The Calcitriol Study

A Randomised Placebo Controlled Clinical Trial to Test the Effects of Calcitriol in Steroid Resistant Asthma

A Thesis presented by

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London SE1 9RT

for the degree of

Doctor of Philosophy

2015

I, Alexandra M Nanzer, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

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For Philip

Abstract

Background

Over five million people in the UK are receiving treatment for asthma. Corticosteroids (steroids) are the mainstay of asthma therapy but some patients do not respond fully to steroid treatment, they are characterised as being steroid resistant (SR) and are at high risk of morbidity and mortality. Earlier data from our laboratory has shown evidence that *in vitro* treatment with the active form of vitamin D, - 1α,25-dihydroxyvitamin D3 or calcitriol - enhanced responsiveness to steroids for induction of the anti inflammatory cytokine IL-10. This thesis discusses the results of a proof of concept clinic trial - 'The Calcitriol Study' - which hypothesized that concomitant *in vivo* treatment with oral calcitriol improves the clinical responsiveness to systemic steroid (prednisolone) therapy. The study further allowed investigation of the *in vitro* cytokine profile of patients with SR and steroid sensitive (SS) asthma. Th17 cells and their hallmark cytokine IL-17A are proposed to play a role in the pathology of severe asthma, including SR asthma, and this work tested the susceptibility of IL-17A and other pro inflammatory cytokines important in asthma to inhibition by steroids and calcitriol *in vivo* and in culture.

Methods

Adult patients with moderate/severe asthma (FEV_1 <80% predicted) with demonstrable reversibility of airways obstruction underwent a two-week, pharmacodynamically standardised course of oral prednisolone (screening phase) to delineate steroid resistance a <10% improvement in FEV_1. Patients were then randomly assigned to receive calcitriol (n=12) or indistinguishable placebo (n=11) for four weeks, with a repeat course of prednisolone during the final two weeks (treatment phase). Changes in lung function (ΔFEV_1) in response to prednisolone were compared between the placebo and calcitriol groups in the treatment phase, and within groups between the screening and treatment phases. Asthma Control Questionnaires and fractional exhaled nitric oxide (FeNo) were scored and analysed as secondary endpoints. All participants had serum 25(OH)D levels measured at baseline. CD8-depleted PBMCs were isolated from SS and SR asthmatics and healthy controls and cultured with or without dexamethasone and / or calcitriol. Cytometric bead array, ELISA, qPCR and intracellular cytokine staining were used to assess cytokine production.

Results

Treatment with calcitriol improved the clinical response to steroids in patients classified as clinically steroid resistant (SR) in a within group comparison of changes in FEV_1. However, there was no significant difference seen between the two groups from screening to the end of the trial.

A striking dichotomy was observed between SR and SS asthma patients in terms of their cytokine profiles; SS patients, who showed the biggest improvement in lung function after a course of prednisolone had the highest levels of IL-10 in culture in response to dexamethasone, whereas SR patients, whose lung function failed to improve, had significantly greater levels of IL-17A. Treatment with steroids appeared to aggravate production of this pro-inflammatory cytokine but *in vitro* and *in vivo* calcitriol not only resulted in a significant reduction of IL-17A levels but also restored the impaired, steroid-induced anti-inflammatory IL-10 response in SR patients. Serum 25(OH)D levels at baseline correlated positively with IL-13 in culture, a Th2 cytokine known to be associated with steroid responsive asthma.

Conclusion

Calcitriol may have the potential to improve the clinical responsiveness of asthma patients to systemic steroid therapy in SR asthma. These data identify immunological pathways that likely underpin the beneficial clinical effects of calcitriol in SR asthma, by directing the SR cytokine profile towards a more SS type, suggesting strategies to characterise vitamin D responder immune phenotypes.

Acknowledgements

I would like to thank my supervisors Professor Chris Griffiths and Professor Catherine Hawrylowicz for an exciting opportunity to conduct this clinical trial and to let me read for this thesis. I would like to thank Professor Hawrylowicz in particular for her mentoring and unswerving encouragement and Professor Chris Griffiths for his support throughout this time. I would also like to thank Professor Chris Corrigan, who provided a wealth of advice and support durig the conduct of this trial.

A special thank you to Dr Emma Chambers, whith whom I shared the scientific work that resulted from this trial, she is a great friend and source of inspiration and education. I would like to thank Grainne Colligan for her help with recruitment and care of the patients enrolled in the study and Dr Anna Freeman for her invaluable help during her three months placements at the laboratory during two consecutive years. Dr Kimuli Ryanna has always offered much appreciated support during the conduct of the trial. I would like to thank Drs Dave Richards, Zoe Urry, Emanuel Xystrakis and Sarah Dimeloe, members of Professor Hawrylowicz's laboratory, everyone was most helpful and provided much appreciated advice.
Ich danke meinen Eltern herzlichst fuer all ihre Unterstuetzung waehrend vielen Jahren.

Most of all I would like to thank Philip, Henry and Isla for their wonderful love, patience and encouragement.

Sadly my dear mother in law, Dr. Joan Kelly, passed away shortly before this thesis was completed. She has always shown a great interest in my work. As a doctor and mother herself, she has been incredibly inspirational and always supportive. We often talked about medicine and motherhood and I shall miss our conversations dearly.

Table of Contents

List of Tables

Abbreviations

1,25-(OH)2D3	-	1,25-DihydroxyVitamin D 3, calcitriol
A467	-	Alexa Fluor 647
ab	-	antibody
APCs	-	antigen presenting cells
C	-	Celsius
CD	-	Cluster of differentiation
Dex	-	Dexamethasone
EAE	-	Experimental Autoimmune Encephalomyelitis
ELISA	-	Enzyme-Linked ImmunoSorbent Assay
FACS	-	Fluorescence-Activated Cell Sorting
FEV_1	-	forced expired volume in 1 second
FITC	-	fluorescein isothiocyanate
FoxP3	-	Forkhead box P3
GATA-3	-	GATA binding protein 3
GM-CSF	-	*Granulocyte-macrophage colony-stimulating factor*
GR	-	glucocorticoid receptor
GRE	-	Glucocorticoid Response Element
HBSS	-	Hanks balanced salt solution

IFN	-	Interferon
IL	-	Interleukin
ILT3	-	Immunolglobulin-like transcript 3
L	-	Litre
M	-	Molar (moles per L)
min(s)	-	minute(s)
n	-	nano
NK	-	Natural Killer
nTreg	-	natural T regulatory
^{o}	-	degrees
OD	-	Optical density
PBMCs	-	Peripheral blood mononuclear cells
PBS	-	Phosphate-buffered saline
POSTN	-	Periostin
PerCp	-	peridinin-chlorophyll-protein complex
pH	-	potential hydrogen
q	-	quantitative
RNA	-	Ribonucleic acid
RNApol	-	RNA polymerase
rpm	-	revolutions per minute

RT	-	Room temperature
RT-PCR	-	Real Time Polymerase Chain Reaction
RXR	-	retinoic X receptor
secs	-	Seconds
SR	-	glucocorticosteroid resistant
SS	-	glucocorticosteroid sensitive
TGFβ	-	*Transforming growth factor β*
TIMP-1	-	Tissue inhibitor of metalloproteinases - 1
Treg	-	*T regulatory cell*
U	-	unit
U.K.	-	United Kingdom
U.S.A.	-	United States of America
V-6	-	1,25-(OH)2D3 at concentration of 10^{-6}M
V-7	-	1,25-(OH)2D3 at concentration of 10^{-7}M
V-8	-	1,25-(OH)2D3 at concentration of 10^{-8}M
VDR	-	vitamin D receptor
VDRE	-	vitamin D3 response element
µ	-	micro

Chapter 1

Introduction

1. 1 Asthma Prevalence

Asthma is a chronic disease and is associated with significant morbidity and mortality. Episodic breathlessness and wheeze has been recognised at least since the time of Hippocrates (circa 460 – circa 370 BC). Exhaustion after exertion, dyspnoea due to heart failure or near the end of life were all described as asthma, but it was the work of Hippocrates and his acolytes, detailed in the Corpus Hippocratrium, that may have given us the first description of asthma: it was more common in the middle aged and in the autumn, identified as a disease of spasm and associated with certain occupations such as anglers, tailors and metalworkers [1]. The word asthma stems from the Greek word αα΄ζειν aazein), which translates as "to breathe with open mouth or to pant" and first appeared in Homer's Iliad [2].

The prevalence of asthma is estimated at 300 million people in the world; in the developed world, it affects up to 10% of adults and 30% of children[3]. 5.4 million people in the UK are currently receiving treatment for asthma and the NHS spends around £1 billion a year treating and caring for people with asthma (asthma.org.uk) [3].

Most asthma originates in childhood following sensitization of the airways to common aero allergens such as pollen, house dust mite, animal dander and fungi and it is typically associated with other atopic diseases such as eczema or rhinitis [4]. In atopic children dermatitis can precede asthma in the 'atopic march' suggesting a role of epicutaneous allergen transfer in asthma development [5]. The rise in childhood asthma and the predominant prevalence in developed countries has been attributed among others to changes in diet and life style; children who grow up in an environment rich of microbial exposure such as farming live stock are less likely to have asthma and

allergies [6]. Studies suggested that the protective effect of a farming environment on asthma development could be largely due to exposure to a wider range of microbes or unpasteurized milk consumption [7, 8] and exposure to pet animals has been found to confer protection against asthma [9]. The data is supported by animal studies showing a strong link between germ free status and a high susceptibility of allergy [10].

These studies have given weight to what is known as the hygiene hypothesis: the rapid global increase in asthma prevalence as a direct result of changes in the intrauterine and infant environment, including changes in maternal diet, smaller family size, reduced infant infections and increased use of antibiotics and immunization [11].

There is consensus though, that the hygiene hypothesis is not the sole explanation for the increase in atopy and asthma. Although the majority of asthma patients are atopic, only a minority of people with allergies will develop asthma[12]. Historically, asthma has been regarded as a disease driven by Th2 cytokines such as IL-4, IL-5 and IL-13. These cytokines drive eosinophilic inflammation and bronchial hyperreactivity [13]. However, several non-allergenic exposures such as bacterial endotoxins, air pollution or viral infections during early life may contribute to the development of non-atopic asthma [14] and studies using sputum induction or bronchoalveolar lavage (BAL) techniques to measure airways inflammation in asthmatics have shown that some patients have severe disease in the absence of eosinophilia, suggestive of non-atopic inflammatory pathways driving pathogenesis [15]. Immune regulation in severe asthma has recently found increased levels of IL-6, IL-8, IL-17A and IFNγ in bronchial biopsies of severe asthmatics shifting the attention from a predominantly Th2 to a Th1/Th17 driven disease [16]. Research has also focused on aspects during fetal growth; the complex interplay between environmental factors including intra uterine smoke exposure, maternal diet, mechanical factors and the large number of genes interacting during human development [17].

Asthma is a heterogeneous disease

There is therefore increasing evidence that asthma is in fact a heterogenous disease encompassing multiple phenotypes or subgroups. A phenotype (from the Greek word *phainein*, "to show" and *typos*, "type") is defined as the observable properties of an organism that are produced by the interactions of the genotype and the environment [18]. It is therefore not surprising that in order to gain control of their asthma symptoms, different patients require different therapeutic approaches and that by defining characteristics of their disease better, with the help of clinical and immunological biomarkers, treatment outcomes can be improved.

Our understanding of asthma pathophysiology has increased considerably over the last decades thanks to the ability to identify biological pathways by measuring mediator cells and cytokines in the lungs and peripheral blood. Including biological markers of inflammation into phenotyping has been referred to as endotyping. Eosinophils, in peripheral blood and sputum, cytokines, serum markers like periostin and fractional exhaled nitric oxide have all become useful and important biomarkers of asthma endotyping. I will discuss asthma endotypes in more detail again in paragraph 1.3 (Novel Strategies for Asthma Management), which outlines why successful therapies are largely based on tailoring therapy to disease phenotype.

1. 2 Asthma Management

Asthma UK estimates that 75% of hospital admissions for asthma are avoidable and as many as 90% of the deaths from asthma are preventable. The recent National Review of Asthma Deaths (NRAD) revealed that in two thirds of cases examined the quality of care was inadequate and clinicians simply failed to adhere to the BTS/SIGN [19] asthma treatment guideline [20]. To date, asthma management remains a challenge for healthcare professionals.

Among the earliest described remedies was to smoke the herb *Datura stramonium* [21]. It has anticholinergic properties and is the forerunner of our current most commonly used inhaled antimuscarinic drugs ipatropium and tiotropium. Other therapies, such as inhalation of the fumes of hydrocyanic acid and inflation of the lungs with bellows, have stood the test of time less favourably and have been abandoned [22]. In the early 20th century more efficient treatments were found, oral ephedrine or, if the patient were *in extremis*, subcutaneous adrenaline. The diuretic theophylline was discovered to be helpful in asthma in the early 1920s and from the 1930s aerosolized solutions of adrenaline were in use right up until the development of the metered dose inhaler in the 1950s.

Asthma was seen as a disease of airway hyperreactivity due to the release of bronchoconstrictor mediators from mast cells and treatment predominantly focused on bronchodilators. Parallel to the continued improvements in performing spirometry - in the 1940s and 1950s measurements of forced expiration became the pre-eminent means of diagnosing and monitoring the disease - was also the recognition that it was an inflammatory condition. This has led to the use of firstly oral glucocorticsteroids (hereafter steroids) in the 1950s, and then from the 1960s inhaled steroids, which remain the most successful treatment for asthma to date. The first British guidelines on asthma management in adults were published in the British Medical Journal in 1990 after a joint initiative between the British Thoracic Society (BTS), the Royal College of Physicians of London, the King's Fund Centre, and the National Asthma Campaign [23, 24]. Figure 1.1 shows asthma management steps according to the current guidelines, which are implemented until control is achieved [19].

