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 (≥ 3 months) symptoms (requiring rescue bronchodilator ≥ 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 β2 agonists, leukotriene receptor antagonists and oral theophylline in a low, anti-inflammatory dose) and / or recurrent severe asthma exacerbations and / or persistent airflow obstruction (post oral steroid, post-bronchodilator Z score < -1.96 for FEV_1 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_3)

$25[OH]D_3$ 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, $25[OH]D_3$ 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 $25[OH]D_3$ 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 $25[OH]D_3$ 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_1) 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_1 was recorded.

Sputum induction

For subjects with a post-bronchodilator FEV1 > 65% predicted, sputum induction was performed using 3.5% saline inhalation for four 5 min periods. For subjects with a post-bronchodilator FEV1 < 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

(Reagena 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 previuosly descirbed (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[l aliquots of the lavage cell suspension, adjusted to 0.5 x 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 (μm) in randomly selected sections (E12). Results are the mean of the 40 measurements per patient in μm. 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:

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

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 isoytpe 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^2 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)

	N	Correlation (r)*	*P* value
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 *Dermatophagoides pteronyssinus*	73	-0.3	0.01
Specific IgE to *Aspergillus fumigatus*	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^2	65 (49-106)
Mast cells in smooth muscle median (range) cells/mm^2	11 (4-40)
Neutrophils in submucosa median (range) cells/mm^2	5 (0-12)
Eosinophils in submucosa median (range) cells /mm^2	14 (3 – 78)

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

	Correlation (r)	***P* 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] (*p* value)	
	Not deficient (serum 25[OH]D_3 levels > 50 nmol/L)	Deficient (serum 25[OH]D_3 levels < 50 nmol/L)	Unadjusted	Multivariate[@]
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)

[@] Multivariate model adjusted for age, body mass index (BMI), % predicted FEV_1 (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_3 levels (*p*=0.07, measured by Kruskal-Wallis test)

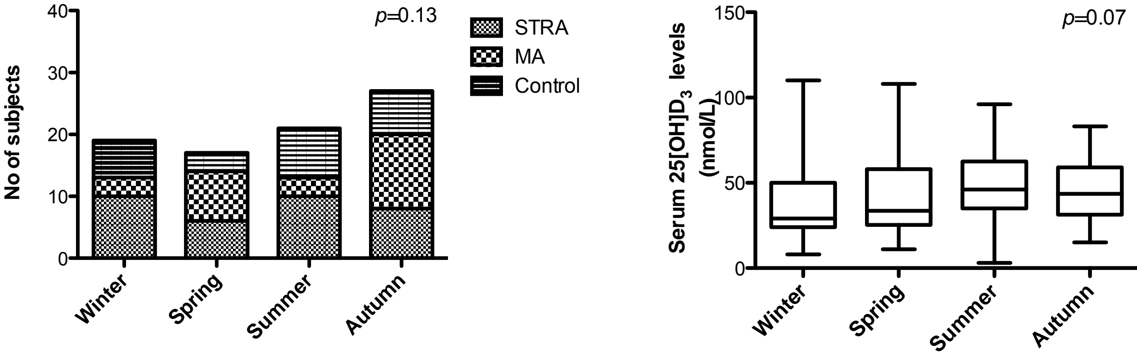


Figure E2. Endobronchial biopsy sections from a child with severe therapy resistant asthma (STRA) stained with haematoxylin and eosin (A) and proliferating cell nuclear antigen (PCNA) (B) (magnification x 200). Smooth muscle volume fraction was quantified by overlaying a weiber grid at x 200 magnification 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).

