pteronyssinus* ( $r=-0.3$ ,  $p=0.01$ ), and *Aspergillus fumigatus* ( $r=-0.36$ ,  $p=0.009$ ) (Table E1). In a *post hoc* analysis, there was a significant inverse relationship between serum 25[OH]D<sub>3</sub> levels and sum of specific IgE to aeroallergen ( $r=-0.28$ ,  $p=0.009$ ) but not to sum of specific IgE to food allergens.

### **Lung function, bronchodilator reversibility (BDR) and vitamin D**

There was a positive correlation between serum 25[OH]D<sub>3</sub> levels and % predicted FEV<sub>1</sub> ( $r=0.43$ ,  $p<0.001$ ) (Figure 2A) and FVC ( $r=0.32$ ,  $p=0.002$ ) (Figure 2B). Serum 25[OH]D<sub>3</sub> was significantly and inversely associated with % BDR ( $r=-0.4$ ,  $p=0.003$ ) and in a *post hoc* analysis, positive BDR (FEV<sub>1</sub> improvement of at least 12%) (Figure 2C & 2D)

### **Asthma control, exacerbations and vitamin D levels**

A positive relationship was found between 25[OH]D<sub>3</sub> level and ACT ( $r=0.6$ ,  $p<0.001$ ) (Figure 3A), whereby higher serum 25[OH]D<sub>3</sub> was associated with better asthma control. Lower serum 25[OH]D<sub>3</sub> levels were associated with increased acute asthma exacerbations in the previous six months ( $r=-0.6$ ,  $p<0.001$ ) (Figure 3B).

### **Medication dose and vitamin D levels**

Of the different therapies received by children with MA & STRA, the use of daily maintenance oral steroids ( $p<0.001$ ), oral theophyllines ( $p=0.02$ ), and leukotriene receptor antagonists (LTRA) ( $p=0.005$ ) were significantly associated with lower

25[OH]D<sub>3</sub> levels, but there was no association between anti-reflux therapy and serum 25[OH]D<sub>3</sub> level (Table 2). Moreover, the daily dose of ICS ( $r=-0.39$ ,  $p=0.001$ ) and oral maintenance corticosteroids ( $r=-0.43$ ,  $p<0.001$ ) were inversely related to serum 25[OH]D<sub>3</sub> levels (Figure 3C & 3D). There was no association between use of anti-reflux medications and serum 25[OH]D<sub>3</sub> levels.

## **Serum vitamin D levels and asthma pathology**

### **i) Airway Inflammation**

Table E3 (OLS) outlines the relationship between serum 25[OH]D<sub>3</sub> levels and measures of airway inflammation in children with STRA. There was no significant correlation between serum 25[OH]D<sub>3</sub> levels and eosinophils or neutrophils in induced sputum ( $n=18$ ), BAL ( $n=22$ ) or endobronchial biopsy ( $n=19$ ). There was also no association between tissue mast cells and serum 25[OH]D<sub>3</sub>.

### **ii) Airway Remodeling**

Endobronchial biopsies were of sufficient quality to quantify airway remodeling in 19 of 21 children with STRA. Median volume fraction of smooth muscle was 0.16 (range 0.07-0.20). There was a significant negative correlation between serum 25[OH]D<sub>3</sub> and volume fraction of ASM ( $r=-0.63$ ,  $p=0.007$ ) (Figure 4A). Median RBM thickness in children with STRA was 8.4 (range 7.9-9.7) $\mu$ m. There was no relationship between serum 25[OH]D<sub>3</sub> and RBM thickness ( $r=-0.12$ ,  $p=0.62$ ) (Figure 4B). Median % epithelial shedding in the biopsies was 98 (range 87.4-99.7)%. There was no significant relationship between epithelial shedding and serum 25[OH]D<sub>3</sub> ( $r=-0.09$ ,  $p=0.69$ ) (Figure 4C). Smooth muscle proliferation was assessed by quantifying the proportion of PCNA positive smooth muscle cells (Figure E2). Median myocyte

proliferation was 35.3% (range 25.3-64.3%). There was no relationship between serum 25[OH]D<sub>3</sub> and % of smooth muscle cells positive for PCNA ( $r=0.007$ ,  $p=0.07$ ) (Figure 4D). There was a significant positive correlation between volume fraction of ASM and BDR ( $r=0.6$ ,  $p=0.009$ ) (Figure 5A) and a negative correlation between volume fraction of ASM and ACT ( $r=-0.7$ ,  $p<0.001$ ) (Figure 5B).

## Discussion

We have shown for the first time that children with STRA have significantly lower serum 25[OH]D<sub>3</sub> levels than MA. Lower serum 25[OH]D<sub>3</sub> levels were associated with worse lung function, poor asthma control and more steroid use in MA and STRA. Within STRA, low 25[OH]D<sub>3</sub> levels were associated with increased ASM mass, but not with other parameters of airway remodeling, nor with airway inflammation despite an association with aeroallergen sensitisation.

Importantly, the children in this study with STRA had been carefully assessed such that their basic management had been optimized, and any 'difficult asthmatics' (whose asthma was uncontrolled because of modifiable factors such as poor adherence to treatment) had been excluded. The detailed multi-disciplinary assessment to ensure as far as possible that basic management is correct is one of the novel features of this study.

In this group with STRA, a significant negative association was present between volume fraction of ASM and 25[OH]D<sub>3</sub> levels. Of note, however, there was no association between RBM thickness or epithelial shedding and vitamin D. Although a

negative relationship between ASM mass and lung function has been reported in pediatric difficult asthmatics (10), this is the first demonstration of an association between low serum 25[OH]D<sub>3</sub>, poor lung function and asthma control, increased BDR and ASM mass. It is therefore plausible that the link between ASM mass and lung function in severe asthma may partly be explained by low 25[OH]D<sub>3</sub> levels. The association between increased ASM mass and low 25[OH]D<sub>3</sub> is supported by *in vitro* studies which have shown that vitamin D inhibits smooth muscle proliferation (11-13). Vitamin D blocked smooth muscle proliferation in a concentration dependent manner in human smooth muscle cells sensitized with asthmatic serum (12), and it inhibited ASM cell proliferation by preventing progression of the cell cycle, not by inducing apoptosis (13). Furthermore, vitamin D inhibits cell growth in muscle cell cultures (13). Moreover, vitamin D increases glucocorticoid bioavailability in bronchial smooth muscle cells (25). In contrast to the published *in vitro* studies, there was no relationship between serum 25[OH]D<sub>3</sub> levels and ASM proliferation assessed by myocyte PCNA staining in our subjects. However, all *in vitro* work is in adult ASM and mechanisms may be different in children. For example, ASM apoptosis may be reduced. Importantly, ASM mass is still increasing as part of normal growth and development in children(26), therefore the influence of superimposed pathological abnormalities are likely to be different to those in adults. Further work is needed to determine the mechanistic effects of 25[OH]D<sub>3</sub> on pediatric ASM.

The cross-sectional nature of the biopsy data prevent us from being certain whether the relationship between increased ASM mass and vitamin D is a result of severe asthma, or whether the increased ASM mass may have been present before the development of disease and caused the asthma. It is possible that a developmental

structural defect of the airway wall, such as ASM hypertrophy in children with STRA results from vitamin D deficiency *in-utero*, and that may have led to asthma in the first place. It may be that exaggerated ASM hypertrophy is a cause of their asthma, rather than a consequence, as a result of *in-utero* (27, 28) and post-natal vitamin D deficiency. The effects of vitamin D deficiency *in utero* (27, 28) could be in addition to, or independent of, airway remodeling. This is especially important as a randomized controlled trial of vitamin D therapy in children with STRA could potentially reverse the ASM hypertrophy and change the course or natural history of these patients' asthma. However, if vitamin D induces a smooth muscle developmental defect *in-utero*, then it may prove more challenging to reverse. Importantly, it should be noted that in a previous study of infants with severe wheeze at a median age of 1 year, (29), there was no increase in ASM mass on biopsy (30). Vitamin D levels were not measured in that study, but the findings mitigate against, although do not exclude, the developmental hypothesis.