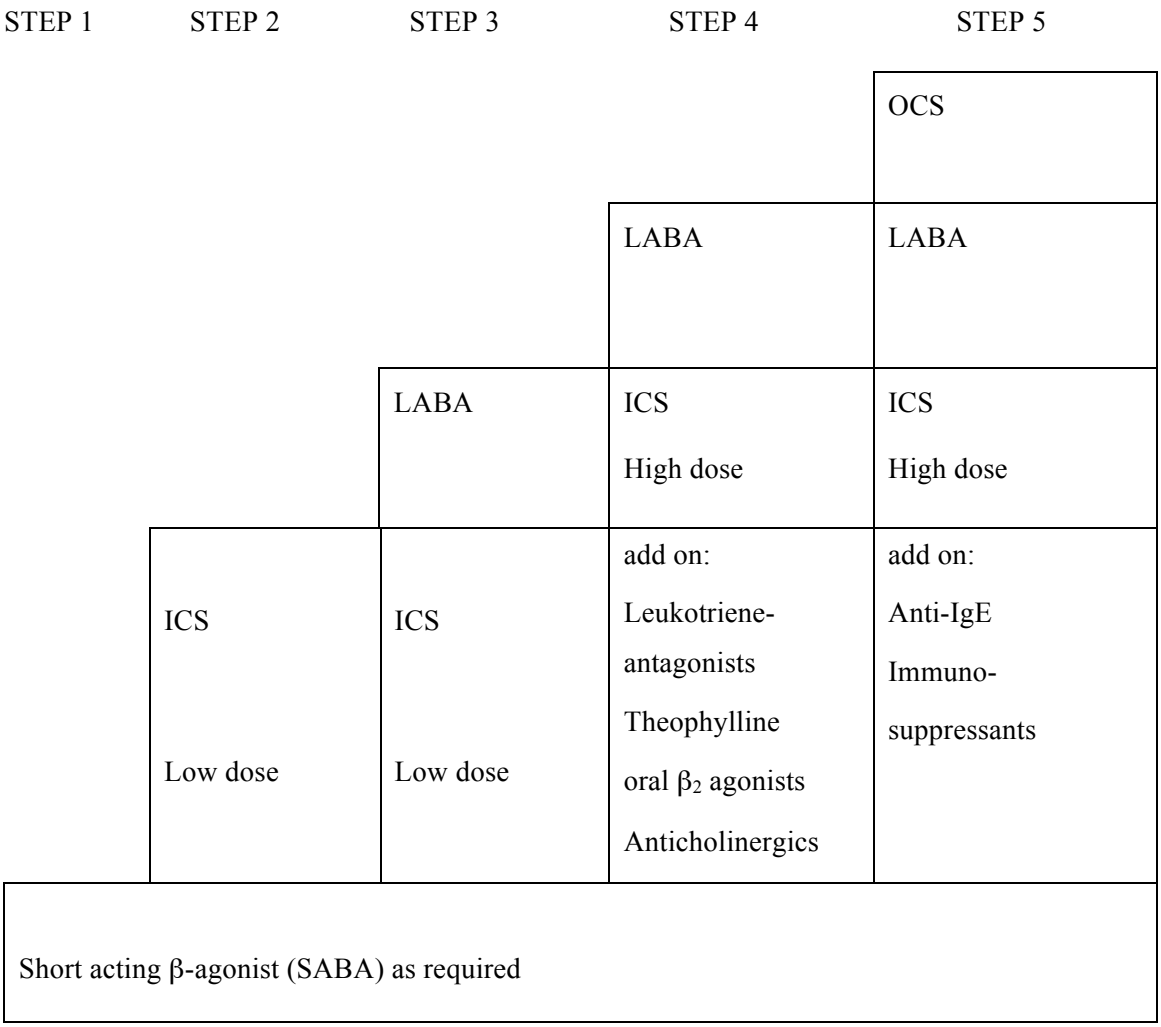


Figure 1.1: Stepwise asthma management (British Thoracic Society (BTS) guidelines November 2014 [19]). ICS = inhaled corticosteroids. Low dose = 200-800mcg/day Beclometasone diproprionate (BDP) or equivalent. High dose ICS = 2000mcg/day BDP or equivalent. LABA = long acting beta agonist. OCS = oral corticosteroids.

Step 1 recommends the use of short acting β-agonists (SABA) as required. The BAGS and BARGE (Beta Adrenergic Response to Genotype) trials, conducted in adults, showed that as-needed use of SABA was just as good as scheduled use [25, 26]. The latter study highlights the importance of pharmacokinetics, showing that participants with the Arg/Arg genotype affecting the 16th amino acid of the β2 adrenergic receptor experienced deterioration in lung function and symptoms when using salbutamol regularly, whereas patients with the Gly/Gly genotype experienced an improvement. Environmental and gene-gene interactions such as smoking, race or polymorphisms in other genes have also been described to affect this [27-29]. Good asthma control is

associated with little need for SABA. If more than 10-12 puffs per week are needed, asthma is poorly controlled and Step 2 (addition of regular inhaled corticosteroid, ICS) should be implemented. ICS remain the most effective anti-inflammatory agents currently available for asthma. The anti-inflammatory mechanisms of steroids include direct effects on gene expression by binding of the steroid receptors to steroid-responsive elements and promotion of anti-inflammatory proteins (transactivation) and inhibition of transcription and synthesis of proinflammatory cytokines (transrepression). This entails inhibition of prostaglandin through activation of annexin I, induction of MAPK phostphatase 1 and repression of transcription of cyclooxygenase 2. Steroids further have indirect effects on gene expression through interactions with transcription factors (i.e. NF-κB and activator protein 1) and glucocorticoid receptor mediated effects on second messenger cascades (i.e. the PI3K-Akt-eNOS pathway) [30]. I will discuss the mechanisms of steroid action and in particular steroid resistance in detail further below. When used appropriately, ICS have few side effects at low-medium doses. Local side effects result from the deposition of the drug in the oropharynx and include hoarseness, candidiasis, dysphonia or cough. Efforts are being made in developing small particle (nanoparticle) size ICS formulations that will improve topical delivery to the lung [31].

The fact that all currently available ICS are absorbed from the lung into the bloodstream, albeit minimally, means they do have the potential for clinically systemic side effects. These include suppression of the hypothalamus-pituitary-adrenal (HPA) axis, Cushing's syndrome, obesity, impaired glucose tolerance, cataract, osteoporosis, dermal thinning and growth suppression in children, to name a few [32]. Anti-inflammatory effects are largely mediated by the inhibition of NF-κB and activator protein 1, whereas systemic side effects are mainly caused by transactivation and by steroid receptor binding to DNA [30, 32, 33]. There have therefore been several attempts to design newer steroids with dissociated activity aiming to separate anti-inflammatory effects from side effects [33-35].

Some patients will benefit more from add-on therapy (step 3) than from increasing ICS doses should their symptoms persist. First choice is the addition of a long acting β2 agonist (LABA). Patients who show no response to LABAs should stop and either increase the inhaled steroid dose to 800 micrograms/day BDP (budenosine diproprionate equivalent) or consider sequential trials of add-on therapy with leukotriene receptor antagonists, theophylline or slow release β2 agonist tablets. Long acting anti-muscarinergic bronchodilators (LAMA) have become widely used in COPD but recent data emerged, showing that the use of two long acting bronchodilators with different modes of action had a significant benefit in patients with moderate to severe asthma. Randomised controlled trials have found that adding tiotropium to pre-existing therapy with ICS or ICS-LABA combination resulted in improved lung function and reduced exacerbation rates in comparison with placebo [36]. Furthemore, tiotropium maintained lung function when ICSs were tapered and LABAs were discontinued [37, 38]. Trials comparing tiotropium with the LABA salmeterol found the beneficial effect to be similar [37, 39]. It is noteworthy though that none of the trials provided significant evidence for an improvement in symptoms or quality of life.

There have been concerns with regards to a possible increase in cardiac toxicity with tiotropium *via* the Respimat® inhaler device [40, 41]. Tiotropium is currently marketed as Handihaler dry powder 18μg once daily or as Respimat® inhaled solution 2.5μg (2 puffs comprise one medicinal dose) once daily. A large well conducted trial involving over 17,000 patients with COPD found no difference in safety profile between the two devices and has provided a great deal of reassurance. It remains to be said though that any type of anticholinergic drug ought to be used with circumspection in patients at high risk of arrhythmias, unstable cardiac disease, and in renal failure.

Patients whose asthma remains uncontrolled despite treatment with high doses of ICS (800 micrograms BDP daily) and an additional drug ought to be treated according to step 4: consider increasing ICS to 2000 micrograms BDP/day, add leukotriene receptor

antagonists, add theophyllines or add slow release β_2 agonists. Oral steroids in the lowest dose providing control are added if patients fail to improve at step 4 [19]. If, at this stage symptoms persist, the patient is likely to suffer from severe or difficult to control asthma. A number of novel biological agents have been in development in the last year to improve severe asthma management.

The patients enrolled into 'The Calcitriol Study', which forms the main part of my thesis, suffered from severe therapy refractory asthma, which is why I would like to discuss severe asthma, and in particular its phenotypes, in more detail here.

1. 3 Novel Strategies for Asthma Management: Targeting the Right Patient

In order to optimise treatment outcomes for patients with ongoing symptoms it is important to further our understanding of severe asthma.

Several approaches have been taken to characterize asthma subgroups. The Severe Asthma Research Program (SARP) identified five asthma sub-phenotypes by unbiased cluster analysis, three of which are severe asthma [42]: the early-onset allergic type and the late-onset eosinophilic phenotype are both orchestrated by Th2 cells. They are clinically distinct, yet overlap immunologically. A Th2-cell signature is believed to play a predominant role in exercise induced asthma (EIA) with mast cells and their mediators understood to be driving inflammation in EIA [43]. A lack of Th2 biomarkers is seen in obesity related asthma and neutrophilic asthma. In another study using cluster analysis, sputum eosinophilia was incorporated. Consequent upon this a phenotype of early onset severe atopic asthma, late onset asthma with persistent eosinophilia and a cluster of obese females with late onset asthma without eosinophilia was identified [4] (see table 1.1).

T-cell signature	Phenotype	Biomarkers	Therapy
Th2-high	Early onset allergic	specific IgE, + SPT	Corticosteroids, Anti-IgE
	Late-onset eosinophilic	sputum eosinophilia, IL-5	Poor response to steroids, Anti-IL-5
	Exercise induced	Mast cells	Leukotriene receptor antagonists, SABA
Th2-low	Obesity related	Mast cells, Adiponectin, Th1 cytokines	Poor response to steroids Weight loss, PPAR agonists
	Neutrophilic	Th17, sputum neutrophilia	Vitamin D, p38 MAPK inhibitors, Macrolides. Poor response to steroids

Table 1.1 Asthma phenotypes according to their biomarker profiles and therapeutic response divided into Th2-high and Th2-low asthma, adapted from *Wenzel*[18]

Molecular phenotyping has led to the development of biomarkers that specifically target Th2 responses in the lung: Woodruff and colleagues identified periostin (*POSTN*), chloride channel regulator 1 (*CLCA1*), and serpin peptidase inhibitor, clade B, member 2 (*SERPINB2*) as epithelial genes that were specifically induced in asthma and directly regulated by IL-13 *in vitro*[44]. They further identified patients with distinctly higher levels of IL-5 and IL-13, termed Th2-high asthma. This was in contrast to patients with cytokine expression similar to healthy controls including Th1 cytokines IL-12 and IFNγ, which were significantly lower in the Th2-high group. The Th2-high and Th2-low groups also differed clinically with the Th2-high group showing significant higher atopy and higher eosinophil levels in peripheral blood and bronchoalveolar lavage (BAL). Of particular interest was their observation of steroid responsiveness: lung function of Th2-low asthmatics failed to improve following treatment with ICS, in fact patients' FEV_1 deteriorated in the first month suggesting a detrimental effect of steroids in Th2 low asthma[44].

1. 3. 1 Th2-high Asthma

Omalizumab remains so far the most successfully applied monoclonal antibody to treat allergic Th2-high asthma. Omalizumab is a humanized IgG1k monoclonal antibody that specifically binds to free human immunoglobulin E but not to IgE that is already bound by the high affinity IgE receptor (FcεRI) on the surface of mast cells, basophils and antigen-presenting dentritic cells. Steric hindrance by the receptor means the receptor is not accessible to omalizumab binding, thus averting anaphylaxis. In a number of studies conducted so far, patients treated with omalizumab reported significant improvements in asthma-related symptoms allowing for a reduction in steroid and rescue inhaler use [45-47]. It appears that patients with blood eosinophilia, high levels of exhaled nitric oxide and serum periostin benefit most from anti IgE treatment [48]. However, evidence is now emerging that omalizumab has a role in non-atopic asthma. A recent trial demonstrated

significantly increased asthma control in 29 non-atopic patients and a trend to reduced exacerbation rates and improved lung function [49, 50]. A small randomized controlled trial comparing treatment with omalizumab and placebo in non-atopic asthmatics over a period of 16 weeks found a trend towards a decrease in exacerbations and a significant improvement in lung function in omalizumab-treated patients, as compared with the placebo group. In addition, the authors showed that the expression of the high-affinity IgE receptor on blood plasmacytoid dendritic cells decreased significantly in the active group but not in the placebo group [50]. Larger trials and in particular, trials shedding light on how best to identify non-atopic patients who are likely going to respond to anti-IgE treatment are needed.

The success of anti-IgE therapy is limited by the fact that IgE production is not affected. Treatment has to be given regularly and on a long term basis. Brightbill et al. have successfully created a monoclonal antibody against the M1′ segment of membrane IgE on human IgE-switched B cells resulting in depletion of IgE-switched B cells *via* apoptosis or/and antibody dependent cell-mediated cytotoxicity [51]. Total IgE levels were reduced without other immunoglobulin isotypes being affected. A phase IIb, randomized controlled trial is currently testing its efficacy in uncontrolled allergic asthma and study results are expected in 2015 (http://clinicaltrial.gov/ct2/show/NCT01582503).

Promising newer treatments targeting the Th2 pathway with steroid sparing potential include the anti-interleukin-5 antibody mepolizumab. The first randomized controlled trials with anti-IL-5 were conducted in patients with mild-to-moderate asthma [52, 53]. These studies failed to show a beneficial effect on lung function. However, after targeting patients with severe asthma and refractory airway eosinophilia and choosing a more appropriate primary outcome (i.e. asthma exacerbations), anti-IL-5 treatment has been shown to significantly reduce exacerbations and oral steroid doses required to control symptoms and has been well tolerated during the study period of over a year [54]. Benralizumab, an IL-5 receptor alpha subunit (IL-5Rα)-antibody, not only reduced

peripheral blood eosinophils but significantly reduced eosinophil counts in airway mucosa/submucosa [55]. However, neither of the two treatments has had any effect on lung function or patient rated asthma control.

Clinical trials have also investigated targeted therapies against the Th2 cytokines IL-4 and IL-13. Pitrakinra is an IL-4 mutein, which binds to the IL-4Rα subunit and prevents the inflammation induced by IL-4 and IL-13. It has been shown to reduce allergen induced airway responses when given in inhaled or subcutaneous form in a study of mild asthmatics and to reduce exacerbation rates in those with eosinophilia [56, 57]. In moderate – severe asthma, the fully human monoclonal IL-4R-α dupilumab improved lung function, symptoms and exacerbation rate [58].

Periostin has proven to be a prognostic biomarker for treatment with the anti-IL-13 antibody Lebrikizumab. A recent study demonstrated that treatment with Lebrikizumab increased FEV_1 in patients with a high serum periostin level[59]. To date, the most successful anti Th2 cytokine therapies have focused on accurate identification of a patient's phenotype to allow for personalized treatment regimes.

1. 3. 2 Non-Th2-high Asthma: a Different Challenge

Treatments targeting other possible inflammatory mediators have shown less clear evidence of clinical benefit and the reason for this might stem from the difficulty of a clear definition: Th2-low asthma remains identified by the absence of Th2 biomarkers. Although the presence of a neutrophilic phenotype of asthma has been suggested, its use as a biomarker is imperfect. In contrast to the significant association between blood eosinophilia and airway eosinophilia in patients with asthma (who are not treated with systemic corticosteroids), there is no correlation at all between blood neutrophilia and airway neutrophilia [60]. Furthermore, there is no consensus as to the level of airway neutrophilia that would define pathology; neutrophils, unlike eosinophils are a normal

component of the cells retrieved in induced sputum. There is robust data however that the later onset, obese non-eosinophilic phenotype is often particularly steroid insensitive and difficult to control. TNF-α (Etanercept, infliximab, Golimumab): anti TNF-α treatment was tested in severe (Etanercept, [61, 62]) and moderate (Infliximab, [63]) asthmatics and resulted in improved asthma control, FEV_1 and bronchial hyperreactivity, however the effect ceased as soon as the drug was discontinued. The fully human anti-TNF-antibody Golimumab did not have any clinical benefit but resulted in an increase of respiratory infections and malignancies leading to an early discontinuation of the trial [64]. Trials on anti-IL-4 [57, 65], anti-IL-13 [66, 67], anti-IL-9 [62], agents targeting TSLP [68], Chemokine inhibitors (CCR3, CCR4 [69], phosphodiesterase and kinase inhibitors [70] and the use of vaccination [71] with the aim to shift from Th2 to Th1 are all under development.