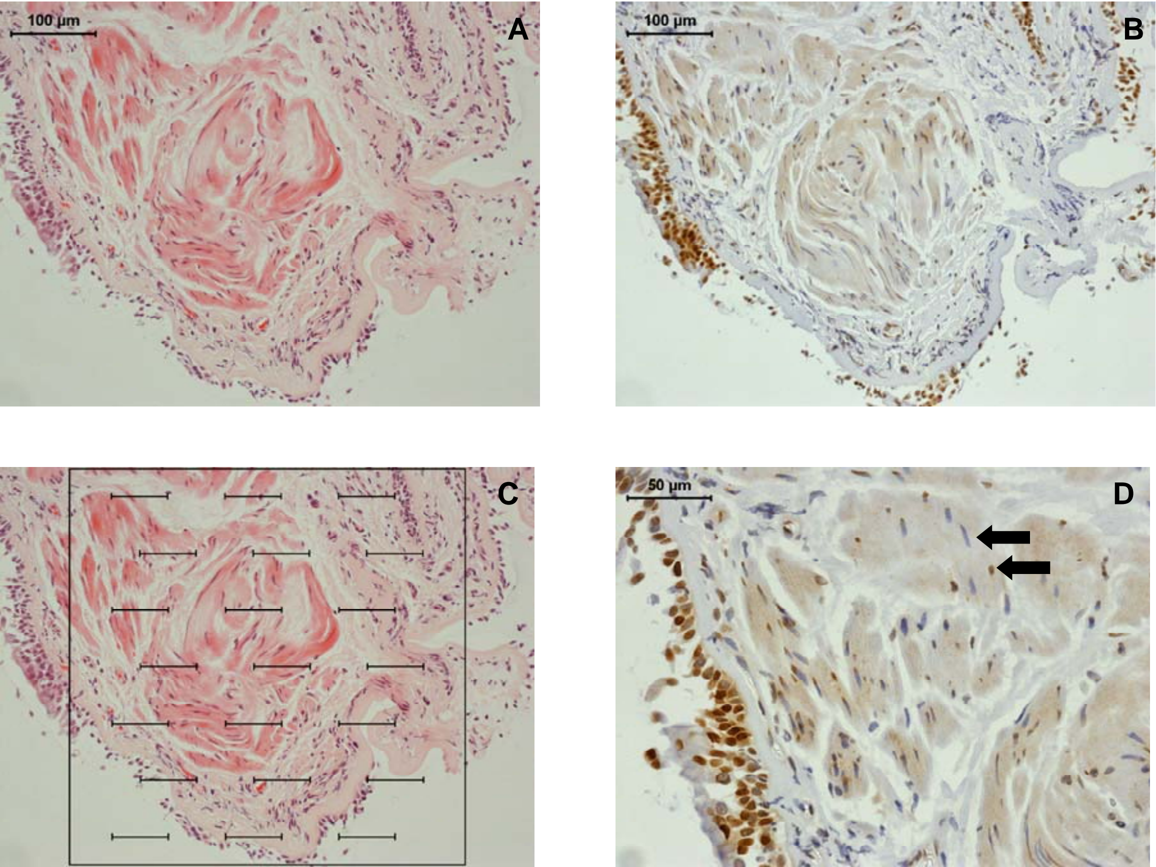
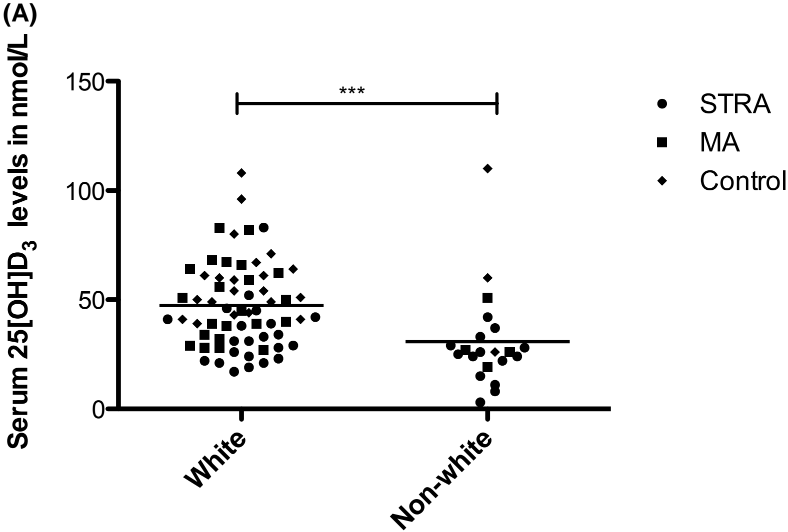


Figure E3. (A) Serum vitamin D levels were significantly lower in ‘non-White’ children than those of ‘White’ ethnic background, (p<0.001 [***] measured by Mann Whitney test). (B) Within the three groups (severe therapy resistant asthma, moderate asthma and controls) there was no difference in serum vitamin 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)