Interestingly, we did not find an association between any of the inflammatory cells quantified (eosinophils, neutrophils or mast cells) and serum 25[OH]D<sub>3</sub> levels. This remained true for both luminal inflammation (BAL and sputum) and tissue inflammation (endobronchial biopsy). It is possible that the substantial anti-inflammatory treatment prescribed for these children may have masked a relationship between vitamin D and airway inflammation in STRA. Having established a link between serum 25[OH]D<sub>3</sub> levels and lung function and asthma control, and importantly, having now seen a novel link between serum vitamin D levels and airway smooth muscle alone, we suggest that vitamin D supplementation in children with STRA and low 25[OH]D<sub>3</sub> levels, may be a novel therapeutic target directed against

some aspects of remodeling. Of note, there are currently no treatments that inhibit or prevent airway remodeling.

Even after adjusting for confounding factors including age, sex, body mass index, FEV<sub>1</sub> and ethnicity, a significant relationship between serum vitamin D levels and asthma control, exacerbations, inhaled and oral steroid use and positive BDR remained. (Table 3) Some of our findings concur with reports in children and adults with much less severe asthma. These include the associations found between low 25[OH]D<sub>3</sub> levels and asthma control and exacerbations (5, 6, 31), lower lung function (6, 32-34), increased reversibility to bronchodilator (5, 34) and greater anti-inflammatory medication (ICS, oral steroid & LTRA) usage (5, 32).

Although total serum IgE levels and specific IgE to cat, dog, pollen, *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* were inversely related to 25[OH]D<sub>3</sub> levels, there was no relationship between 25[OH]D<sub>3</sub> and serum, BAL or biopsy eosinophils. Some, (5, 32) but not all (6) investigators have found correlations between lower 25[OH]D<sub>3</sub> levels and markers of allergy in childhood asthma. A U-shaped association between low and high 25[OH]D<sub>3</sub> levels and serum total IgE levels has been demonstrated (35). An association between 25[OH]D<sub>3</sub> deficiency and increased sensitization to 11 of 17 environmental and food allergens in children (n=3136), but not in adults (n=3454) in the National Health and Nutrition Examination survey has also been shown (36). Our results are in agreement with this report (36) that children with low serum 25[OH]D<sub>3</sub> levels are more likely to have allergic sensitization. Interestingly, we have only found an association between sensitization to aero-allergens and 25[OH]D<sub>3</sub> levels, but not food allergens.



In terms of ethnicity, because numbers are small we divided the children into 'White' and 'non-White' groups, but did not attempt to classify ethnicity in more detail. There were more non-White children in the STRA group (36%) compared to MA (16%) and controls (12%) ( $p=0.056$ ). Children with non-White skin had lower serum vitamin D levels (Figure E3A), as reported by others (4). Moreover, non-White children had more severe asthma compared to White children (Figure E4). However the ANOVA results have shown that there is no interaction between ethnicity and disease severity for levels of vitamin D ( $p=0.20$ ). This could be due to the small number of subjects in the study. Importantly, race is a proxy for skin color, and although skin color is a proxy for low Vitamin D levels, our conclusions relate to the relationship between low serum Vitamin D levels, whether driven by diet, sunlight, ethnicity or another factor, and asthma severity and pathology.

The cross-sectional design of this study made it impossible to determine whether low 25[OH]D<sub>3</sub> levels result in severe asthma in children, or whether children with severe asthma have low 25[OH]D<sub>3</sub> levels because, for example, they are unable to go outside and exercise normally. Also, because of the ethics of performing bronchoscopy for research in children, we could not perform invasive endobronchial biopsies in controls and MA, and this is a potential source of information bias. One of the other limitations of this study was that BDR and ACT were not performed in normal controls. Whilst the potential to correlate ASM mass with the results of airway challenge would be of interest, given the subjects' disease severity, this was thought to be both unsafe and unethical.

It is challenging to propound a unifying hypothesis to account for ASM changes being the sole manifestation of vitamin D deficiency. In terms of remodeling, we speculate that this may be due to a heightened sensitivity of ASM to vitamin D deficiency as compared to other airway wall components. The lack of any effect on inflammation may in part be due to high dose inhaled and oral steroid therapy. Although vitamin D deficiency causes a degree of steroid insensitivity, the high doses used in the STRA children may have overcome this. However, we acknowledge that these ideas remain speculative at the present time and the determination of the exact mechanism between low 25[OH]D<sub>3</sub> and airway remodeling in STRA will require intervention studies.

In summary, in this cross-sectional study, children with genuine severe asthma had significantly lower serum vitamin D levels than MA and controls. Lower serum vitamin D levels were associated with worse parameters of asthma severity, and we propose that a contributory mechanism may be via an effect on ASM. As numbers are small, and represent a selected population, the conclusions drawn must be tentative. However, these findings suggest that detecting and treating low serum vitamin D levels in children with STRA may aid in treatment of specific structural airway changes.

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**Table 1. Demographic characteristics of subjects** <sup>□</sup>

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

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

Decimal values were approximated to closed integer for ease of exposition.

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

♦ Sum of specific IgE to nine allergens: cat, dog, egg, milk, peanut, grass, tree pollen, *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus*

+One or more positive allergen-specific IgE responses

\$ Rise in FEV<sub>1</sub> post bronchodilator (%)

^ Score out of 25

% Beclomethasone equivalent

& *P* value calculated by Mann Whitney test.

£ *P* value calculated by Chi-square test.

<sup>a</sup>*P* value calculated by Kruskal- Wallis test.

**Table 2. Serum vitamin D levels and medication use in all asthmatics (moderate asthma and severe therapy resistant asthma)**

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

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

\*The Wilcoxon test was used for median value differences for categorical variables.

**Table 3. Serum vitamin D levels and disease severity in all asthmatics (moderate asthma and severe therapy resistant asthma). (A) Linear regression was used for continuous measures of disease severity (B) Logistic regression was used for categorical variables.**

**(A)**

Outcome	@Beta coefficient [95% confidence interval] ( <i>p</i> value)	
	Unadjusted	Multivariate model <sup>^</sup>
ACT	0.18 [0.13 to 0.24] (<0.001)	0.15 [0.09 to 0.20] (<0.001)
Daily Inhaled Corticosteroid dose (microgram/day)	-0.006 [-0.01 to -0.003] (0.001)	-0.004 [-0.007 to 0.0001] (0.05)

@ Beta coefficient is for each nmol/L increase in serum vitamin D levels

<sup>^</sup> Multivariate model adjusted for age, sex, BMI, FEV<sub>1</sub> and ethnicity.

ACT= asthma control test

**(B)**

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

\* Odds ratio are for each nmol/L increase in serum vitamin D levels

<sup>^</sup> Multivariate model adjusted for age, sex, BMI, FEV<sub>1</sub> and ethnicity.

<sup>%</sup> Positive Bronchodilator response (BDR) = FEV<sub>1</sub> improvement of at least 12% after inhalation of 1,000 µg of salbutamol

<sup>\$</sup> CS= Corticosteroid; use of maintenance daily oral corticosteroids

<sup>£</sup>Exacerbation = any acute exacerbations in the last 6 months requiring oral steroids  
Decimal values were approximated to closed integer for ease of exposition.