In a randomized controlled trial the human anti-IL-17 receptor monoclonal antibody brodalumab was not superior to placebo in terms of asthma control [72]. Interestingly, the authors observed a nominal significance in a subgroup of patients with high bronchodilator reversibility and further studies of brodalumab in this asthma subpopulation are warranted.

Macrolide antibiotics have anti-inflammatory and immune-modulatory effects. They also increase gastrointestinal motility and to that extend might prove beneficial in patients with significant GERD. They have been proven effective in chronic respiratory diseases such as cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). They have also been shown to reduce severe exacerbations in patients with non-eosinophilic asthma and to have significantly improved Asthma Quality of Life Questionnaire scores [73]. However, chronic antimicrobial use is associated with the risks of population resistance, and treatment should be restricted to severe asthma patients at greatest unmet need despite optimal asthma management.

Methotrexate has proven a valid agent in patients who, despite long term treatment with oral steroids, fail to gain satisfactory control of their asthma [74]. Due to considerable side effects, close monitoring is needed and treatment should only be initiated in specialist centers.

1. 3. 3 Bronchial Thermoplasty

Smooth muscle hyperplasia is a distinctive feature of asthma. Applying radiofrequency energy to sub-segmental airways has been shown to reduce muscle mass at the site of thermoplasty. Trials demonstrated a reduction in the number of severe asthma exacerbations and improvement in asthma–specific quality of life, [75] and a recent study found no adverse events after a five-year follow up period [76]. Although guidelines recommend bronchial thermoplasty for adults with severe asthma that is not controlled on ICS and long acting $beta_2$ agonists (LABA), it is currently unclear which phenotypes respond best to the treatment. Studies are needed to identify the phenotype of patients that will derive significant clinical benefit most from this invasive procedure.

In summary, a well-defined, unbiased approach defining clinical characteristics that specify distinct phenotypes of asthma is critical for a better understanding of disease pathogenesis and thus successful management. New drug therapies are expensive and this substantiates the need to recognize responder patients and to select these patients for clinical trials.

1. 4 Steroid Resistance

Despite the availability of effective therapies a substantial number of patients with asthma appear to suffer from continuous symptoms. Poor compliance to treatments may be a reason for this. However, it is noteworthy that studies assessing asthma control observed persistent symptoms in over 20% of patients despite treatment adherence in clinical trials, where compliance is assumed as exceptional and often monitored closely [77]. It is therefore widely acknowledged that a small proportion of patients have an indisputable poor response, in particular to steroids. Furthermore, even when taken regularly, ICS do not appear to significantly modify the course of the disease and are not curative, asthma symptoms recur when treatment is discontinued.

Although the concept that some asthma patients show no benefit to steroids has been described for nearly 50 years [78], there is no absolute consensus on how to define clinical steroid resistance.

Pioneering work in this field demonstrated that cells from patients known to be clinically sensitive to steroid therapy did not differ significantly from those of clinically resistant patients in terms of their immuno-phenotype or the number of colonies generated by culture in the presence of phytohaemagglutinin[79]. However, *in vitro* steroids inhibited colony growth from cells of steroid sensitive (SS) patients, whereas there was less inhibition seen from cells from steroid resistant (SR) patients. In their work, *Corrigan et al* confirmed these findings and showed that proliferation of peripheral blood T cells from SS but not from SR asthma patients was significantly inhibited by dexamethasone and that there was a correlation between the degree of sensitivity to dexamethasone *in vitro* and the *in vivo* response to prednisolone with respect to improvement in lung function [80].

Research into the exact mechanisms leading to steroid resistance remains topical. Genetic disease specific factors and the environment have been reported to add to steroid resistance: gene expression studies have linked gene modification, for example

in p50 (a component of NF-κB), FKBP51, a GR chaperone protein or corticotrophin releasing hormone receptor-1, with the degree of responsiveness of asthmatics to steroids [81].

Steroid resistance is also associated with an increased expression of the transcriptionally inactive glucocorticoid receptor beta (GR-) ß [70, 82, 83]. Further, accounting for corticosteroid insensitivity are defects in glucocorticoid receptor binding, activation of transcription factors such as AP1, which is activated by pro-inflammatory cytokines such as tumor-necrosis-α (TNFα) or through failure to induce regulatory cytokines like IL-10 [84]. There is also an association between steroid resistance and IL-17, which is detailed further below.

Cigarette smoke has been implicated in steroid resistance: oxidative stress has been shown to impair nuclear translocation of GR and to increase pro-inflammatory transcription factors such as NF-κB and AP-1, and to reduce histone deacetylase (HDAC2), a protein suppressing pro-inflammatory genes [85, 86]. PBMCs from smoking asthmatics also have an elevated GR-β to GR-α ratio [87]. Allergen exposure decreases the binding affinity of GR and viruses and bacteria have been shown to impair GR nuclear translocation [88, 89].

Obesity related asthma is poorly responsive to steroids. Factors such as insulin resistance, altered adaptive and innate immunity, changes in mechanical loading of the chest wall and abdomen and increased airway hyper responsiveness secondary to low lung volume breathing have all been linked to obesity related asthma [90]. Furthermore it has been shown that obese asthmatics have impaired induction of MKP-1 expression in response to dexamethasone in both PBMCs and BAL cells [91]. Glucorticoids inhibit pro-inflammatory gene expression, in part through negative regulation of mitogen-activated protein kinase (MAPK) signaling pathways by molecules such as MAPK phosphatase (MKP)-1.

Steroid resistance has also been associated with airway remodeling: TIMP-1 is a tissue inhibitor of the matrix metalloproteinases (MMPs), which degrade collagen. MMP-9 and TIMP-1 have both been shown to be increased in asthmatic patients but interestingly steroid resistant patients expressed a higher ratio of MMP-9/TIMP-1 because of an inability of steroids to enhance TIMP-1 production [92 93].

In The Calcitriol Study we have chosen to define SR asthma based on the previous work by Professor Chris Corrigan, who also acted as one of the Chief Investigators in the study [80]. Steroid sensitivity was defined by an improvement of FEV_1 of > than 10% following a two week course of oral prednisolone at 40mg/1.73m^2 body surface area (BSA).

1. 5 IL-17A

Although the production of IL-17A by activated CD4+ T cells, the pro-inflammatory cytokine accredited for the recruitment, activation and migration of neutrophil granulocytes, had been known for a while, the recognition of Th17 cells as an independent and important subtype driving autoimmunity has been fairly recent [94, 95]. Experiments in mice with experimental autoimmune encephalomyelitis (EAE, the murine model of multiple sclerosis), an inflammatory disease historically associated with a Th1 immune response, revealed that administration of antibodies to the classical Th1 cytokine IFN-γ unexpectedly resulted in worsening of the disease [96]. Further investigations into these apparent discrepancies established that the p40 subunit shared with IL-12 could pair with another subunit p19, which would then form the cytokine IL-23 [97]. This heterodimeric cytokine is critical for IL-17A production by CD4+ cells and it is worth mentioning here. *Cua* and colleagues were able to show that EAE did not develop in *Il23a*$^{-/-}$ mice, making IL-23 a key cytokine in Th17 driven autoimmunity [94]. Subsequent experiments by the same group showed how IL-23 is crucial for differentiation and maintenance of Th17 cells and is responsible for the phosphorylation of STAT3, a factor involved in transcriptional regulation of Th17 cell development [98].

These findings showed how Th1 cells alone are not fully accountable for autoimmune disease. To highlight the distinction of Th17 cells from Th1 cells, it was further demonstrated that their differentiation was not dependent on IL-12/Tbet or IL-4/GATA3/c-Maf respectively, the main cytokines and transcription factors involved in Th1 and Th2 differentiation. In fact, forced expression of T-bet or overexpression of c-Maf resulted in reduced IL-17A production [99]. CD4+ T cells isolated from mice lacking Tbet and Gata3 retained the ability to differentiate into Th17 cells *in vitro* when activated in the presence of IL-23 [100, 101].

Studies comparing gene expression profiles of activated T cells stimulated with the

cytokines IL-23 and/or IL-12 showed that while Th1 cells greatly expressed T-bet, Th17 cells expressed *rorcgamma*, the gene encoding for RORγt. Expression of RORγt in naïve T cells is both necessary and sufficient to induce IL-17A. *Ivanov* demonstrated that CD4+ T cells from RORγt-deficient animals were unresponsive to IL-23, had reduced numbers of Th17 cells and were resistant to EAE induction [99]. These findings established the association between IL-17 expressing cells and the transcription factor RORγt.

The balance between different Th cell subsets is controlled by various pro-inflammatory cytokines: three independent groups demonstrated that in mice TGF-β was required for initiation and IL-6 was a critical co-factor for Th17 cell differentiation from naïve T cells [102-104]. This was surprising as TGF-β has so far been accredited anti-inflammatory activity and required for peripheral induction of FoxP3+ Tregs known for their anti-inflammatory activity. Further studies highlighted the importance of the co-factor IL-6, which was shown to have inhibitory action on Treg development and to upregulate IL-21, a key player in Th17 cell induction, amplification and development [105, 106]. In humans, key cytokines involved in the development and differentiation are less clearly defined: Some studies suggest an inhibitory role for TGF-β in Th17 cell differentiation, whereas others found that TGF-β was necessary and sufficient to induce Th17 cells from naïve CD4+ T cells. [107-112]. Both IL-23 and IL-1β have been shown to drive Th17 cell differentiation, the effect of IL-1β being enhanced by IL-23 and/or IL-6. The importance of IL-1β in the induction of IL-17 production is unique to human T cells. Furthermore, it has been shown that the presence in culture medium of natural agonists for aryl hydrocarbon receptors (Ahr) is critical for optimal Th17 cell differentiation [113]. Aryl hydrocarbon receptors respond to a broad range of chemicals resulting in receptor nuclear translocation and binding to gene promoters containing dioxin-responsive elements. Tissues that come in contact with the external environment express high levels of the Aryl hydrocarbon receptor (Ahr) and it has been shown that Ahr activation boosts

TH17 differentiation and increased the severity of autoimmune disease in mice [114, 115].

The hallmark cytokine produced by Th17 cells is IL-17 (also referred to as IL-17A), a member of the IL-17-family consisting of the six members IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25) and IL-17F. Most knowledge has so far been gained on IL-17A, IL-17F and IL-25. IL-17A and IL-17F share >50% homology and bind to the same receptor, a transmembrane receptor ubiquitously expressed on various organs and cells. Expression of IL-17A was first described by memory CD4+ T cells but it has since been found to be secreted by CD8+ T cells, NK cells, γδ T-cells, CD3+ cells, lymphoid-tissue inducer (LTi)-like cells and neutrophils under certain conditions [116-119]. Each of these cells has an important role in tissue surveillance, notably at sites of infection in the gut, skin and lung. Cellular response to IL-17R-mediatied signaling induces production of granulocyte colony stimulating factor (G-CSF), IL-6, IL-8 and neutrophil recruitment, required for bacterial and fungal clearance, and highlights the protective role of IL-17A against extracellular bacterial infection at mucosal interfaces [120]. In the lungs γδ T-cells were identified as an invaluable source of IL-17A in promoting efficient antibacterial immunity, as shown for example in patients with tuberculosis [121]. IL-17A has also been shown to augment the expression of antimicrobial peptides, induce production of IL-12 in dendritic cells and drive Th1 immunity and therefore support intracellular bacterial control [122, 123]. Dominant-negative mutations in human STAT3 lead to the development of hyper-IgE syndrome, which compromises the generation of IL-17A producing cells and patients suffer from mucocutaneous candidiasis and pulmonary infections with Staphylococcus aureus [124]. In cystic fibrosis, a disease characterized by bronchoalveolar neutrophilia, studies have shown an increased amount of IL-17A+ CD4+ T cells, γδ T-cells and NK T cells [125].

IL-17A can have both beneficial (host defense) and harmful effects: although the mechanisms that underlie pathogenicity against self are still not entirely known, it has become clear that the immune response elicited by Th17 cells, *i.e.* activation of the

innate immune system and neutrophil influx can lead to damage of the surrounding tissue. IL-17A is associated with several autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, psoriasis or lung inflammatory disease like asthma [100, 126-128]. Higher levels of IL-17A are found in sputum and BAL of patients with asthma compared to control subjects and the severity of airway hypersensitivity correlates with airway neutrophilia and levels of IL-17A expression [129-131]. Barczyk reported almost a decade ago that higher levels of IL-17A correlated with increased AHR in response to methacholine in patients with asthma [132].

Animal models have demonstrated in the past a role for IL-17A in Th2 cytokine-mediated asthma by promoting an allergen driven immune response in the sensitization phase or by inhibiting a local allergic response in the challenge phase, suggesting that the timing of IL-17A administration or neutralization may play a critical role on outcome and function [133,134].

It has also been reported that IL-17A can induce antigen-presenting cells (APCs) to produce inflammatory cytokines and in this way lead to generation of antigen-specific Th17 cells and exacerbate autoimmunity [120].

Interestingly, studies in mice and humans suggest an association between steroid resistance and Th17 mediated disease: adoptive transfer of Th17 cells resulted in increased levels of CXC chemokines and G-CSF in the BAL fluid of SCID mice. Treatment with dexamethasone resulted in increased neutrophil numbers but no improvement of airway hyperresponsiveness [135]. *Molet* reported increased levels of IL-17A in chronic sinusitis that is resistant to steroids [129].

Steroid resistance is associated with an increased expression of the transcriptionally inactive glucocorticoid receptor beta (GR-)ß [70, 82, 83]. (GR)-ß increases in response to exogenous IL-17A and IL-17F, an effect that is more prominent in asthmatics than in healthy controls. It has also been shown that dexamethasone significantly decreased the

expression of IL-6 in epithelial cells from healthy controls but failed to do so in asthmatics [136].

To date, there have been few trials assessing the clinical utility of targeting IL-17A in asthma. Busse recently published a report of a clinical trial of an IL-17RA antibody in patients with moderate – severe asthma and found improved asthma control in a small subgroup of patients with high beta-agonist reversibility [72]. In keeping with this, an earlier study found higher IL-17A levels in patients with reversible severe airflow obstruction as compared to chronic persistent obstruction, suggesting that there might be a role for anti-IL-17A treatment in this particular asthma phenotype [137]. Interestingly the same monoclonal anti-IL-17A antibody has previously been shown to be effective in psoriasis but has had no benefit in rheumatoid arthritis[138]. Targeting IL-17A is an attractive option. However, when designing future trials researchers must take into account not only its pathogenic effect in autoimmune disease but also its importance in host defense. I will discuss the role vitamin D might play in this context in paragraph 1.8.2 below.