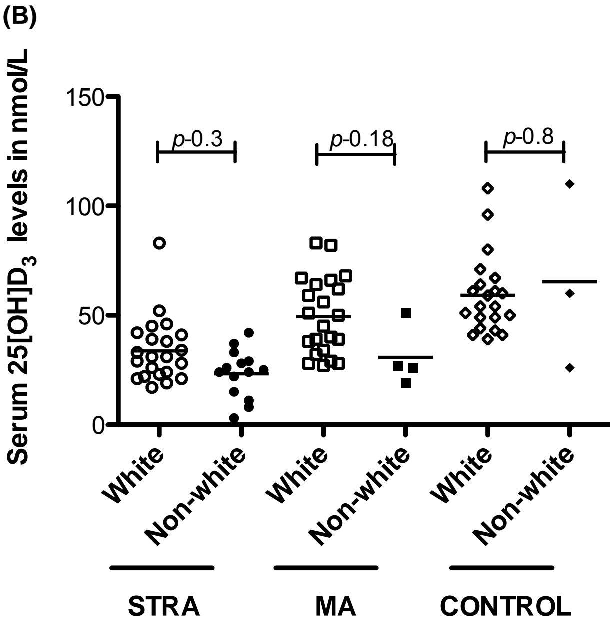
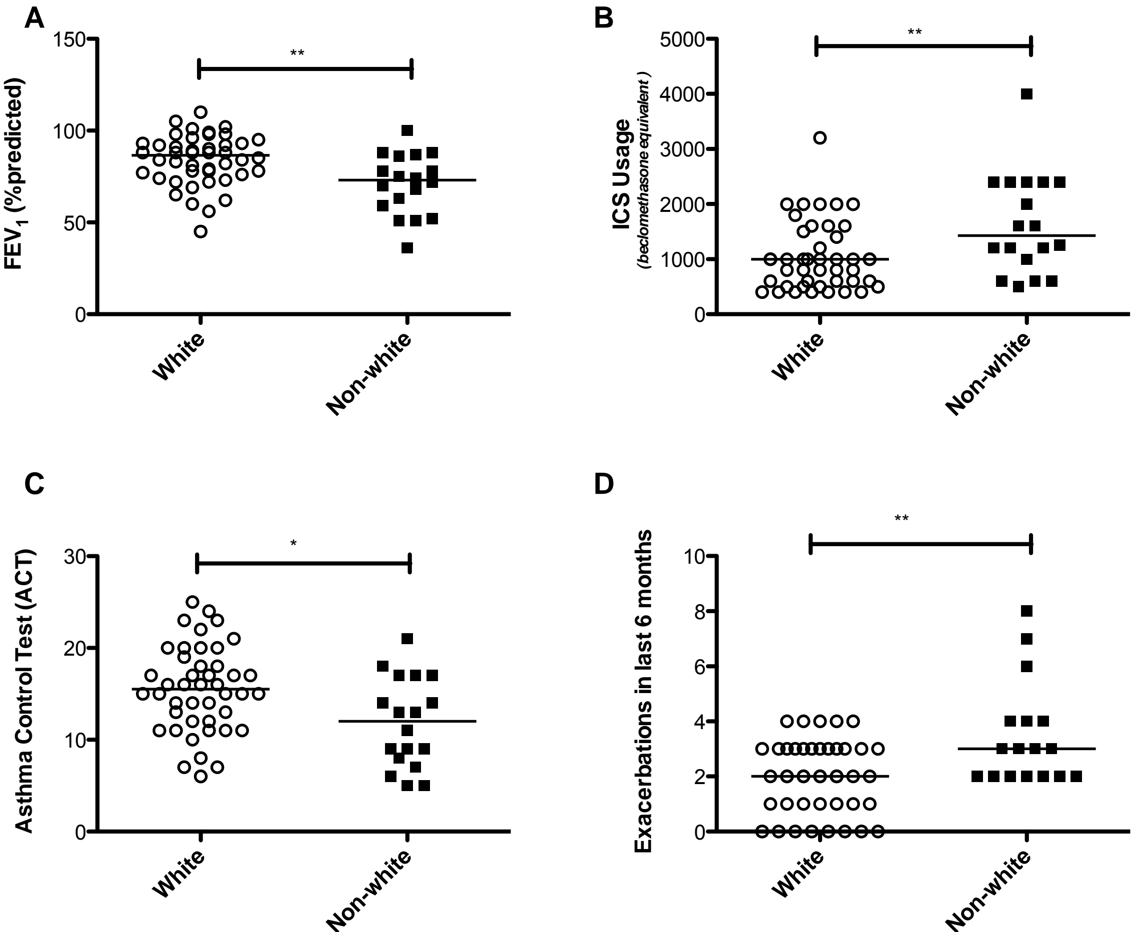