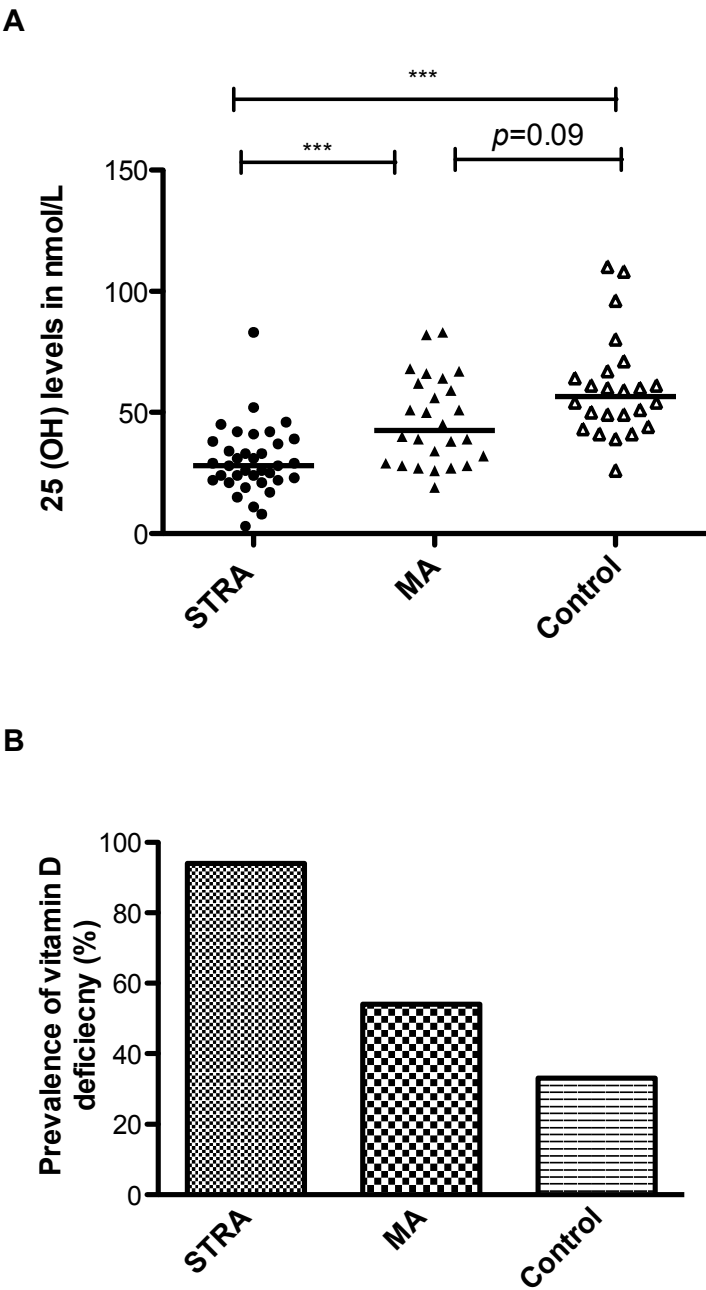


Figure 1A. Serum vitamin D levels in severe therapy resistant asthma (STRA), moderate asthma (MA) & controls. Serum 25-hydroxyvitamin D levels were lower in STRA and MA than controls (Kruskal-Wallis test  $p<0.0001$ ). Bar represents median values. Mann Whitney U test, followed by a Bonferroni correction was used to compare differences between groups. Figure 1B shows a higher prevalence of vitamin D deficiency (serum 25-hydroxyvitamin D levels less than 50 nmol/L) in STRA compared to MA and controls ( $p<0.001$ , calculated by Chi-square test)

\*\*\* $p<0.001$

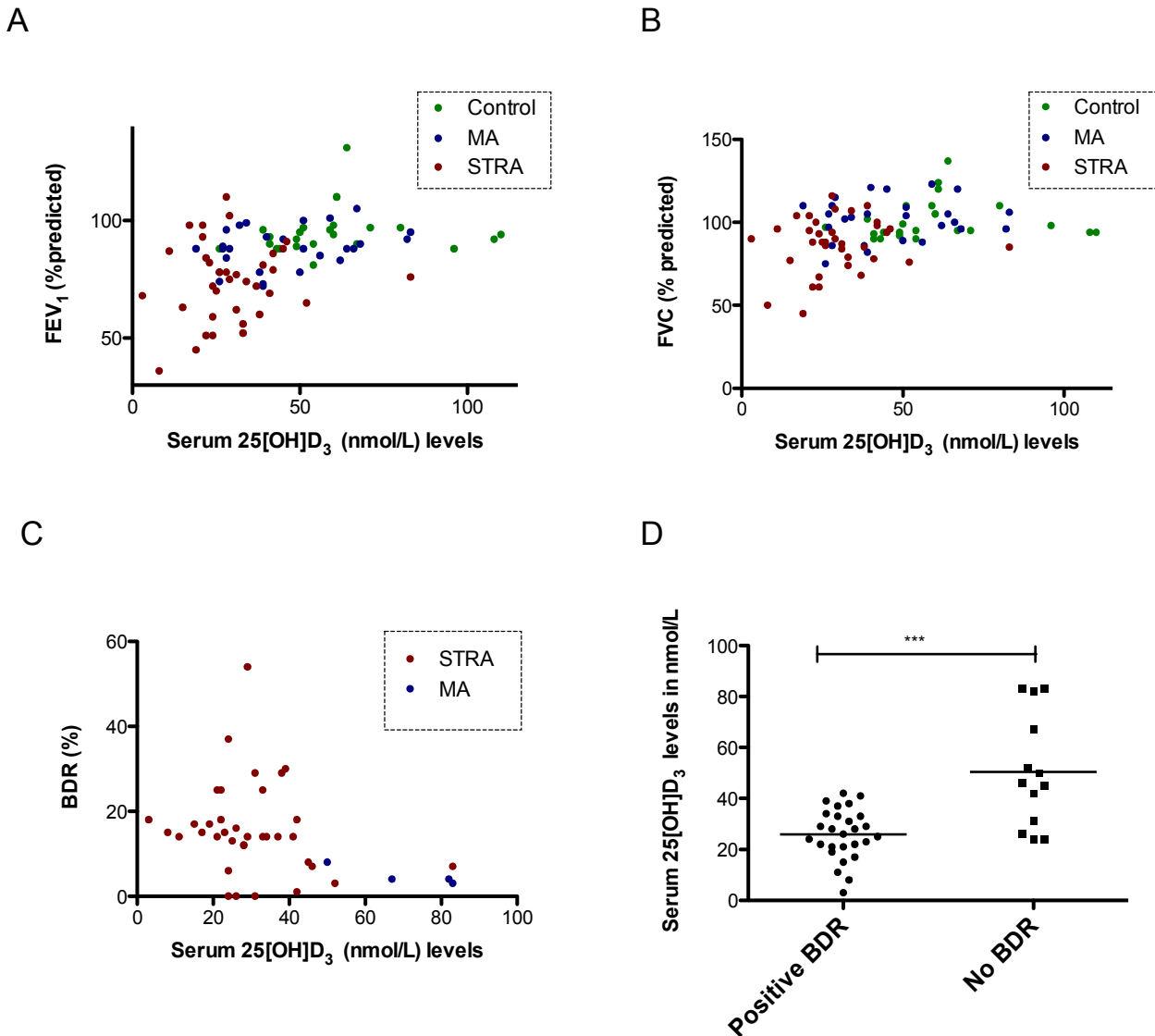


Figure 2 Positive association between serum vitamin D levels and %predicted FEV<sub>1</sub> ( $R=0.43$ ,  $p<0.001$ ) (A) and FVC ( $R=0.32$ ,  $p<0.002$ ) (B). Lower serum vitamin D levels were associated with higher bronchodilator response (BDR), ( $r=-0.40$ ,  $p=0.003$ ) (C) and positive BDR (FEV<sub>1</sub> improvement of at least 12%) ( $p<0.001$ ) (D). BDR (%) = percentage increase of FEV<sub>1</sub> after inhalation of 1,000  $\mu$ g of salbutamol. Correlation was determined by the Spearman rank correlation coefficient. Mann Whitney U test, was used to compare differences between groups