Comparable to IL-17, another leukocyte-derived cytokine – IL-22 - has been suggested to be important in sustaining inflammation in autoimmune disease and to predominantly have regenerative function. IL-22 is a member of the IL-10 cytokine family. Biological activity of IL-22 depends on tissue location and cytokine environment [139].

The IL-22 receptor complex is composed of the heterodimer IL-22R1 and IL-10R2 [140]. Although IL-10R2 is found on various types of immune cells, IL-22R1 is expressed by non-hematopoietic cells, predominantly by epithelial cells of the respiratory and digestive tract, and skin keratinocytes. IL-22 ligation to its receptor activates STAT3 signaling pathway leading to the expression of genes involved in inflammation, chemotaxis, host defense and mucosal protection [141]. Among the different T cell populations, Th17 (mouse and human) and Th1 and Th22 (human) are the main producers of IL-22. [111]. IL-22 is also regulated by the transcription factor aryl

hydrocarbon receptor (Ahr) [142].

In the lung, IL-22 plays a crucial role in innate immune defense. Mouse models have shown an increase in antimicrobial peptides and recruitment of neutrophils by IL-22 [143]. Mice deficient in IL-22 have reduced airway hyperreactivity, eosinophil recruitment and mucus secretion. Neutralization of IL-22 antibody during immunization in an OVA-induced model of allergic asthma inhibited airways inflammation. Deletion of IL-22 attenuated collagen-induced arthritis [144]. In contrast, IL-22 has also been shown to have a protective role. Neutralisation during antigen challenge caused enhanced allergic lung disease and analogously, recombinant IL-22 given during challenge protected mice from lung inflammation [145-147]. This tissue protective activity has also been shown in murine models of hepatic inflammation, inflammatory bowel disease and experimental autoimmune myocarditis [143, 148, 149].

Similarly to IL-17, the bioactivity of IL-22 in animal models appears to depend on the timing during which those cytokines are activated as well as the cellular source and whether they elicit a protective or pro-inflammatory response depending on the phase of disease.

In summary, data from human and animal models demonstrate that IL-17A and IL-22 are both crucial players in inflammatory lung disease. Their pathogenicity makes them a complex but attractive target for therapeutic intervention and I will be discussing the expression of IL-17A and IL-22 in healthy individuals and in steroid sensitive and steroid resistant asthma patients and investigate the cytokines' response to vitamin D and dexamethasone treatment.

1. 6 IL-10

Unlike IL-17A and IL-22, IL-10 is an immunosuppressive cytokine with broad anti-inflammatory properties and is thought to play a key role in maintaining immune homeostasis in the lung [150]. It is produced by a wide range of cell types, including B

cells, macrophages, dendritic cells, mast cells, and eosinophils. Many T cell subsets synthesize IL-10, such as CD8+ T cells, CD25+FoxP3+ T reg cells, and effector CD4+ T cells namely Th1 cells, Th2 cells and Th17 cells.

IL-10 affects many functions relevant to asthma; it inhibits mast cell and eosinophil function and favourably modulates IgE to IgG_4 ratios. IL-10 acts on macrophages and dendritic cells to inhibit pro-inflammatory cytokine production and the capacity to activate T cells, including Th2 cells [154].

In comparison to healthy controls, patients with asthma have been found to have reduced IL-10 mRNA and protein in bronchoalveolar lavage fluid [151]. Furthermore, studies of polymorphisms in the IL-10 gene promoter suggest that individuals who make low IL-10 suffer from more severe asthma[152-154]. Similar observations have been made in terms of atopy: *Akdis* noted, that allergic patients had a significant reduction in the frequency of allergen-responsive IL-10-positive T cells in the peripheral blood as compared to non-atopic individuals [155].

However, IL-10 may also enhance immune function and has been shown to activate mast cells, CD8 T cells and natural killer (NK) cells [156, 157]. It promotes humoral immune responses and induces immunoglobulin production.

The diverse functions of IL-10 have clinical implications as studies using recombinant IL-10 have shown: whilst early trials showed reduction of pro-inflammatory cytokines in the peripheral blood mononuclear cells of patients with psoriasis and Crohn's disease, both chronic auto-immune disorders like asthma, larger studies failed to see a therapeutic success due to side effects such as fever, which suggests immune stimulatory effects of the treatment [150]. Several established therapies for asthma have been shown to promote IL-10 in patients [156]. Both, inhaled and systemic steroid therapy increases mRNA for FoxP3 and IL-10 synthesis. Our laboratory has previously shown that the steroid dexamethasone dose-dependently induced IL-10 synthesis by CD4+ T cells in culture

and that these cells were able to inhibit autologous effector T cells in an IL-10-dependent manner [158]. Enhanced IL-10 activity might therefore promise greater anti-inflammatory activity, which is of particular benefit to patients who remain symptomatic on current available treatments.

Allergen immunotherapy is associated with the induction of IL-10-secreting T cells and APCs [159]. Subcutaneous (SCIT) or sublingual (SLIT) immunotherapy (IT) is potentially very successful in terms of symptom relief of, for example, allergic rhinitis, and hence leads to better asthma control. However, it requires administration over a lengthy period and works in highly atopic individuals only and carries the risk of severe allergic reactions. IT needs to deliver better efficacy before widespread application in asthmatic patients can be considered.

A study in mice has shown that co-administration of the active form of vitamin D, calcitriol significantly augments the beneficial effects of IT on Ag-induced allergic asthma manifestations in a mouse model. Calcitriol has further been shown to induce tolerogenic dendritic cell phenotype with increased IL-10, which acts directly on CD4+ T cells to induce IL-10+ Tregs cells [160]. An interesting associating exists between IL-10 and serum 25(OH) levels. In children with both moderate and severe, therapy resistant asthma systemic vitamin D status directly correlated with airway IL-10 [161]. Vitamin D supplementation in patients with multiple sclerosis enhanced IL-10 synthesis [162] and in our own study of steroid resistant asthma, *Xystrakis* showed that the active form of vitamin D, calcitriol, restored the defective IL-10-production of CD4+ T cells [163]. This has led to the hypothesis that vitamin D may contribute to pulmonary homeostasis *via* induction of the anti-inflammatory IL-10. Coupled with the antimicrobial activity vitamin D is know to have (see next section), this might promise a safer approach of IL-10 enhancing therapies than that which we have seen so far.

1. 7 Vitamin D

The pathology of asthma and in particular severe and steroid-refractory asthma remains complex and new therapeutic approaches could have profound consequences for the patient and healthcare professionals.

Our laboratory has had an interest in vitamin D and its effect on respiratory health for a number of years. The role of vitamin D and its metabolites in bone and calcium metabolism is well established but there is increasing awareness of its importance in immune regulation [164-166]. The overlap in the epidemiology of vitamin D insufficiency and asthma has led to intense interest in its role(s) in inflammatory lung disease.

1. 7. 1 Metabolic Pathway

Vitamin D can be derived from the diet, however the majority of it is produced in the skin: once 7-dehydrocholesterol is photolysed into pre-vitamin D3 and processed into vitamin D3 it is converted in the liver to 25-hydroxyvitamin D by CYP27A1 (25(OH)hydroxylase) and subsequently in the kidneys by CYP27B1 (25-hydroxyvitamin-D-1-hydroxylase) to its biologically active form 1,25-dihydroxyvitamin D (hereafter calcitriol), a mechanism tightly regulated by PTH (Figure 1.2). Calcitriol is transported to target cells bound to vitamin D binding protein (DBP) or deactivated by 24-hydroxylation by CYP24A1 which catabolizes calcitriol to its water-soluble inactive form calcitroic acid, excreted in the bile. It further catabolizes 25(OH)D to 24,25(OH)D. 1α-hydroxylation of 25-hydroxivitamin D also occurs at extrarenal sites such as brain, breast, colon, prostate and cells of the immune system. At site of inflammation, local synthesis of calcitriol (subject to availability of 25-hydroxyvitamin D substrate) can modulate immune responses in a paracrine fashion [167].

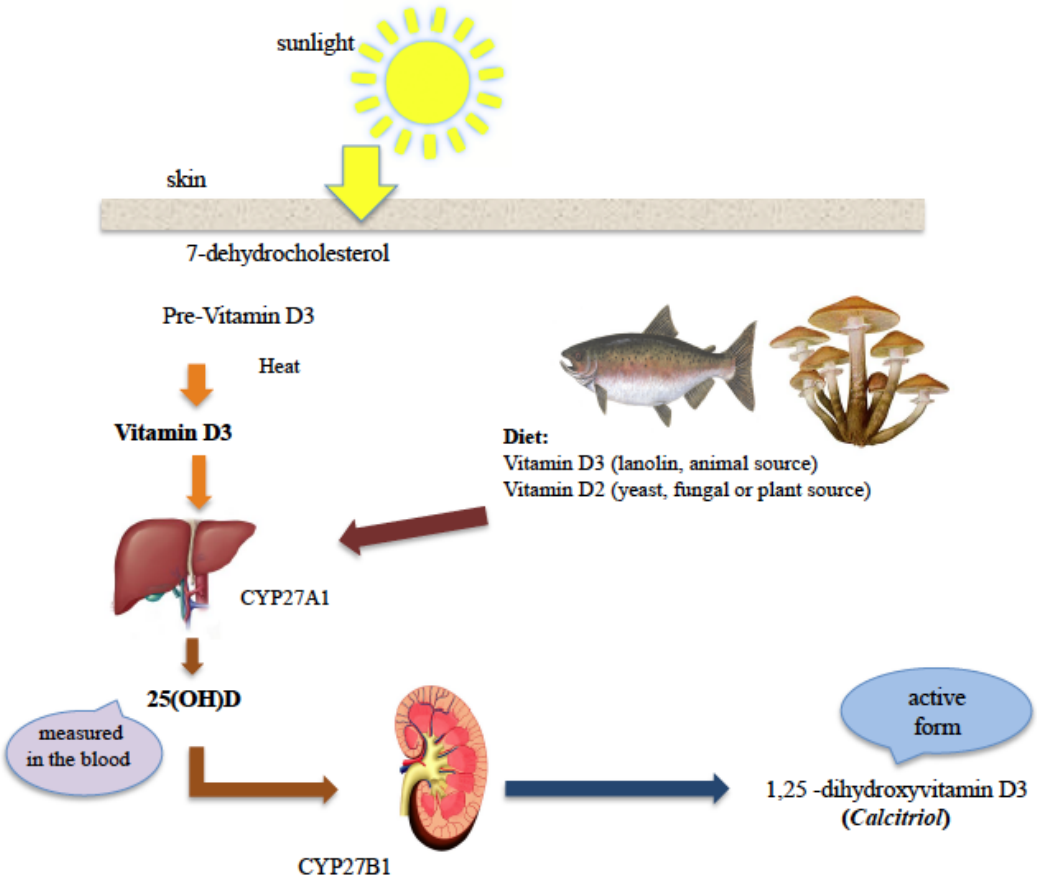


Figure 1.2 Synthesis of vitamin D:

During exposure to UVB 7-dehydrocholesterol in the skin is converted to pre-vitamin D3, which is converted to vitamin D3 in a heat-dependent process.

Vitamin D2 and D3 from dietary sources and vitamin D from the skin enter into the venous circulations, bind to vitamin D binding protein and are transported to the liver, where vitamin D is converted by CYP27A1 to 25-hydroxyvitamin D_3 (25(OH)D). This is the major circulation form and is used to determine vitamin D status. 25(OH)D is biologically inactive though and is converted in the kidneys by CYP27B1 to the biologically active form 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$, *calcitriol*).

Calcitriol binds to the nuclear vitamin D receptor (VDR), a member of the superfamily of nuclear hormone receptors, which is found in most organs as well as monocytes, and activated T- and B-cells [168]. The pleiotropic activity of the calcitriol – VDR complex has been widely studied and receptor polymorphism has been associated with various immune diseases [169-172]. VDR is located within the region of q13-26 on chromosome 12, a region linked to asthma in genome-wide linkage analysis [173]. Binding of calcitriol to

the VDR induces a conformational change allowing the VDR to bind to and form a heterodimer with the retinoid X receptor (RXR). This VDR-RXR complex then acts as a transcription factor and binds to vitamin D3 response elements (VDREs) within the promoter region of genes that respond to vitamin D3. Calcitriol is catabolised by CYP24A1 and excreted in the urine.

1. 7. 2 Bioavailability

25(OH)D serum levels are greatly influenced by exposure of the skin to sunlight with maximum synthesis achieved when UVB reacts with 7-dihydrocholesterol in the skin at wavelengths of 295nm. Very little vitamin D is produced in areas at beyond a latitude of 35° from October to March and synthesis is greatly influenced by skin pigmentation, sun protection, (a sun protection factor of >15 will absorb UVB light to 99%), age and coverage by clothing [167].

Numerous studies emerge reporting hypovitaminosis D in widespread areas of the world including temperate climates [174-179]. Whilst serum levels of 25(OH)D as low as 25nmol/L will prevent rickets in children, it is often suggested that much higher levels are required for an adequate immune response.

Only a few foods, such as oily fish, contain moderate amounts of 25(OH)D and unlike countries like the United States or Finland, dairy products in the UK and the rest of Europe are no longer fortified with vitamin D [167].

It is therefore not surprising that Vitamin D deficiency is common and widespread. Over half of the British population has been found to have low levels of vitamin D during the winter months [180]. Several reports are showing vitamin D deficiency not only in adults but in pre-pubertal populations: studies conducted in Buenos Aires, Southern Tasmania, Turkey, the US and Spain found vitamin D insufficiency (25(OH)D < 50nmol/L) in 10 – 80% of the children with concerns raised over bone growth health and immune function

[153, 181-183]. Moreover, hypovitaminosis D has been linked to predisposition to chronic inflammatory lung disease, such as asthma and viral respiratory infections with higher rates of hospital admission for chest related diseases [184, 185]. There is a high prevalence of low levels of vitamin D in medical inpatients, where an increased risk of bone fracture and muscle weakness potentially adds to a slower recovery [186]. Also of interest are studies demonstrating an increased prevalence of cancers in countries at higher latitude including cancer of the colon, breast, oesophagus and prostate among others, suggesting an antiproliferative activity of vitamin D [125, 187, 188]; similarly, latitude seems to confer an increased risk for certain autoimmune conditions such as multiple sclerosis [189], rheumatoid arthritis [190], inflammatory bowel diseases[63] and type I diabetes mellitus [191].

The importance of vitamin D for pulmonary health has been highlighted in many studies so far (reviewed in [192]). Deteriorating asthma control and 25(OH)D levels have a similar seasonal oscillation and both have seen an increase in prevalence in the last decade. These observations have generated interest in exploring the link between the two conditions with a study by Black being one of the first to demonstrate a positive correlation between serum 25(OH)D and lung function [185]. Many studies followed, and table 1.2 gives an overview of a number of observational studies examining the relationship between serum 25(OH)D levels and asthma. These studies vary in design, sample size and assessment of 25(OH)D status, which might explain the differing results.