Figure E4. Clinical markers of asthma severity in 'White' and 'non-White' asthmatic children (severe therapy resistant asthma and moderate asthma). 'Non-White' asthmatics had lower % predicted FEV_1 (A), more inhaled corticosteroid (ICS) use (B), poor asthma control (C) and more acute exacerbations in last six months needing oral steroids (D)

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



References

E1. Bush A, Saglani S. Management of severe asthma in children. *Lancet* 2010;376:814-825.

E2. Holick MF. Vitamin d deficiency. *N Engl J Med* 2007;357:266-281.

E3. Liu AH, Zeiger R, Sorkness C, Mahr T, Ostrom N, Burgess S, Rosenzweig JC, Manjunath R. Development and cross-sectional validation of the childhood asthma control test. *J Allergy Clin Immunol* 2007;119:817-825.

E4. Schatz M, Sorkness CA, Li JT, Marcus P, Murray JJ, Nathan RA, Kosinski M, Pendergraft TB, Jhingran P. Asthma control test: Reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *J Allergy Clin Immunol* 2006;117:549-556.

E5. Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, Murray JJ, Pendergraft TB. Development of the asthma control test: A survey for assessing asthma control. *J Allergy Clin Immunol* 2004;113:59-65.

E6. Stanojevic S, Wade A, Stocks J, Hankinson J, Coates AL, Pan H, Rosenthal M, Corey M, Lebecque P, Cole TJ. Reference ranges for spirometry across all ages: A new approach. *Am J Respir Crit Care Med* 2008;177:253-260.

E7. Lex C, Payne DN, Zacharasiewicz A, Li AM, Wilson NM, Hansel TT, Bush A. Sputum induction in children with difficult asthma: Safety, feasibility, and inflammatory cell pattern. *Pediatr Pulmonol* 2005;39:318-324.

E8. Payne D, McKenzie SA, Stacey S, Misra D, Haxby E, Bush A. Safety and ethics of bronchoscopy and endobronchial biopsy in difficult asthma. *Arch Dis Child* 2001;84:423-426.

E9. Weibel ER, Hsia CC, Ochs M. How much is there really? Why stereology is essential in lung morphometry. *J Appl Physiol* 2007;102:459-467.

E10. Ochs M. A brief update on lung stereology. *J Microsc* 2006;222:188-200.

E11. Regamey N, Ochs M, Hilliard TN, Muhlfeld C, Cornish N, Fleming L, Saglani S, Alton EW, Bush A, Jeffery PK, Davies JC. Increased airway smooth muscle mass in children with asthma, cystic fibrosis, and non-cystic fibrosis bronchiectasis. *Am J Respir Crit Care Med* 2008;177:837-843.

E12. Sullivan P, Stephens D, Ansari T, Costello J, Jeffery P. Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies. *Eur Respir J* 1998;12:811-815.

E13. Barbato A, Turato G, Baraldo S, Bazzan E, Calabrese F, Panizzolo C, Zanin ME, Zuin R, Maestrelli P, Fabbri LM, Saetta M. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;174:975-981.

E14. Niimi A, Torrego A, Nicholson AG, Cosio BG, Oates TB, Chung KF. Nature of airway inflammation and remodeling in chronic cough. *J Allergy Clin Immunol* 2005;116:565-570.

E15. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, Larche M, Robinson DS, Durham SR, Kay AB. Expression of il-4 and il-5 mrna and protein product by cd4+ and cd8+ t cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997;158:3539-3544.

E16. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, Jeffery PK. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med* 2007;176:858-864.