\*\*\* $p<0.001$

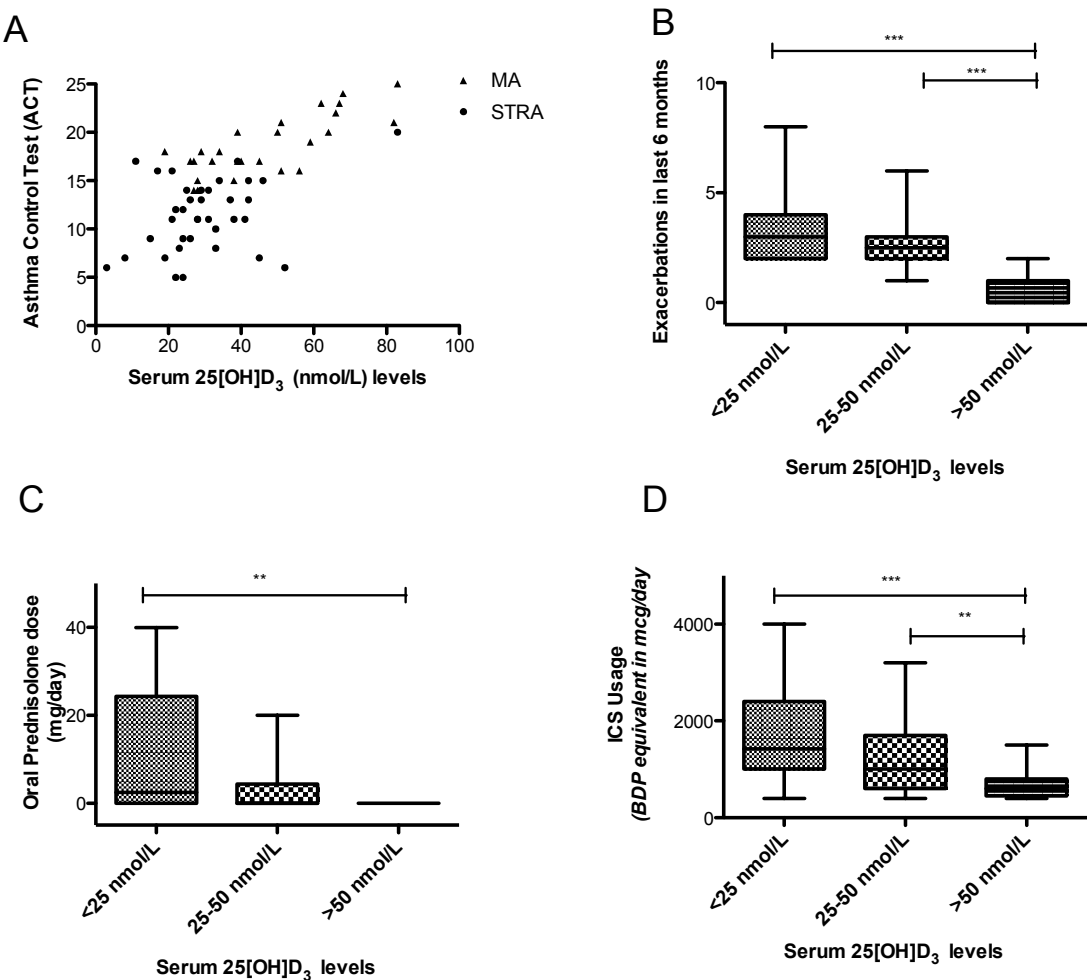


Figure 3. Positive association between serum vitamin D level and asthma control test (ACT) ( $r=0.6$ ,  $p<0.001$ ). Children with higher serum vitamin D levels had less asthma related symptoms (A). Correlation was determined by the Spearman rank correlation coefficient. Children with lower serum vitamin D levels had more acute exacerbations in the last 6 months (B). Lower serum vitamin D levels were associated with increased oral (B) and inhaled (C) corticosteroid usage.

Kruskal-Wallis test and Mann Whitney U test, followed by a Bonferroni correction was used to compare differences between groups.

\*  $p<0.05$

\*\*  $p<0.01$

\*\*\*  $p<0.001$

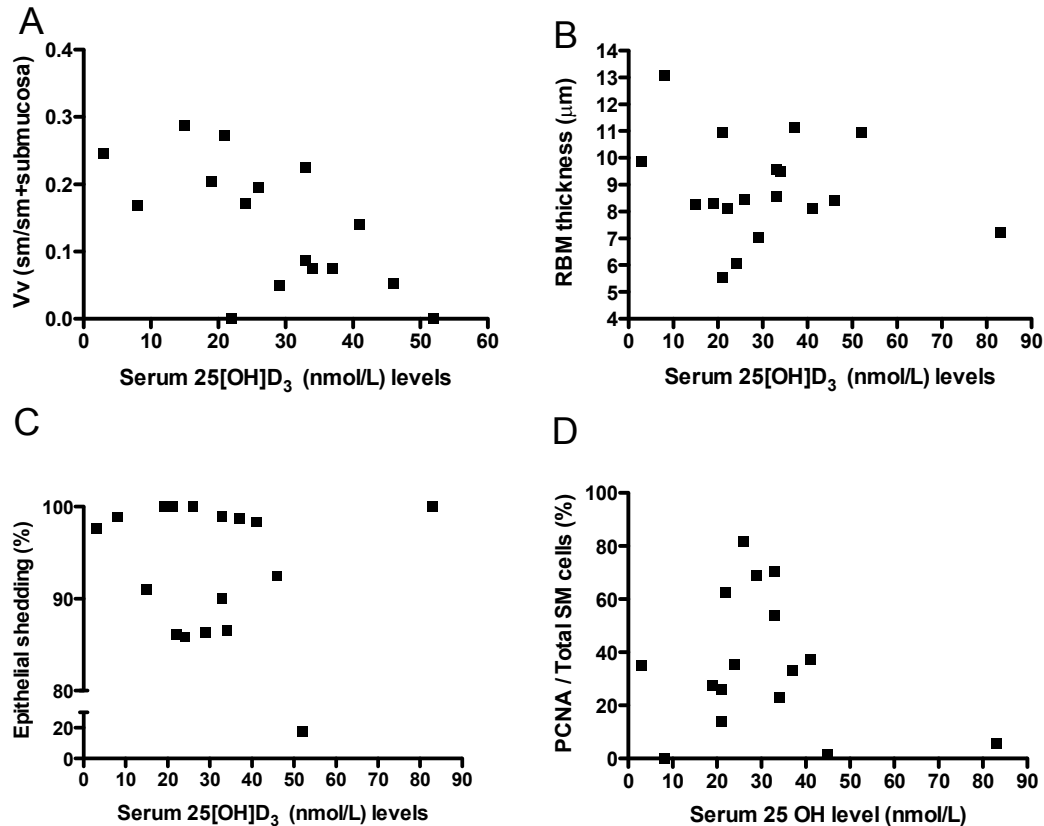


Figure 4. Relationships between serum 25[OH]D<sub>3</sub> levels and airway remodeling. There was a significant negative correlation between serum 25[OH]D<sub>3</sub> and volume fraction of ASM ( $r = -0.6$ ,  $p = 0.008$ ) (A). There was no significant correlation between serum 25[OH]D<sub>3</sub> and RBM thickness (B) or epithelial shedding (C). There was no relationship between serum 25[OH]D<sub>3</sub> and proliferating cell nuclear antigen (PCNA) positive smooth muscle cells (D). Correlation was determined by the Spearman rank correlation coefficient.