Author, Reference & Study Design	Effect
	Beneficial
Camargo [193] BC	Cord blood vit D inversely associated with wheeze but not with asthma by age 5
Camargo [194] BC	High vit D intake during pregnancy associated with reduced risk of wheeze
Miyake [195] BC	High maternal vit D intake associated with reduced risk of wheeze
Erkkola [196] BC	High maternal vit D intake associated with reduced risk of wheeze
Devereux [197] BC	High maternal vit D intake associated with reduced risk of wheeze
Tolppanen [198] BC	Vit D levels negatively correlate with wheeze
Brehm [199] L	Vit D levels negatively correlate with asthma severity and use of anti-inflammatory medicine
Brehm [200] CS	Vit D levels negatively correlate with asthma exacerbations
Chinellato [201] CS	Vit D levels positively correlate with asthma control
Searing [202] CS	Vit D levels positively correlate with lung function and negatively with IgE and use of steroids
Sutherland [203] CS	Vit D levels positively correlate with lung function and glucocorticoid response
Li [121] CS	Vit D levels positively correlate with lung function
Gupta [204] CS	Vit D levels positively correlate with lung function in children with severe asthma
Tolppanen [205] CS	Vit D levels positively correlate with lung function
Alyasin [206] CS	Vit D levels positively correlate with lung function
Montero-Arias [207]	Vit D is negatively associated with asthma severity and hospitalization.

CS	The association with lung function was not significant
Wu [208] CS	Vit D levels positively correlate with lung function
Freishtat [209] CC	Vit D levels negatively correlate with asthma
Korn [37] CC	Vit D levels negatively correlate with asthma control and severity
	No or adverse effect
Menon [210] CC	Vit D levels are not associated with asthma severity
Pike [211] BC	No association between maternal vit D levels and childhood asthma
Gergen [212] CS	No association between vit D levels and asthma
Krobtrakulchai [213] CC	No association between vit D levels and asthma
Devereux [214] BC	No association between vit D levels and asthma
Gale [215] BC	Maternal vit D levels *positively* correlate with risk of childhood asthma
Hollams [216] BC	No association between vit D levels and asthma

Table 1.2 Observational studies showing beneficial, no or adverse effect of vitamin D status on asthma

Vit D: vitamin D: 25(OH)D. BC – birth cohort; L – longitudinal; CS – cross sectional; CC – case control

Although birth cohort studies allow us to prospectively assess the relation between an vitamin D exposure and disease prevalence, significant loss to follow up and confounding factors will need to be taken into account when interpreting these results. Although most epidemiological studies show an association between lower vitamin D levels and increase asthma severity, there are alternative explanations for these findings. First, reverse causation is a potential concern in correlation studies. The vitamin D hypothesis states that lower vitamin D levels are causally associated with increased

asthma severity. It is possible that the association is causal, but in the opposite direction from the vitamin D hypothesis. For example, since vitamin D is synthesized in the skin by exposure to UV-B radiation, it is possible that children who are sicker due to their asthma may spend more time indoors and therefore have lower vitamin D levels. Brehm et al looked at the association between vitamin D levels and asthma exacerbations using a multivariate model, adjusting for time spent outdoors, racial ancestry as estimated from genetic data, and atopy (ref) All of these are potential confounders of the relationship between vitamin D and asthma, however the group found that even after adjusting for these exposures, there was still a strong relationship between vitamin D insufficiency and asthma exacerbations (ref) .

Another potential issue is that vitamin D may be a negative acute phase reactant. Several studies have reported an acute drop in serum 25-hydroxy vitamin D levels with elective knee or hip arthroplasty, implying that the changes in vitamin D levels are simply a marker of inflammation Curr Allergy Asthma Rep (2014). While this is one possible explanation, there is no clear biological mechanism for how this would occur as an unrelated phenomenon. Another explanation, consistent with the vitamin D hypothesis, is that serum 25-hydroxy vitamin D is converted to active 1,25-dihydroxy vitamin D as an appropriate response to acute inflammation, acutely depleting the serum supply of 25-hydroxy vitamin D (ref) .

To answer this question, we need well-designed randomized clinical trials of vitamin D supplementation..

There are abundant studies, both epidemiological and immunological, reporting the detrimental effect vitamin D insufficiency may have on our health. Simultaneously, a vast number of reports across all medical sub-specialties suggest a potentially beneficial effect of normal – high serum 25(OH) levels. However, there is no absolute consensus on what physiological serum vitamin D levels are, or indeed what defines vitamin D deficiency. In fact, a recent comprehensive review of intervention studies in a broad

range of diseases showed no beneficial effect apart from in elderly people where supplementation seemed to slightly reduce all cause mortality [217]. It is noteworthy though that his review focused only marginally on respiratory health.

To date there are few studies that investigated the effect of supplementing vitamin D in asthma and they vary considerably in terms of dose and treatment duration (see table 1.3). One study, the VIDA trial of vitamin D supplementation in vitamin D insufficient asthmatics recently reported its outcomes [218]. Although the primary outcome, time to first asthma treatment failure, was not met, patients in the treatment arm were significantly more likely to be able to reduce the dose of inhaled corticosteroids. One could conclude from this that supplementation with vitamin D might prove more successful when applied to a selected patient group, possibly in populations with a high prevalence of vitamin D deficiency at baseline, and using doses sufficient to induce sustained elevation of 25(OH)D concentrations. The aforementioned study proved successful in terms of steroid requirements to control disease and this is certainly interesting in relation to our interest and The Calcitriol Study.

	Population	Treatment	Outcome
Majak [219]	Mild asthma, aged 5-18 years, n=48	ICS[$] +/- 500IU D3 daily for 6 months	Decreased exacerbation in D3 group
Arshi [220]	Mild-moderate asthma, aged 10-50, n=108	ICS[*] +/- LABA +/- 100'000 D3 i.m. Bolus, 50'000 orally weekly for 24 weeks	Improved FEV_1 at 24 weeks in D3 group
Castro [218]	Moderate asthma, aged >18, n=408	ICS[$$] +/- 100'000 D3 i.m. 4'000 daily for 28 weeks	No change in FEV_1 but D3 group able to reduce ICS further
Nageswari [221]	Moderate-severe asthma, aged 35-65, n=63	ICS[*] +/- 1000 D3 daily for 90 days	Improved FEV_1 in D3 group
Yadav [222]	Moderate asthma, aged 3-14, n=82	ICS +/- 60'000 D3 orally per month for 6 months	Improved Peak Flow, reduced ICS requirements and reduced exacerbation rate in D3 group

Table 1.3 Randomised controlled interventional studies in asthma and their outcomes

[$] 800 mcg/d Budenoside; [*] Budenoside 400mcg twice daily +/- fermoterol 12mcg twice daily; i.m. intramuscular, FEV_1 forced exspiratory volume in 1 second; [$$] Ciclesonide 320mcg/day; D3: cholecalciferol

There are a number of ongoing trials of vitamin D supplementation to prevent asthma or reduce asthma morbidity still to publish their results (see table 1.4).

Title / Design	Target Population	Hypothesis	Treatment
VDAART NCT00920621 RCT	Pregnant women and their children	Vit D supplementation during pregnancy = reduced incidence of asthma during first 3 years of life	D3 4000 IU/d vs placebo
ABCvitaminD NCT00856947 RCT	Pregnant women and their children	Vit D supplementation during pregnancy and 1 week after delivery prevents asthma symptoms in the first 3 years f life	D3 2400 IU/d vs placebo
ViDiAs NCT00978315	RCT	Vit D supplementation will influence time to severe asthma exacerbation in adult and adolescents over 1 year follow up	D3 2-monthly oral doses vs placebo

Table 1.4 Ongoing trials of vitamin D supplementation to prevent asthma or asthma exacerbations (taken from clinicaltrials.gov.identifier)

RCT: randomised controlled trial. Vit D: vitamin d, D3: cholecalciferol. IU: international units

Supplementation studies not only stem from the association of low vitamin D status and potentially poor respiratory health, but also from abundant evidence that vitamin D and its active metabolites have important immuno-modulatory functions - questions therefore arose as to how vitamin D might influence the innate and adaptive immune system. Research has focused on the immuno-modulatory properties of vitamin D following the observation that monocytes/macrophages from patients affected by the granulomatous disease sarcoidosis constitutively synthesize the active form of vitamin D from the precursor 25(OH)D and that the receptor for vitamin D (VDR) is detectable in

activated, proliferating lymphocytes. The next paragraph discusses the immunological effects of vitamin D with a focus on asthma.

1. 7. 3 Immunological effects of vitamin D and its proposed effect in asthma

There is abundant evidence that the active form of vitamin D is important for respiratory health [192]. Chest (upper and lower respiratory tract) infections are the most common trigger for asthma exacerbations and are associated with a decline in lung function over time. The incidence of infections, in particular viral induced ones, peaks in the winter months when cutaneous vitamin D synthesis is weakened paving the way for many research studies to link vitamin D to the innate and adaptive immune system.

Active vitamin D, ($1,25(OH)_2D_3$, hereafter calcitriol) induces antimicrobial activity *in vitro* in monocytes/macrophages [223], neutrophils [224], keratinocytes [225] and also respiratory epithelium [226] and has been shown to upregulate *hCAP18*, a human antimicrobial peptide precursor of the family of cathelicidins. Other antimicrobial peptides regulated by vitamin D are the cationic peptides defensin-β 2 and 4 [224]. The induction of cathelicidin by calcitriol results in autophagy, important for the killing of Mycobacterium tuberculosis in infected cells [227]. Calcitriol upregulates the pattern recognition receptor Nucleotide-binding oligomerization domain containing 2 (NOD2) which, when activated in the presence of bacteria, leads to release of the antimicrobial peptide defensin-β 2 [228]. M. tuberculosis has also been shown to induce the expression of matrix metalloproteinases (MMPs), which degrade components of pulmonary extracellular matrix; calcitriol has been found to decrease the production of MMPs, thus reducing destruction of the lung by the mycobacteria [229].

Treatment with calcitriol down-regulates monocyte expression of the toll-like receptors (TLRs) TLR2 and TLR4, which recognise non-specific pathogen-associated molecular patterns and are vital for the induction of early innate immune responses. This

downregulation reduces production of the pro-inflammatory cytokine Tumour Necrosis Factor-α (TNFα) when these TLRs are ligated [230].

Calcitriol reduces dendritic cell (DC) expression of the antigen presenting molecules CD1a and MHC class II, and the co-stimulatory molecules CD40 and CD86. Upon antigen stimulation, these DCs cannot upregulate MHC class I and II, CD80, CD86 and CD40 in order to fully mature, and their production of the pro-inflammatory cytokine IL-12 is impaired. Production of the anti-inflammatory cytokine IL-10 is enhanced. Consequently, calcitriol-treated DCs stimulate reduced T cell proliferation and appear to induce a state of hyporesponsiveness. Calcitriol-treated DCs have been described as being 'tolerogenic' with respect to T cells [231-233].

Vitamin D has not only been linked to infection but also to autoimmunity. Several reports acknowledge an important role of calcitriol and VDR in Th2 driven disease, such as asthma. However, variable effects have been reported with some studies in mice describing reduced airway eosinophila, chemokine-induced T-cell migration and lung inflammation after injection with calcitriol [234, 235], whereas others reported no effect of calcitriol on lung pathology [236]. Other in vitro studies have shown that calcitriol inhibits T cell proliferation [168, 237] and IL-2 and IFN-γ production by CD4+ T cells [238].

The effect of vitamin D on cytokines key to asthma pathogenesis, Il-4, IL-5 and IL-13 is less clear: one study in mice observed that calcitriol reduced the frequency of IFN-γ secreting CD4+ T cells but promoted IL-4, IL-5 and IL-13, suggesting calcitriol enhanced Th2 cell activity [239] whilst a human study reported an increase in IL-5 and IL-13 following *ex vivo* treatment of PBMCs with calcitriol[240]. On the other hand, subsequent mouse and human studies found either no effect or an inhibitory effect of calcitriol on Th2 cells [238] among them an *ex vivo* cord blood study from neonates demonstrating inhibition of IL-4, IL-13 and IFN-γ by calcitriol [241]. These doscrepancies have partly been explained by a dosage effect with studies reporting that very high

concentration of 1calcitriol may fail to inhibit [160] or enhance [240] Th2 cytokine production.

One way calcitriol may influence asthma pathogenesis is through modulation of CD4+ regulatory T cells. *In vitro* studies have demonstrated the ability of calcitriol to induce peripheral CD4+CD25- T cells to express FoxP3. Induced T regulatory cells are able to suppress the proliferation of effector CD4+CD25- T cells [242]. *In vivo* studies in mice and humans reported similar findings: treatment with a calcitriol analogue resulted in disease regression in adult non-obese mice, autoimmune skin disease, rheumatoid arthritis and prevented transplant rejection by enhancing T regulatory cell activity [190].

CD39 is an ectonucleotidase that catalyses the dephosphorylation of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) into adenosine monophosphate (AMP). AMP is subsequently converted into the immuno-regulatory adenosine by CD73 [243]. CD39 has been shown to be down-regulated in severe asthma and also to negatively correlate with IL-17A. Our previously published work has shown that in vitro calcitriol increases CD39 and hence reduces pro inflammatory IL-17A [244].

There have been various reports about the immuno-modulatory and steroid sparing effect of vitamin D on Th17 mediated disease in *in-vivo* mouse and human models of experimental allergic encephalitis (EAE) [125], inflammatory bowel disease [245], rheumatoid arthritis [246] and autoimmune uveitis [247]. To that extent several publications reported that vitamin D reduced the risk of MS with studies correlating the suppressive effect of vitamin D on Th17 cells with prevention of EAE [248]. Joshi et al have shown that paralysis in EAE was reduced following treatment with active vitamin D3, which correlated with a reduction in IL-17A producing CD4+ cells [249]. IL-22 is another pro inflammatory cytokine associated with asthma as described in paragraph 1.5 of this chapter. We and others have shown that calcitriol downregulates IL-22 [244, 250]. One mechanisms by which calcitriol exerts it inhibitory effect on IL-17A could be by limiting expression of transcription factors RORC, RUNX1, and NFAT as well as the cytokine IL-6, all of

which promote the differentiation and maintenance of Th17 cells [251]. Vitamin D has further been shown to affect the pro inflammatory IL-8, a key neutrophil attractant and relevant in severe, therapy resistant asthma. A study in colorectal cancer patients showed that daily supplementation with vitamin D significantly reduced IL-8 after 6 months.

Vitamin D has also shown to modulate expression of matrix metallo-proteinases (MMP), such as MMP-7, MMP-9 and MMP-10. Extracellular matrix proteins are implicated in airway remodeling, which can lead to irreversible airways obstruction, often seen in longstanding and severe asthma, that is less responsive to steroid treatment. Increased airway smooth muscle mass (ASM) is also seen in airway remodeling and mouse models of asthma have shown that increased levels of vitamin D reduced ASM mass, subepithelial deposition and goblet cell hyperplasia [252]. Serum 25(OH)D inversely correlates with ASM mass in paediatric asthmatics and addition of calcitriol to human ASM in vitro reduces cell proliferation[204, 253].

Our laboratory's focus of interest has been the therapeutic response to steroids in asthmatics. *In vitro* dexamethasone inhibits Th2 associated cytokines via the proposed mechanism of IL-10 upregulation. CD4+ T cells from steroid resistant asthma patients fail to produce IL-10 when cultured in the presence of dexamethasone. However, when used in conjunction with calcitriol, dexamethasone induced a population of IL-10 producing regulatory cells [163]. In healthy controls, calcitriol alone did not lead to an increase in the expression of GRα but it prevented the dexamethasone dependent downregulation of GRα [163]. These data provided a potential mechanism through which calcitriol enhanced dexamethasone induced IL-10 response and they led to the design of the proof of concept study termed 'The Calcitriol Study', which formed the base of this thesis.

Chapter 2

Rationale and Hypothesis

Steroid resistant asthma has been the focus of our laboratory's research for a number of years. The work presented here discusses the results of a proof of concept clinical trial aimed at investigating the effect of calcitriol in steroid resistant and sensitive asthma patients *in vivo* and *in vitro.*

It was hypothesised that calcitriol enhances clinical responsiveness to systemic (glucocorticoid) prednisolone therapy in asthma patients who are clinically insensitive to steroids.

The immunological aim of the study was to utilise PBMC samples from the patients in this trial to further assess the immunophenotypes of SS and SR asthmatics at baseline, and following calcitriol therapy in the SR patients in order to identify potential immunophenotypes not only of steroid resistance but also the likely impact of treatment with calcitriol.

Based on the association of severe, steroid resistant asthma and the cytokine IL-17A and in order to extend the laboratory's earlier findings on the immunomodulatory effects of calcitriol on T cells and cytokines involved in asthma pathogenesis the study further hypothesised that calcitriol not only enhances IL-10 in culture but also inhibits elevated IL-17A production by blood Th17 cells from severe asthmatics independently of glucorticoids and independently of their clinical responsiveness to glucorticoid therapy *in vivo*.

Chapter 3

Materials and Methods

3. 1 Clinical Trial

Recruitment into the "Calcitriol study" started in April 2009 and finished in July 2012. The trial was conducted in compliance with the principles of the Declaration of Helsinki and the principles of GCP. The study was approved by the NHS research ethics committee London Bridge (08/H0804/84) and to the MHRA EudraCT (2008-002244-42) .

We recruited patients form the asthma clinics at two local hospitals: Guys and St Thomas' NHS Foundation Trust and Barts Health NHS Trust. In an attempt to increase patient numbers we extended recruitment to the Homerton University Hospital NHS Foundation Trust, but were unable to enroll any patients into trial.

Patients were approached under the following inclusion and exclusion criteria:

3. 1. 1. Inclusion Criteria

1. Male or female adults aged18 to 75 years.

2. Documented history and typical symptoms of asthma for ≥ 6 months prior to screening.

3. Pre-bronchodilator FEV1 < 80% predicted and documented variability in airways obstruction of 12% or greater within the previous 5 years or diurnal Peak Flow variability of > 20%.

4. Corticosteroid refractory asthma, as defined by a < 10% improvement in FEV1 following a 14 day course of prednisolone 40mg/1.73m^2/ day29-31.

5. Written informed consent received.

3. 1. 2 Exclusion Criteria

1. Past or present disease, which, as judged by the investigator, may affect the study outcome (other than asthma, rhinitis or eczema).

2. Serum corrected calcium >2.66mmol/L

3. Clinically significant deviation from normal (physical examination or laboratory parameters) as judged by the investigator at the screening visit.

4. Current smoker or an ex-smoker of less than 5 years with a greater than 5 pack year history.

5. Pregnant or lactating females or those at risk of pregnancy (women of childbearing age were offered a pregnancy test prior to recruitment).

6. History of a respiratory tract infection and/or exacerbation of asthma within 4 weeks of the screening visit requiring oral corticosteroid tablets.

7. Participation in a study involving an investigational medicinal product in the previous 3 months or blood donation within the last year.

8. Current or previous allergen immunotherapy.

9. Concomitant treatment with lithium carbonate or calcium supplements.

10. Inability to understand or comply with the research protocol

Subjects were withdrawn from investigational product treatment if they developed hypercalcaemia (corrected serum calcium >2.66 mmol/l confirmed on two samples) during the course of the trial. This was monitored at each follow-up time point.

Patients requiring rescue medication for exacerbation of asthma in the form of corticosteroids immediately before and during the trial were excluded or withdrawn from the trial (this included the four week wash out period).

Throughout the study the participants were allowed to continue their usual asthma medication as prescribed by their doctor (for example, short and long acting beta agonists (inhaled and oral form) including slow release, anticholinergics, inhaled corticosteroids, leukotriene receptor antagonists, theophyllines or antihistamines).

3. 1. 3 Primary Outcome Measure/End Point:

The primary outcome measure was the change in FEV_1 at baseline compared to the end of the treatment period.

3. 1. 4 Secondary Outcome Measures/End Points:

The secondary outcome measures were

1. Level of the *ex-vivo* production of cytokines by T cells
2. Fraction of nitric oxide in exhaled air
3. ACQ score (We obtained permission to use the Asthma Control Questionnaire by the author Professor Elisabeth Juniper.)

3. 1. 5 Trial Design

Screening Visit One

Informed consent to take part was obtained before the screening start. Suitable subjects underwent full assessment of their medical history (including a complete smoking history). Up to 100ml of venous blood was drawn for *ex-vivo* experiments. In addition, differential full blood counts were performed using a LH750 haematology analyser (Beckman Coulter, Brea, CA, USA). Albumin, phosphate and total serum calcium concentrations were determined using an Architect ci8200 analyser (Abbott Diagnostics, Chicago, IL, USA). Calcium concentration was corrected for serum albumin concentration using the formula: corrected calcium (mmol/l) = total calcium (mmol/l) + 0.02 × (40 – albumin [g/l]). Concentrations of 25(OH)D2 and 25(OH)D3 were determined by isotope-dilution liquid chromatography–tandem mass spectrometry and summed to give values for total 25(OH)D at the biochemistry laboratory under consultant Dr. Peter Timms at the Homerton University Hospital NHS Foundation Trust. Sensitivity for this assay was 10 nmol/l. Spirometry was performed using a PC based spirometer and software (MIR Medical International Research, Italy / WinspiroPRO). Reversibility was assessed by giving the subject 400mcg short acting beta agonist (salbutamol) followed by measuring the FEV_1 pre- and 15 – 30 mins post-bronchodilator. We measured FeNO using a Aerocrine NIOXMINO monitor, following the ATS/ERS guidelines. All these procedures were done in accordance to departmental Standard Operating Procedures. Eligible subjects were entered into the second part of the screening process. These subjects received prednisolone (Wockhardt UK Ltd; 40mg/1.73m^2/day, where m2 = Body Surface Area, BSA). The dose was calculated as (patient's BSA (m2)/1.73)x 40mg and rounded down to the nearest 5 mg, to be taken at home.

Screening Visit Two

The subject's medical history and adverse events were reviewed at the start of the screening visit 2. Spirometry was performed and the FEV_1 and FVC obtained as described for Screening Visit 1. Subjects demonstrating an increase of > 10% in the FEV_1 when compared to baseline (Screening Visit 1) were excluded. Subjects who were still eligible were entered into the main part of the trial. We repeated FeNO in exhaled air as described for Screening Visit 1. Up to 120ml of venous blood was taken to check that haematological and biochemical blood parameters were within normal range as described for Screening Visit 1, and for *ex-vivo* experiments.

Treatment Visit One - Day One

Following a 4-8 week washout period (from Screening Visit 2) participants attended Treatment Visit Day 1. The subject's medical history and adverse events were reviewed at the start of this visit. Up to 100ml of venous blood was taken for safety haematology and biochemistry and for ex-vivo experiments as described for Screening Visit 1. Participants were randomised to receive either calcitriol 0.25µg soft capsules (Rocaltrol®; Roche) or organoleptically identical lactose placebo generated in house (Pharmacy Production Unit, St. Thomas' Hospital NHS Trust, London, UK) twice daily. Patients were randomised in a 1:1 ratio using a computerised random plan generated by a physician not involved in the trial. Patients and trial investigators were blinded to treatment allocation.

Treatment Visit Two - Day Fifteen

On day 15 of treatment subjects attended Treatment Visit Day 15. The subject's medical history and adverse events were reviewed at the start of this visit. Prior to dosing, up to 120ml of venous blood was taken for safety haematology, biochemistry as described for Screening Visit 1 and for *ex-vivo* experiments. Subjects performed spirometry and fraction of nitric oxide in exhaled air (FeNO) pre-dose as described for Screening Visit 1. A standard Asthma Control Questionnaire (ACQ) was completed by the subject

before prednisolone dosing. Subjects were then commenced on prednisolone 40mg/1.73m^2/day as a single daily dose for 14 days as described for Screening Visit 1.

Final Visit - Day Twenty-Eight

On day 28 of treatment subjects attended Treatment Visit Day 28. They had completed a 28 day dosing period of treatment article and a 14 day dosing period of prednisolone. Medical history and adverse events were reviewed at the start of this visit. Following this, up to 120ml of venous blood were taken for safety haematology, biochemistry, as described for Screening Visit1 and for *ex-vivo* experiments. Subjects performed spirometry, and fraction of nitric oxide in exhaled air (FeNO) as described for Screening Visit 1. A standard Asthma Control Questionnaire (ACQ) was also be completed by the subject.

Follow up - Day Fifty-Six

After 1 month subjects were interviewed either during a routine visit to the asthma clinic or *via* telephone or email call to check medical history and adverse events. Following this, subjects were discharged from the study.

Figure 3.1 shows the study flowchart:

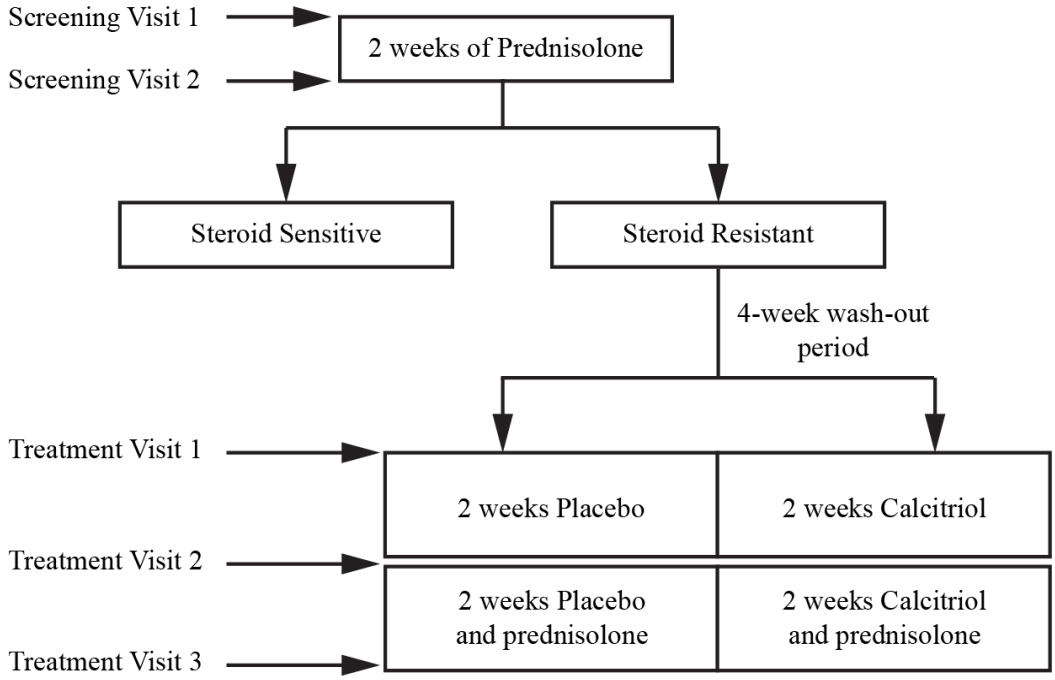


Figure 3.1: Calcitriol Study Flowchart

Participants who fulfilled all inclusion criteria on Screening Visit 1 commenced a 2 week course of oral prednisolone (Wockhardt UK Ltd; 40mg/1.73m^2/day, where m2 = Body Surface Area, BSA). At screening visit 2 lung function tests were repeated and patients who had a > 10% improvement in FEV_1 were excluded. All others were randomized after a 4 week wash out period to start either 0.25µg soft capsules (Rocaltrol®; Roche) or organoleptically identical lactose placebo generated in house (Pharmacy Production Unit, St. Thomas' Hospital NHS Trust, London, UK) twice daily. After 2 weeks participants additionally took a further course of prednisolone the same dose as before for the remaining 2 weeks of the trial.

Table 3. 1 shows the tests performed each visit:

	Screening visit 1	**Screening visit 2**	**Treatment visit 1**	**Treatment visit 2**	**Final visit**
Informed consent	**x**				
Spirometry	**x**	**x**		**x**	**x**
Exhaled FeNo	**x**	**x**		**x**	**x**
Blood test	**x**	**x**	**x**	**x**	**x**
ACQ				**x**	**x**
TREATMENT:					
Calcitriol			**x**	**x**	
Prednisolone	**x**			**x**	

Table 3. 1: Calcitriol Study: interventions at each visit.

FeNO= fraction of exhaled nitric oxide. ACQ = Asthma Control Questionnaire.

3. 1. 6 Trial Statistics

Sample Size calculation

Although we could not anticipate the actual magnitude of enhancement of the FEV_1 response to prednisolone produced by calcitriol therapy in this study, we readily estimated our power to detect theoretical enhancements based on a previous study in which 23 moderate to severe asthmatics underwent a similar prednisolone trial[31]. Mean (SD) percent improvement in FEV_1 was 15.0 (32.5%). Power calculations predicted that the study of 40 glucocorticoid resistant participants would enable detection of an improvement in change in FEV_1 from 0.4% to 10% with 80% power, tested at the 2-sided 5% significance level. Because of difficulties with patient recruitment, however, the decision was made to terminate the study after 24 patients completed, giving 65% power.

Statistical methods

The differences in % predicted FEV_1 at 28 days post randomisation between treatment groups was assessed using a linear regression model adjusted for screening values of % predicted FEV_1 value and vitamin D status. The analysis was performed on the complete case population and statistical significance was assessed at the 5% level.

For subsequent sub-analyses, data were assessed for Gaussian distribution and equality of variance after which the appropriate statistics were performed (see individual figure legends). Data were presented as the mean ± 95% confidence intervals (CI).

3. 1. 7 Randomisation

A clinician not involved in the trial generated a randomised list with the help of a computerised randomisation plan generator (randomization.com). This was kept in Guy's pharmacy and treatment was allocated accordingly. Allocation concealment was maintained and investigators and scientists as well as patients were blinded to the nature

of treatment. Guy's and St Thomas' NHS Foundation Trust and the clinician not involved in the trial kept a copy of the randomisation code. The investigator or treating physician was to unblind a participant's treatment assignment only in the case of an emergency, when knowledge of the study treatment is essential for the appropriate clinical management or welfare of the subject. This situation did not arise.

3. 1. 8 Investigational Medicinal Product and Placebo

The investigational medicinal product in this study was calcitriol produced by Roche Products Limited, Marketing Authorisation Number PL/0031/0122, 0.25μg: soft capsules. The soft capsule was encapsulated into a hard capsule to have the same appearance as the placebo.

Patients randomised to receive the investigational medicinal product received 0.25μg twice a day orally for 28 days.

The placebo used was matching hard capsules with the same appearance and texture as the Investigational Medicinal Product and was produced by Guy's Hospital pharmacy. Patients randomised to receive placebo received 1 capsule of matching placebo twice a day orally for 28 days.