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

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



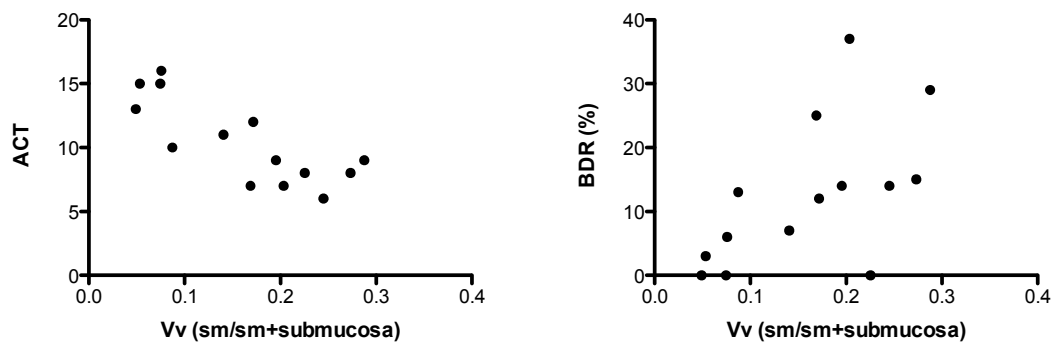


Figure 5. Relationship between volume fraction of airway smooth muscle (ASM) in endobronchial biopsies from children with STRA, and asthma control test (ACT) and bronchodilator response (BDR). A significant negative relationship was found between volume fraction of ASM and ACT ( $r=-0.7$ ,  $p<0.001$ ) (A). A significant positive relationship was present between BDR and volume fraction of ASM ( $r=0.6$ ,  $p=0.009$ ) (B).

BDR (%) = percentage increase of  $FEV_1$  after inhalation of 1,000  $\mu\text{g}$  of salbutamol;  
Vv (smooth muscle/smooth muscle + submucosa) = Volume fraction of smooth muscle indexed to volume of submucosa tissue  
ACT= asthma control test

## **Online supplement**

### **Title**

Relationship between serum vitamin D, disease severity and airway remodeling in children with asthma

### **Authors**

Atul Gupta, Alies Sjoukes, David Richards, Winston Banya Catherine Hawrylowicz, Andrew Bush, Sejal Saglani

## **Materials and Methods**

### **Subjects**

Children referred for 'beyond the guidelines' therapies were categorized as 'problematic severe asthma' (E1). They underwent a staged investigation protocol to exclude a wrong diagnosis, asthma with important co-morbidities and difficult asthma (in which potentially modifiable factors have not been identified and remedied). This included a formal home assessment and resulted in approximately half the referrals being classified as difficult asthmatics, in whom basic management needed to be optimized rather than therapy escalated. The remaining children with STRA had persistent ( $\geq 3$  months) symptoms (requiring rescue bronchodilator  $\geq 3$  days per week) despite treatment with high dose inhaled corticosteroids (at least 800 microgram/day of beclomethasone equivalent) and trials of add on drugs (long acting  $\beta_2$  agonists, leukotriene receptor antagonists and oral theophylline in a low, anti-inflammatory dose) and / or recurrent severe asthma exacerbations and / or persistent airflow obstruction (post oral steroid, post-bronchodilator Z score  $< -1.96$  for FEV<sub>1</sub> despite above therapy) ; all children had been through a detailed assessment to optimize adherence and other aspects of basic management, as far as possible (E1).

### **Exclusion criteria**

Subjects were excluded if they were taking vitamin D supplements or had additional chronic pulmonary conditions (e.g. bronchiectasis or cystic fibrosis)

## **Recruitment**

### **STRA**

All newly diagnosed STRA children (n=22) who underwent clinically indicated bronchoscopy at Royal Brompton Hospital from October 2009 to April 2011 were recruited after informed consent. Parents of 18 children with STRA were approached randomly in pediatric respiratory out-patient clinics, and 14 of these consented to take part in the study. The reason for refusal was needle anxiety (3/4) and uninterested (1/4)

### **MA**

Children with MA were randomly recruited from pediatric respiratory and allergy out-patient clinics at Royal Brompton Hospital. Parents of 32 children with MA were approached, and 26/32 agreed to participate. Again, the most common reason for refusal was needle anxiety (3/6), the other 3/6 families gave no reason of refusal.

### **Controls**

Non-asthmatic controls comprised either children with no respiratory disease whose parents had consented for a blood test during an elective surgical procedure (n=18) or children undergoing a clinically indicated bronchoscopy for upper airway symptoms (n=6) at Royal Brompton Hospital between October 2009 and April 2011. Parents of 36 children were approached, 24 of these agreed to participate. Again, the most common reason for refusal was procedural / general anesthesia anxiety (7/12), the remaining 5/12 gave no reason for refusal.

## **Serum 25-hydroxyvitamin D (25[OH]D<sub>3</sub>)**

25[OH]D<sub>3</sub> was chosen as it reflects total vitamin D from dietary intake and sun exposure, as well as the conversion of vitamin D from adipose stores in the liver. Also, 25[OH]D<sub>3</sub> has a longer half-life (2-3 weeks) than 1,25-dihydroxyvitamin D (4 hours). Vitamin D deficiency is defined by most experts as a 25[OH]D<sub>3</sub> level of less than 50nmol/L (20ng per milliliter) (E2). Based on changes in parathyroid hormone levels and intestinal calcium transport values of less than 75nmol/L have been suggested as insufficient (E2).

The date of the blood test from which the 25[OH]D<sub>3</sub> level was measured was recorded to assess seasonal variation between subjects. Subjects were grouped as follows; winter (December to February), spring (March to May), summer (June to August) or autumn (September to November).

### **Serum total and allergen-specific IgE**

Serum total IgE was analyzed by the Beckman Access 2 immunoassay analyzer and specific IgE to nine allergens (cat, dog, grass, tree pollen, *dermatophagoides pteronyssinus*, egg, milk, peanut and *Aspergillus fumigatus*) were measured by the Phadia Immunocap 250 analyzer.

### **Asthma Control Test (ACT)**

Asthma control in MA and STRA was assessed using the childhood ACT(E3). (see OLS for details). The ACT is a 5-point questionnaire marked out of a total of 25. It is a simple tool to assess control. It is used to compare asthma control over a 4-

week period. This scoring system has been well validated (E3, 4, 5) and correlates well with specialists' evaluation of asthma control (E4).

### **Pulmonary Function Testing**

Spirometry was conducted using interactive computerized incentive spirometry (Vitalograph Pneumotrac, Spirotrac® IV software). At least 3 spirometric manoeuvres were performed, with at least 2 reproducible manoeuvres required for each test. The best forced vital capacity (FVC) and forced expired volume in 1 second (FEV<sub>1</sub>) of the 3 manoeuvres was selected for data analysis. All spirometry results were compared to appropriate recent reference ranges (E6). If clinically indicated bronchodilator response (BDR) was assessed by repeating spirometry 15 minutes after the administration of 1mg salbutamol via a large volume spacer in subgroup of children with STRA & MA. Percentage increase in FEV<sub>1</sub> was recorded.

### **Sputum induction**

For subjects with a post-bronchodilator FEV<sub>1</sub> > 65% predicted, sputum induction was performed using 3.5% saline inhalation for four 5 min periods. For subjects with a post-bronchodilator FEV<sub>1</sub> < 65% predicted, sputum induction was performed with 0.9% saline. After each inhalation period subjects were encouraged to cough and expectorate any sputum. Spirometry was repeated 30 sec after each induction interval or earlier in the event of troublesome symptoms.