Patients also received prednisolone 40mg/1.73m2/day (where m2 = Body Surface Area, BSA) for 14 days, to be taken at home. The dose was calculated as (patient's BSA (m2)/1.73)x 40mg and rounded down to the nearest 5 mg. Prednisolone was produced by Wockhardt UK Ltd, Marketing Authorisation Number PL 29831/0178: flat faced tablets for 14 days.

3. 2 Experimental Work

3. 2 .1 Isolation of Cell Populations from Human Peripheral Blood

Peripheral blood (60-120mls) was collected by venepuncture into the anti-coagulant sodium citrate (Sigma-Aldrich Co. Ltd, Poole, UK) at a ratio of 10:1 and diluted 1:1 in Hank's balanced salt solution (HBSS; Life Technologies, Gaithsburg, MD). This was layered at a ratio of 3:1 onto Lymphoprep® density gradient (Axis-Shield, Oslo, Norway) and centrifuged at 800g for 20 minutes at 4ºC. The peripheral blood mononuclear cells (PBMC) were removed from the Lymphoprep®:plasma interface and washed twice in HBSS supplemented with 2% foetal bovine serum (FBS; PAA laboratories, Pasching, Austria) (2%FBS/HBSS), first 600g for 10 minutes at 4ºC, then 200g for 10 minutes at 4ºC. PBMC pellets were then centrifuged at 6000rpm for 6 minutes at room temperature, the supernatant was discarded and the pellets were snap frozen in liquid nitrogen and stored at -80°C.

3. 2. 2 Isolation of CD4+ and CD8+ T cells by Positive Selection and CD4+ & APC cells by Negative Selection using Antibody coated Beads

CD4+ and CD8+ T cells were isolated using Dynal® CD4 or CD8 Positive Isolation Kits respectively (Dynal, Oslo, Norway). PBMCs were incubated with CD4 or CD8 coated Dynabeads® (CD4 beads: 50µl per 10^7 PBMC, CD8 beads: 25µl per 10^7 PBMC) for 20 minutes at 4ºC under continuous rotation. The sample was then placed on a magnet for 2 minutes. The supernatant containing the CD8 depleted cells was centrifuged at 200g for 10 minutes at 4ºC. The cells were then resuspended in 10ml 10%FBS/HBBS. A 1:1 dilution of 50µl of cell suspension and 50µl Trypan Blue was made and placed under a Haemocytometer. Viable cells were counted in 16 squares. The viable cell number was then calculated using the formula: Cell number x Dilution Factor x 10^4 = viable cells/ml.

The bead-bound cells were washed six times with 2%FBS/HBSS by application to the magnet and were then resuspended in 500µl of 2%FBS/HBSS. DETACHaBEAD® (Dynal) was added (CD4 beads: 250µl, CD8 beads: 125µl) and the cells were incubated for 45 minutes at room temperature under continuous rotation. The sample was applied to a magnet and the supernatant containing the released, purified CD4+ or CD8+ cells was transferred to a fresh tube. The beads were washed twice in 2%FBS/HBSS to collect residual cells. The CD4+ or CD8+ cells were then washed twice in 2%FBS/HBSS at 200g for 10 minutes at 4ºC to remove any of trace of the DETACHaBEAD® solution. Purity was routinely assessed by FACS analysis as 99% for CD4+ cells and 90% for CD8+ cells.

Figure 3.2 shows representative plots demonstrating the purity of CD4+ & APC cells isolated by bead-based selection:

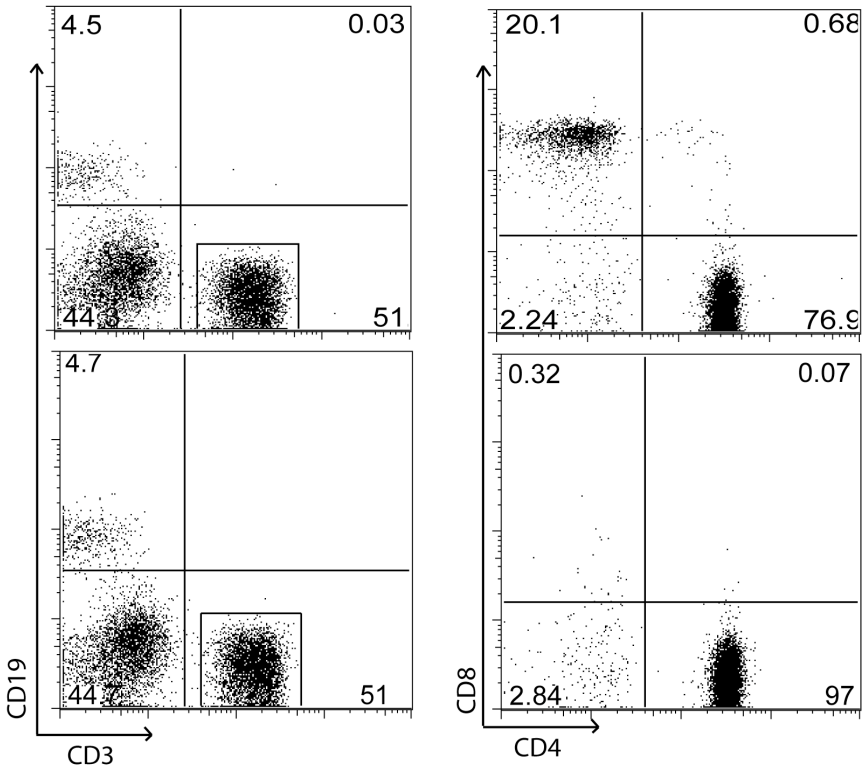


Figure 3.2: Purity of CD4+ & APC cells isolated by bead-based selection

CD4+ & APC T cells were isolated from PBMCs by negative selection using antibody-coated Dynal beads. Purity was assessed by surface staining using CD19-FITC, CD3-PerCP, CD8-PE, CD4-APC antibodies. 10000 live cells were analysed for fluorescence by FACS. Values shown are indicative of the percentage of positive cells within the relevant quadrant.

Isolated cells were counted using a haemocytometer with dead cells excluded by the use of Tryphan Blue staining. Purified cells were resuspended at a concentration of $1x10^6$cells/ml 10%FBS/RPMI.

3. 2. 3 CD4+ & APC cell Cultures

On day 0, cells were stimulated with plate-bound anti-CD3 (1mg/ml) (clone OKT3; purified in-house) and rIL-2 (50U/ml; Eurocetus, Harefield, UK). Cells were incubated at 37ºC/5%CO_2. Feeding occurred on day 3; half the volume of culture medium was removed and replaced with 10%FBS/RPMI with 50U/ml rIL-2.

On day 7 counting was performed by FACS analysis, using a FACScalibur (BD Biosciences). 0.4mg Propidium iodide (PI; Sigma-Aldrich) was added to exclude dead cells from the analysis. Cells were then washed twice in 2%FBS/HBSS by centrifugation at 200g for 10 minutes at 4ºC and resuspended at $0.5x10^6$cells/ml in 10%FBS/RPMI. Cells were restimulated with plate-bound anti-CD3 (1mg/ml) and rIL-2 (50U/ml). On day 9 supernatants were collected and stored at -20°C for CBA analysis.

1,25-dihydroxyvitaminD3 (also known as calcitriol, the active form of vitamin D3; BIOMOL Research Laboratories, Exeter, UK) and Dexamethasone was added to cultures on day 0 at the concentrations indicated. IL-4 was added to the cultures at 10ng/ml; (NBS biological, Huntingdon U.K.).

3. 2. 4 Quantification of mRNA Expression

3. 2. 4. 1 RNA Extraction and Quantification

Total RNA was isolated from cell pellets (previously 'snap' frozen in liquid nitrogen and stored at -80ºC) using the Qiagen RNeasy mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturers instructions. RNA was treated with RNase-free DNase (Qiagen) to remove residual genomic DNA, before being re-purified using the RNeasy kit and eluted into 30ml of nuclease-free H_20. RNA concentration was determined with a NanoDrop® ND-1000 spectrophotometer using ND-1000 software version 3.2.0. The purity and integrity of the RNA was assessed by A_{260}/A_{280} spectrophotometric measurements.

3. 2. 4. 2 Reverse Transcription

Two Hundred and Fifty nanogram RNA was reverse-transcribed into cDNA in a 30ml reaction. 0.2mg random hexamers (Amersham Biosciences, Buckinghamshire, UK) and 250ng RNA were heated to 70ºC for 5 minutes, and allowed to cool on ice. A master mix containing a final concentration of 5x reverse transcriptase reaction buffer (MBI Fermentas, Sunderland, UK), 1mM 4dNTP (MBI Fermentas), 40U RNAguard™ (Amersham) and 200U of Revertaid Mouse Moloney Leukaemia Virus (M-MuLV) reverse-transcriptase enzyme (MBI Fermentas) was added per reaction. The samples were incubated at 25ºC for 10 minutes, 42ºC for 10 minutes, 70ºC for 10 minutes and held at 4ºC. The cDNA generated was stored at -20ºC until subsequent analysis by Real Time RT-PCR.

3. 2. 4. 3 Real time RT-PCR

Transcriptional expression of target mRNA transcripts were determined by PCR amplification, quantified by 5'-nuclease assay using fluorescent labelled TaqMan® probes and analysed using an ABI PRISM 7900HT Sequence Detection System thermal

cycler (Applied Biosystems, Foster City, USA). All primers/probes sets were purchased from Applied Biosystems. The sets used and the probe labels were IL-10 (Hs00174086_m1), IL-17A (Hs00174383_m1), RORc (Hs01076112_m1) and FoxP3 (Hs00203958_m1), BATF (Hs00232390_ml), IRF4 (Hs01056533_ml), STAT3 (Hs00253714_ml). Reactions were performed in triplicate in 1 x TaqMan® Universal PCR MasterMix (Applied Biosystems) with 5ng reverse transcribed RNA, 0.5 x gene specific primer/probe set and 0.5 x 18S primer/probe set in a total volume of 10ml. Cycle parameters: 50ºC for 2 minutes; 95ºC for 10 minutes for 1 cycle followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

3. 2. 4. 4 Validation of Multiplexed Reactions

A TaqMan® probe has a fluorescent reporter dye (i.e. FAM or VIC) covalently linked to its 5'-end and a downstream non-fluorescent quencher. Fluorescence quenching depends upon the spatial proximity of the reporter and quencher. During the course of the PCR, *Taq* polymerase cleaves the reporter dye from the probe, thereby releasing it from the quencher and allowing it to fluoresce. The fluorescent dye released directly corresponds to amplification of the specific target sequence. The Real time RT-PCRs were internally controlled by an endogenously expressed gene (18S). The 18S probe has a VIC reporter dye whereas all of the other probes have a FAM reporter dye. These distinguishable reporter dyes allow amplification of 18S rRNA and the target gene of interest in a single well; known as a multiplex reaction. Validity of this approach was confirmed by performing serial dilutions of cDNA template in singleplex reactions with 18S or target primers/probe alone or in multiplex reactions with both 18S and target primers/probes. The relative quantification approach was used as the amplification efficiencies of both target and 18S primers/probes were found to be similar; both having slopes of within 0.5 of -3.3 and an R^2 value of greater than 0.98. Multiplexing was performed only where it was determined to have no effect on the relative efficiencies of the primers/probe sets.

3. 2. 4. 5 ΔΔCt Analysis

For each sample assayed, the Threshold Cycle (Ct) were determined for both the target gene and the 18S endogenous control, from the corresponding amplification plot according to guidelines from Applied Biosystems, using SDS software version 2.1 (Applied Biosystems). Briefly, the baseline is set to incorporate the range of cycles in which the PCR product is not amplified. The threshold line is set in the centre of the exponential phase of amplification to avoid the results being affected by the reaction components becoming limited in the plateau phase. The Ct value for each reaction is the cycle number at the point where the amplification curve crosses the threshold.

The data is expressed as arbitrary units and calculated by first normalising the data by subtracting the Ct value of the endogenous control from the target gene Ct (ΔCt). Samples from cell lines cultured with IL-2 but no VitD3 were chosen as reference samples. The ΔCt of the reference or control sample was next subtracted from the ΔCt of the other samples to give the ΔΔCt. Finally, mRNA relative quantity of target genes was calculated using the equation: $2^{-(DDCt)}$.

3. 2. 5 Flow Cytometry

Antibodies used for cell surface phenotyping were CD3 (BD Biosciences), CD19, CD4 and CD8 (BD Biosciences). Please see graph below for gating strategy. For intracellular cytokine staining on day 7, cells were restimulated for 4 hours with 0.25 ng/ml PMA (Sigma-Aldrich) and 25 ng/ml ionomycin (Calbiochem), with monensin (Sigma-Aldrich) added for the final 2 hours. Cells were washed, fixed, and permeabilized using Cytofix/Cytoperm kit (BD Biosciences) and triple-stained with fluorescently labeled monoclonal antibodies to IL-22 (ebiosciences 12-7229-42 PE anti-IL-22), IFNγ (BD Pharmigen FITC anti-IFNγ 554551), IL-13 (ebioscience PE anti-IL-13 55471), TNFα (BD Pharmingen PE anti-TNFα 554513), IL-10 (BD Pharmingen APC anti-IL-10 554707), IL-17A (ebioscience APC anti-IL-17A 17-7179-42), IL-2 (BD Pharmingen

APC anti-IL-2 554567). Dead cells (7-aminoactinomycin D [7-AAD] positive; Sigma-Aldrich) were gated out and were analysed using a FACSCalibur flow cytometer (Becton Dickinson). Figure 3.3 shows the gating strategy chosen:

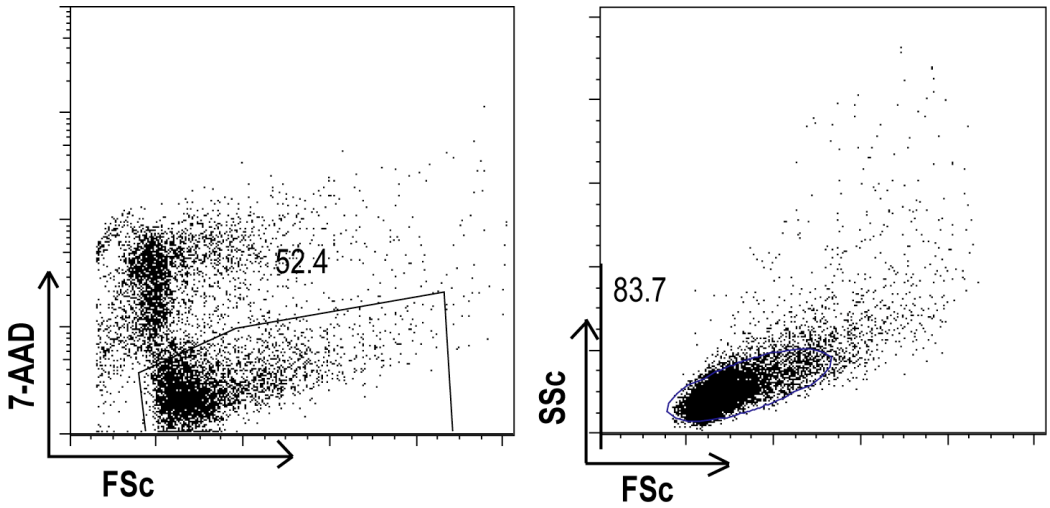


Figure 3.3: Gating strategy

Two representative plots showing the gating strategy. Firstly live cells were collected by 7-AAD negative, lymphocytes were collected on forward (FSc) and side (SSc) scatters.