### **Sputum processing**

Selected sputum (plugs separated from saliva) was processed within two hours and stained with as with a modified Wright Giemsa stain Reastain® Quick-Diff staining kit

(Reagentia Ltd. Toivala, Finland) as previously described (E7). Differential cell counts were expressed as a percentage of 400 cells, excluding squamous cells.

### **Protocol for flexible bronchoscopy**

All bronchoscopies were performed under general anaesthesia as previously described (E8). Bronchoscopy was only performed in STRA because of the ethics of performing bronchoscopy under general anesthetic, just for research, in healthy children and well-controlled asthmatics (MA). Olympus BF-XP40 BF-MP60 (4.0 mm videobronchoscope) or BF-P20D (4.9 mm) bronchoscopes (KeyMed, Southend-on-Sea, Essex, UK) were used as appropriate to the size of the child. Bronchoalveolar lavage (BAL) was performed using 3 aliquots of 1ml/kg 0.9% sterile saline (to a maximum of 40mls per aliquot) instilled into the right middle lobe or an area of radiographically-defined abnormality and the returns pooled. The larger single use forceps (FB-231D, KeyMed) were used with the 4.0 and 4.9mm bronchoscopes. Up to 4 biopsies were taken per subject, a total of 26 biopsies were assessed. Median number of biopsies per subject was 1 (range 1-2).

### **Processing of BAL**

Cytospin was performed as previously described (Lex, Blue). BAL fluid was centrifuged at 300 g at 4°C for 10 minutes. The supernatant was removed and the cell pellet resuspended in RPMI-1640 medium (Sigma) with 10% fetal calf serum. Slide preparations for differential percentage counting of cells were made in a Shandon cytocentrifuge (Cytospin II; Shandon Ltd, Runcorn, Cheshire, UK) using 100[μl] aliquots of the lavage cell suspension, adjusted to  $0.5 \times 10^6$  cells/ml.

Preparations were stained with May-Grunewald Giemsa. Differential counts were made from a minimum count of 400 cells.

### **Processing of endobronchial biopsy**

Biopsies were fixed in formal saline, and processed to paraffin within 24 hours. 5µm sections were stained with haematoxylin and eosin (H&E) and assessed for adequate quality.

### **Evaluable biopsies**

To be categorized as “evaluable”, a biopsy had to fulfill the following criteria (haematoxylin and eosin staining): (i) presence of identifiable epithelium, reticular basement membrane (RBM) with associated submucosa; (ii) good orientation; (iii) minimal crush, edema or blood within the biopsy.

### **Tissue morphometry**

Tissue morphometry was performed on haematoxylin and eosin stained sections using equations from design-based stereology (E9, 10), as described previously (E11).

### ***Morphometry of airway remodelling***

Evaluable H&E stained sections were used to quantify airway remodeling, including RBM thickness (E12), epithelial shedding (E13) and smooth muscle mass (E11).

### ***Reticular basement membrane thickness***



RBM was measured in sections stained with H&E using computer aided image analysis at x 400 magnification. Forty point-to-points- repeated measurements were taken of RBM thickness at right angles to the basement membrane, at regular intervals of 20 micrometer ( $\mu\text{m}$ ) in randomly selected sections (E12). Results are the mean of the 40 measurements per patient in  $\mu\text{m}$ . The mean intra-observer coefficient of variation for measurement of RBM thickness was 6.7%.

### ***Epithelial shedding***

The length of incomplete epithelium was measured as a percentage (%) of the total epithelial length (assessed by the length of basement membrane) at x 200 magnification. The epithelium was considered incomplete when the basement membrane was completely denuded or when it was only covered by a single layer of basal cells with no intact ciliated cells or goblet cells (E13). A minimum length of 1mm epithelium was assessed. The mean intra-observer coefficient of variation for measurement of epithelial shedding was 0.6%.

### ***Smooth muscle volume fraction***

Smooth muscle quantification was performed using equations from design-based stereology. The volume fraction of smooth muscle (sm) was measured using a weiber grid at x 200 magnification. Stereological data were calculated as follows: (E11)

Volume fraction of sm indexed to volume of submucosa tissue:

$$V_v(\text{sm/submucosa}) = \frac{\sum \text{points on sm}}{\sum \text{points on sm} + \text{points on submucosa}}$$

The mean intra-observer coefficient of variation for measurement of volume fraction of ASM was 16.2%.

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

The deparaffinised sections were washed in phosphate buffered saline (PBS). PBS with 0.5% Tween was used for the staining protocol. Antigen retrieval was performed by microwaving the sections in sodium citrate (2.941 g/L, pH 6.0) for 3 x 3 minutes. Sections were cooled and air dried, washed 2 x 5 minutes with PBS, before incubating with avidin blocking solution for 15 minutes and then again for 15 minutes with biotin blocking solution (Vector Laboratories, Avidin/Biotin Blocking kit, cat. no. SP2001). Sections were then blocked with horse serum (Vector Laboratories, cat. no. S2000) for 20 minutes. Excess of horse serum was drained off onto a paper towel, and the sections were then incubated for 1 hour with antibodies for mouse monoclonal PCNA antibody (Dako, cat. no. M0879) at a concentration of 327 mg/ml PCNA in a 1:200 dilution in PBS. To confirm specificity of the primary antibody, tandem sections were stained with mouse isotype IgG. The sections were then washed 2 x 5 minutes in PBS. The slides were incubated with biotinylated mouse IgG (Vector Laboratories) at dilution 1:200 in PBS, containing 1% normal horse serum, for 45 minutes. The sections were washed 2 x 5 minutes in PBS and then incubated with avidin/biotin complex (Vector Laboratories, Vectastain ABC Standard Elite kit, cat. no. PK-2100) for 40 minutes following manufacturer's instructions. After washing twice for 5 minutes with PBS the sections were incubated with 3,3'-diaminobenzidine (DAB) (Vector Laboratories, DAB Peroxidase Substrate kit, cat. no. SK-4100). The sections were washed in distilled water for 5 minutes, then counterstained with 20% haematoxylin for 5 minutes, washed in tap water for 5 minutes, dehydrated through

70% to 100% ethanol, and finally histoclear before mounting cover-slips with DPX mounting medium. Positively stained smooth muscle nuclei were counted in every biopsy at x 400 magnification and divided by the total number of smooth muscle nuclei and expressed as a percentage (%).

### ***Quantification of tissue inflammation***

Mucosal inflammation was quantified in sections stained with congo red (eosinophils), and immunohistochemistry was used to assess neutrophils (neutrophil elastase) (E14) and mast cells (mast cell tryptase) (E15). All cells with positive nuclear staining were counted in the submucosa in every biopsy at x 400 magnification. Mast cells were also counted within the smooth muscle in every biopsy. The data is presented in mm<sup>2</sup> per area of submucosa or smooth muscle (for mast cells alone).

### **Statistical analysis**

Categorical data were analysed using the Chi squared or Fishers exact tests. Between group differences for normally distributed data were analysed using the student's t test, or the Mann-Whitney U test for non-normally distributed variables.

### **Power calculation**

Our study has shown that the mean vitamin D levels for the controls, moderate asthmatics and severe therapy resistant asthmatics were 59.9, 46.5 and 29.8 respectively and the within group mean square was 304.66 giving a pooled standard deviation of approximately 17.5. Based on these means and SD, the power to

achieve the effect size reported in this study is greater than 90%. Our study was therefore adequately powered for the data relating to serum vitamin D and clinical status. For the results of airway remodeling and serum vitamin D levels, there are no published data with which to inform a power calculation, so sample size is opportunistic. However, we know from previous pediatric biopsy studies that groups of  $n=15-20$  are sufficient to show significant and meaningful results (E11, E16).

**Table E1. Association between serum vitamin D levels and clinical variables in all subjects (severe therapy resistant asthma, moderate asthmatics and controls)**

	<b>N</b>	<b>Correlation (r)*</b>	<b>P value</b>
Age	86	-0.16	0.14
BMI	86	0.2	0.08
Serum Eosinophil count %	84	-0.08	0.4
IgE (IU/ml)	86	-0.3	0.01
Specific IgE to cat	79	-0.27	0.01
Specific IgE to dog	75	-0.29	0.01
Specific IgE to tree pollen	67	-0.28	0.02
Specific IgE to <i>Dermatophagoides pteronyssinus</i>	73	-0.3	0.01
Specific IgE to <i>Aspergillus fumigatus</i>	51	-0.36	0.009
Specific IgE to grass	57	-0.14	0.3
Specific IgE to egg	37	-0.04	0.7
Specific IgE to milk	35	-0.09	0.58
Specific IgE to peanut	34	-0.07	0.7

\*Correlation (r) was determined with the Spearman rank correlation coefficient

**Table E2. Biopsy cell count results, Median (Interquartile range (IQR)).**

	STRA
Patients with good quality biopsies	19 (out of 21)
Smooth muscle volume fraction measurements Median (range)	0.16 (0.07-0.20) – smooth muscle points / total points 0.18 (0.07 – 0.26) – smooth muscle points / submucosa points
Proliferating smooth muscle cells in % median (range)	34.1 (20.8-56.1)
Epithelial loss in % median (range)	98 (87 -100)
RBM thickness in micrometer median (range)	8.4 (7.9 - 9.7)
Mast cells in submucosa median (range) cells/mm <sup>2</sup>	65 (49-106)
Mast cells in smooth muscle median (range) cells/mm <sup>2</sup>	11 (4-40)
Neutrophils in submucosa median (range) cells/mm <sup>2</sup>	5 (0-12)
Eosinophils in submucosa median (range) cells /mm <sup>2</sup>	14 (3 – 78)

**Table E3. Association between serum vitamin D levels and airway inflammation in children with STRA. Correlation was determined by the Spearman rank correlation coefficient.**

	Correlation (r)	<i>P</i> value
Sputum Eosinophils	-0.35	0.11
BAL Eosinophils	-0.24	0.15
Mucosal Eosinophil	0.01	0.61
Sputum Neutrophils	0.5	0.6
BAL Neutrophils	-0.17	0.24
Mucosal Neutrophils	0.02	0.97
Mast cells within the smooth muscle	0.34	0.38

BAL; broncho-alveolar lavage

**Table E4. Serum vitamin D status in children with severe therapy resistant asthma (STRA), moderate asthma (MA) and controls.**

	Vitamin D status		Odds Ratio [95% CI] ( <i>p</i> value)	
	Not deficient (serum 25[OH]D <sub>3</sub> levels > 50 nmol/L)	Deficient (serum 25[OH]D <sub>3</sub> levels < 50 nmol/L)	Unadjusted	Multivariate <sup>@</sup>
Control (n=24)	15 (63%)	9 (37%)	1	
MA (n=26)	11 (42%)	17 (58%)	2.27 [0.73 to 7.07] (0.16)	1.51 [0.44 to 5.14] (0.5)
STRA (n=36)	2 (6%)	34 (94%)	28.33 [5.45 to 147.25] (<0.0001)	13.15 [2.19 to 78.82] (0.005)

<sup>@</sup> Multivariate model adjusted for age, body mass index (BMI), % predicted FEV<sub>1</sub> (forced expiratory volume in 1 second), % predicted FVC (forced vital capacity), ethnicity.



Figure E1. No significant difference in season and sample collection in the three groups ( $p=0.13$ , measured by Chi-square test) and serum 25[OH]D<sub>3</sub> levels ( $p=0.07$ , measured by Kruskal-Wallis test)

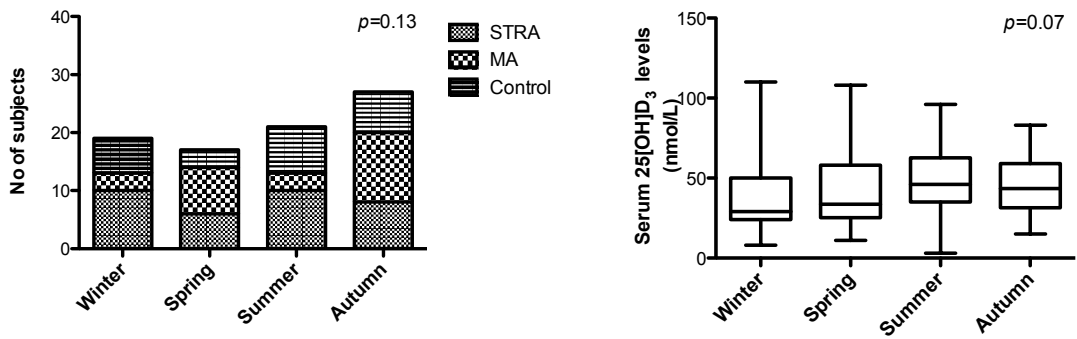


Figure E2. Endobronchial biopsy sections from a child with severe therapy resistant asthma (STRA) stained with haematoxylin and eosin (A) and proliferating cell nuclear antigen (PCNA) (B) (magnification x 200). Smooth muscle volume fraction was quantified by overlaying a weiber grid at x 200 magnification over the section and performing point counting. The volume fraction of smooth muscle was indexed to volume of submucosal tissue (C) (10). Smooth muscle proliferation was assessed by quantifying the proportion of PCNA positive smooth muscle cells and dividing by the total number of smooth muscle nuclei, expressed as a percentage (magnification x400) (D). Arrows indicate a positively stained nucleus (dark brown) and a negative nucleus (light blue).

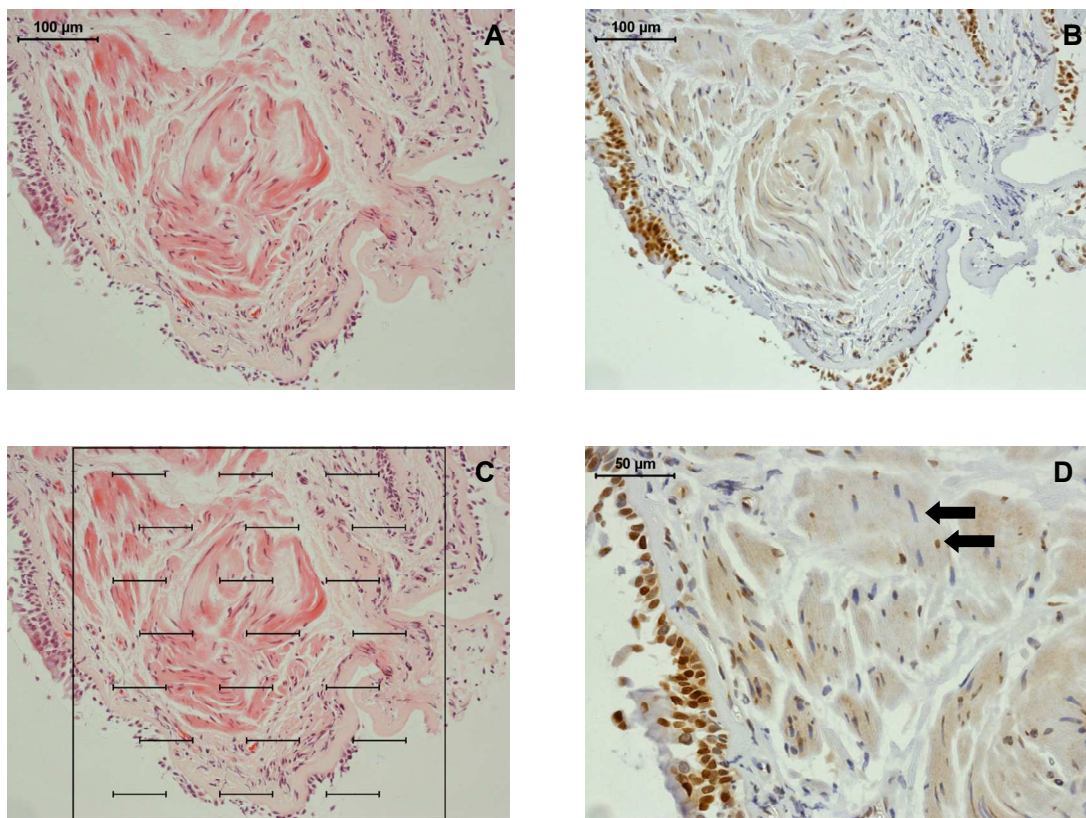


Figure E3. (A) Serum vitamin D levels were significantly lower in ‘non-White’ children than those of ‘White’ ethnic background, ( $p<0.001$  [\*\*\*] measured by Mann Whitney test). (B) Within the three groups (severe therapy resistant asthma, moderate asthma and controls) there was no difference in serum vitamin D levels between ‘White’ and ‘non-White’ children (Kruskal-Wallis  $p<0.001$  followed by Mann Whitney test for inter-group differences and then a Bonferroni correction for multiple comparison)

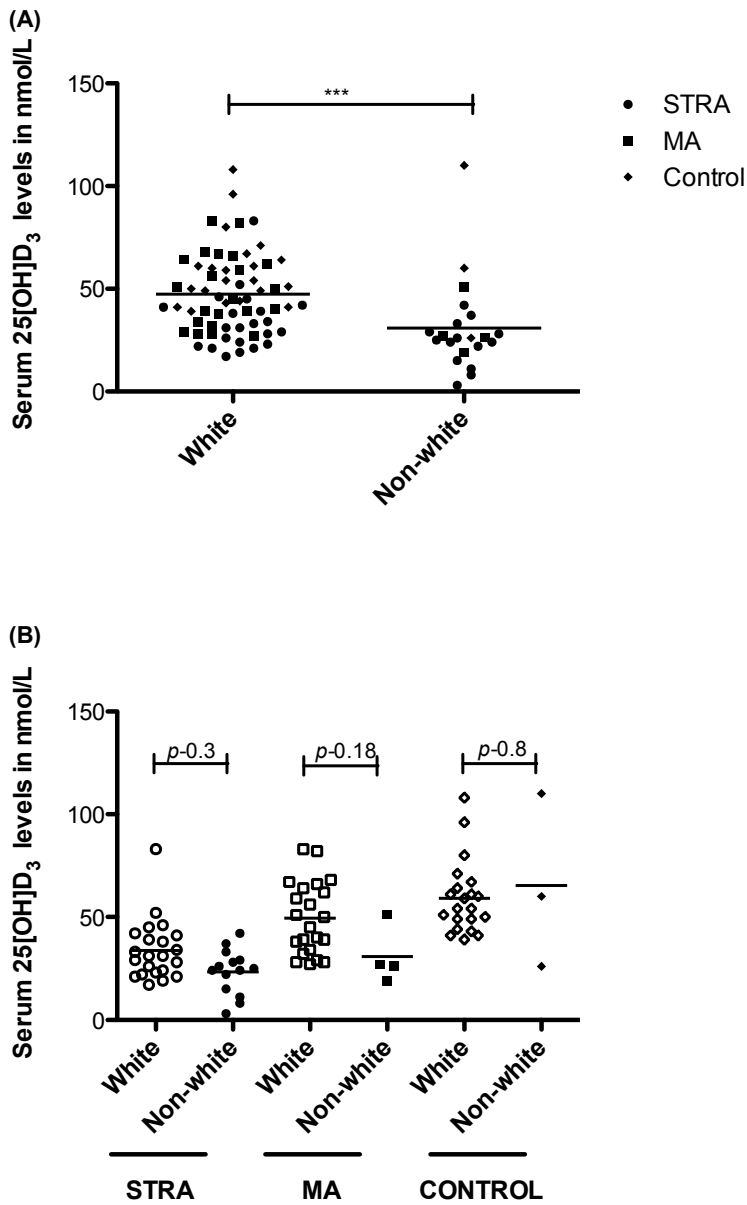
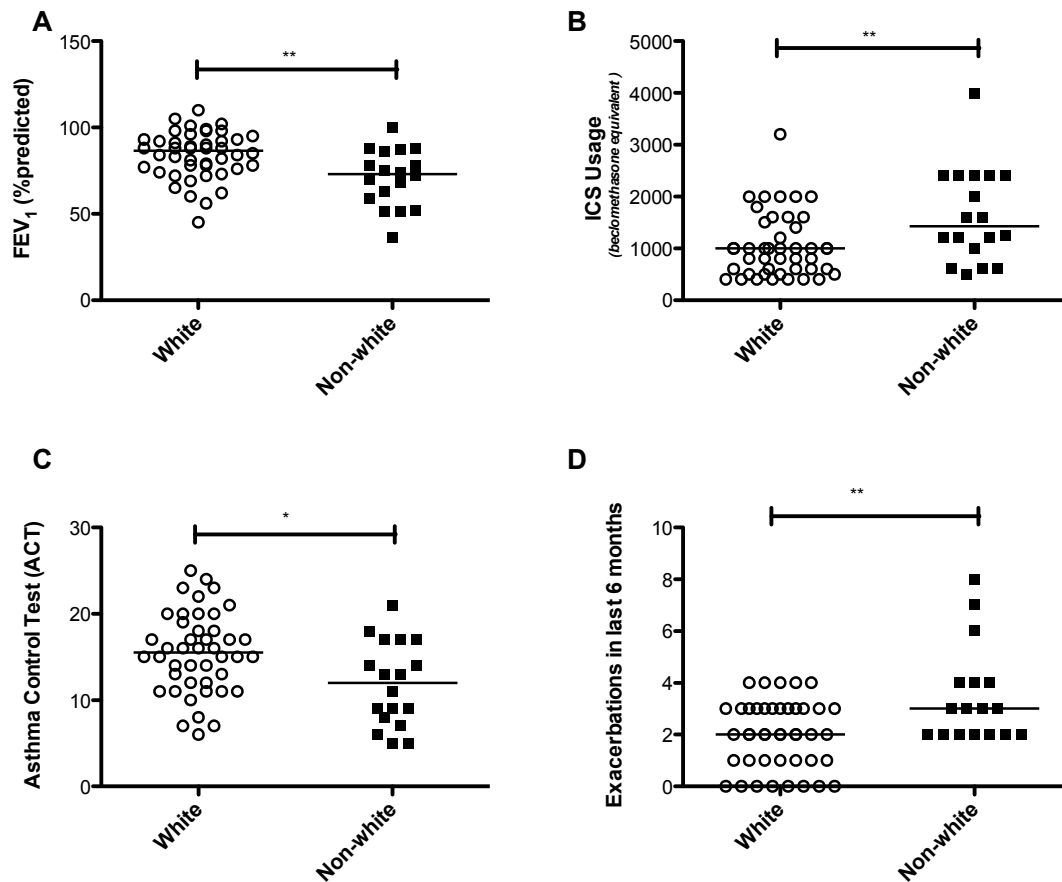


Figure E4. Clinical markers of asthma severity in 'White' and 'non-White' asthmatic children (severe therapy resistant asthma and moderate asthma). 'Non-White' asthmatics had lower % predicted FEV<sub>1</sub> (A), more inhaled corticosteroid (ICS) use (B), poor asthma control (C) and more acute exacerbations in last six months needing oral steroids (D)

\* $p < 0.05$ , \*\* $p < 0.01$  \*\*\* $p < 0.001$ , measured by Mann Whitney test



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