3. 2. 6 Enzyme-Linked Immunosorbent Assay (Elisa)

One of the techniques used to measure secreted cytokine levels from cell culture was ELISA (enzyme-linked immunosorbent assay), using matched antibody pairs (BD Biosciences). Antibody used: IL-22 (capture antibody 841990; biotinylated detection clone anti-hIL-22 clone 142928). 96 well plates were coated overnight with 1mg/ml specific catch antibody in coating buffer (0.1M bicarbonate buffer, pH9.7) at 4ºC. After which, the plates were washed four times with PBS buffer (PBS, 0.05% Tween-20 (Sigma-Aldrich). The relevant cytokine was added to the plates at a final concentration of 0.5g/μml, along with the samples in duplicate, and incubated overnight at 4ºC. Standards were prepared in 10%FBS/RPMI or AIM V medium by serial 1:10 dilutions from the top standard 125000pg/ml. Each plate was then washed four times in PBS buffer and incubated with the appropriate biotin labelled detection antibody at 1mg/ml in detection buffer (PBS, 0.5% mouse serum (Sigma-Aldrich), 0.5% Tween-20, pH7.4) for 2 hours at room temperature. Following four further washes, Streptavidin HRP was diluted 1/200 in detection buffer and added to the plates for 30mins at room temperature. After that the plate was washed three times and TNB (Invitrogen 002023). The cytokine levels were measured using a Fusion Plate reader (Packard, UK) at an absorbance at 450nm with 690nm control filter.

The cytokine levels were measured using a Fusion Plate reader (Packard, UK) at an absorbance at 450nm with 690nm control filter.

3. 2. 7 Cytometric Bead Array (CBA)

An alternative method used to measure secreted cytokines in multiplex was by cytometric bead array (CBA), using the CBA flex set (BD Biosciences). CBA is a flow cytometry based application using matched antibody pairs that allows for the quantification of multiple cytokines simultaneously within a sample. Samples or

standards are firstly incubated with antibody-coated beads, which capture secreted cytokines, and following this step a fluorochrome-labeled detection antibody is incubated with the beads to bind to and quantify captured cytokine. The beads fluoresce with a unique intensity within the allophycocyanin (APC) emission spectrum, to allow for discrimination between beads and the detection antibodies fluoresce within the phycoerythrin (PE) emission spectrum at an intensity determined by the quantity of captured cytokine. A 16-point standard curve containing all relevant cytokines (rIL-5, riL-9, rIL10, rIL-13, rIL-17, rIFN-g; all R&D systems) was prepared in CBA sample buffer (BD Biosciences) by serial 1:2 dilutions from the top standard, which was 50000 pg/ml for all cytokines. Standards or samples (50µl) were added to a 96-well plate and incubated with 50µl of capture beads (diluted 1/250 in 'in house' CBA buffer; FACSflow (BD Biosciences), 10% Bovine Serum Albumin (BSA, Fischer Scientific), 0.5% TWEEN-20 (Sigma) and 2mM EDTA (Sigma)) for 3 hours at room temperature in the dark, with gentle shaking (500rpm) for the first 15 minutes. After 3 hours the plate was centrifuged at 500g for 5 minutes at 4°C, the supernatant decanted and the beads resuspended with 200µl 'in house' CBA buffer. The plate was then centrifuged for a further 5 minutes at 500g / 4°C and the buffer decanted. The beads were then incubated with 50µl antibody-detection reagent (diluted 1/250 in 'in house' CBA buffer) for a further 2 hours at room temperature in the dark with gentle shaking (500rpm) for the first 15 minutes. Following this 2 hour incubation, beads were washed twice as described above and finally were resuspended in 150µl FACSflow (BD Biosciences) for analysis using a BD Fortessa flow cytometer. Data were analysed using FlowJo (version 9.2, TreeStar Inc). The lower limit of detection for all samples was 1.5pg/ml.

3. 2. 8 Statistics

Data were presented as the mean ± 95% confidence intervals (CI). Data were assessed for equivalence to a Gaussian distribution and equality of variance after which the appropriate parametric or non-parametric test was performed (see individual figure

legends). Differences were considered significant at the 95% confidence level.). Box-and-whisker plots represent the median, the box as inter-quartile range, and error bars as 10th to 90th percentiles, with outliers outside the 10th to 90th percentiles shown as individual points.

Chapter 4

‘The Calcitriol Study’: A Randomized Placebo Controlled Trial to Investigate the Effects of Calcitriol Treatment in Glucocorticosteroid Resistant Asthma

4. 1 Introduction:

Steroids improve the clinical features of asthma and airways inflammation in a majority of patients; however they are not curative and they cause considerable side effects, particularly when taken at a high dose and over lengthy periods of time [130].

Some patients with asthma do not improve with steroid therapy, many do so incompletely, even within a clinical trial, where clinical symptoms are seen in up to 20% of patients despite monitored adherence[77]. In clinical practice, account must be taken of poor compliance, incorrect diagnosis, poor inhaler technique, co-morbidity or continuous exposure to external trigger factors for inadequate asthma control. Considering this, there remains a small number of patients, who are clinically not responsive to steroid therapy. Patients with steroid resistance have a high risk of hospitalisation and suffer considerable morbidity and mortality and this group needs other agents or strategies both to control their disease and spare them from the side effects of systemic steroids.

Overcoming steroid resistance, to improve the clinical response, is the focus of much basic and clinical research with many ongoing clinical trials. A number of mechanisms have been proposed to contribute to steroid resistant (SR) asthma (see also chapters one and five) [80, 83, 136, 254-258].

Our laboratory has had an interest in the immune-modulatory effects of both IL-10 and vitamin D for a number of years: *Richards et al* showed that steroids induced T cell populations to synthesize high levels of IL-10 and greatly reduced amounts of disease-promoting IL-4 and IL-5 [158]. Yet, CD4+ T cells from SR asthma patients showed an impaired *in vitro* response to steroids for the induction of IL-10 in comparison to cells from steroid sensitive (SS) asthmatics [259]. Subsequently, *Xystrakis* showed that the active form of vitamin D (1,25-dihydroxyvitamin D3, cholecalciferol; $1,25(OH)_2D_3$,, thereafter calcitriol) when used in combination with dexamethasone restored the CD4+

T cell response for the induction of IL-10 in SR asthma patients both *in vitro* and *ex vivo* [163].

The importance of vitamin D on pulmonary health has been highlighted in many studies so far (see chapter one and [192]). Deteriorating asthma control and serum 25(OH)D levels peak during winter / early spring and both have seen an increase in prevalence in the last decade. These observations have generated interest in exploring the link between the two conditions. *Black* et al first demonstrated a positive correlation between serum 25(OH)D and lung function [185] and many studies followed and Table 1.2 in Chapter one gives an overview of a number of observational studies examining the relationship between serum 25(OH)D levels and asthma. Notably, the results differ, which might be due to variations in design, sample size and assessment of 25(OH)D status. Nevertheless, it is interesting to see that those who focused on the role of 25(OH)D in *improving* disease control and treatment response to corticosteroids found that children with 25(OH)D levels below 75 nmol/L had increased use of inhaled and oral steroids relative to children with levels above 75 nmol/L, suggestive of a potential for steroid enhancing properties of 25(OH)D [202]. A different study in adults with persistent asthma found a significant association between reduced serum 25(OH)D levels and steroid sensitivity [203], a factor that considerably influences asthma severity and prognosis.

Our laboratory's earlier findings, together with epidemiological evidence that low levels of circulatory serum 25(OH)D is associated with a poor clinical response to steroid treatment in asthma[174, 204, 260, 261], provided the rationale for this proof-of-concept trial termed 'The Calcitriol Study'. We hypothesized that treatment with calcitriol would improve the clinical responsiveness to steroids in adult SR asthma patients.

The primary outcome of the study was a change in lung function (ΔFEV_1) from screening to the end of treatment period.

As secondary endpoints we choose to monitor asthma management *via* the asthma control questionnaire (ACQ) and measurements of fractional exhaled nitric oxide (FeNo). The primary goal of asthma management is to optimise asthma control (minimisation of symptoms, activity limitation, and rescue β_2-agonist use) and thus reduce the risk of life-threatening exacerbations and long-term morbidity. The Asthma Control Questionnaire (ACQ) measures both - the adequacy and a change in asthma control, which occurs either spontaneously or as a result of treatment [262]. There are many different questionnaires available to assess asthma control and studies have shown only a moderate agreement between five commonly used asthma controls scores. The ACQ is one of only a few fully validated questionnaires and has been shown to be particularly responsive to change in asthma control[263]. We chose the ACQ for its strong evaluative and discriminative properties is assessing asthma control.

Measurement of FeNo is a quantitative method of measuring airway inflammation. Whilst FEV_1 reversibility or provocation tests are only indirectly associated with airway inflammation, measurements of FeNo offer the advantage of detecting eosinophilic airway inflammation and to that extend allow monitoring of corticosteroid responsiveness or treatment adherence [264].

4. 2 Results

4. 2. 1 Patient Characteristics

Fifty patients were screened for eligibility between April 2009 and September 2012 (for inclusion and exclusion criteria see chapter two Materials and Methods). Figure 4.1 shows a schematic of the trial design. All patients were seen by the principal investigator (myself) at their first screening visit and at their first treatment visit. Follow up visits were managed by myself or a clinical nurse specialist (Grainne Colligan). For an overview of the tests performed at each visit see table 3.1.

Twenty-five patients were excluded before or during the screening period, which included a two-week course of oral prednisolone (at 40mg/1.73m^2 body surface area; bsa): seven patients did not meet inclusion criteria, three declined to participate, three were non-compliant with the protocol and 12 were steroid sensitive (> 10% improvement in FEV_1 compared to baseline). 24 severe asthmatics met the inclusion criteria for the study. They were randomly allocated, following a four-week washout period, to receive either twice daily 0.25μg calcitriol or identical placebo for a further four weeks. In the final two-weeks steroid treatment was repeated. 11 patients were allocated to placebo therapy and 13 to calcitriol. One patient in the calcitriol group withdrew from the study, due to constipation and back pain, leaving 11 placebo and 12 calcitriol patients in each arm for full analysis.

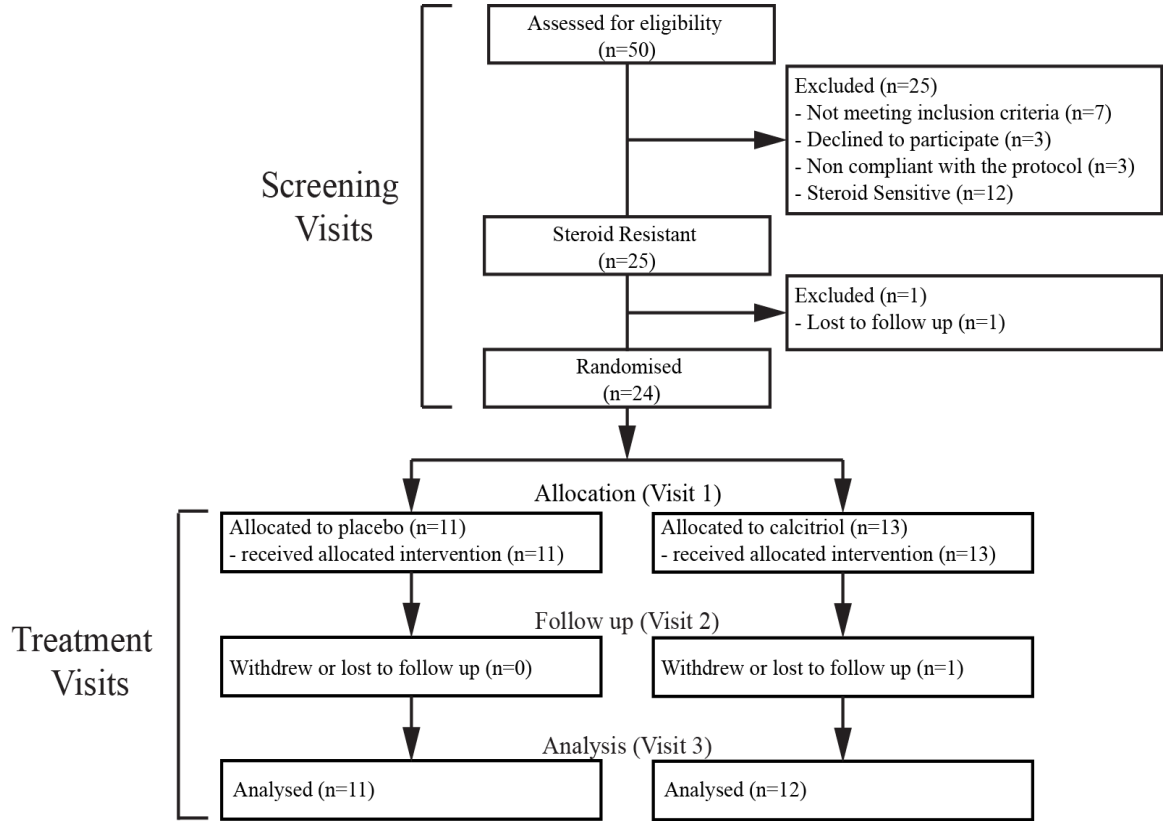


Figure 4.1 Schematic of Clinical Trial Design

Participants who fulfilled all entry criteria were screened at visit 1 then given a 2 week course of oral prednisolone. Lung function testing at screening visit 2 excluded all glucocorticoid sensitive asthmatics. Following a wash out period of 4 weeks, glucocorticoid resistant asthmatics began study treatment: At visit 1 the patients were commenced on either calcitriol or placebo, and at visit 2 (2 weeks later) the patients were additionally prescribed prednisolone at the same dosage as during the screening period. Two further weeks later, at visit 3, patients were seen again for assessment and lung function testing. At each visit blood tests were performed for safety monitoring and immunological analysis.

Patients did not differ in terms of age, gender, FEV_1, fractional exhaled nitric oxide (FeNo) and atopic status at screening. All had moderate to severe asthma with a mean

FEV_1 of 56% (steroid sensitive patients) or 61.3% (steroid refractory patients) (table 4.1).

In our study SS patients had on average a 15% improvement of their FEV_1 following an oral dose of prednisolone for 14 days, whereas SR patients had a mean deterioration on -1.22%. Table 4.1 lists baseline characteristics of all patients completing the screening period and the patients who were randomized to receive placebo or calcitriol. There were no statistically significant differences in patient demographics including age, body mass index (BMI), atopic status (assessed by skin prick testing in the asthma clinic within the last five years), baseline FEV_1 and inhaled steroid usage at randomisation.

		Steroid Sensitive	Steroid Resistant	P value
Age		49.0 (40.7-57.3)	51.8 (45.9-57.8)	
Ethnic Origin	Caucasian	9 (75.0)	17 (73.9)	
	African	3 (25.0)	5 (21.7)	
	Asian	0	1 (4.4)	
Sex	Male/ Female	4 (33.3)/ 8 (66.6)	14 (60.8)/ 9 (39.2)	
Atopic		10 (83.3)	19 (82.6)	
BMI (Kg/m^2)		31.8 (28.0-35.6)	28.2 (26.2-30.2)	p = 0.06
Inhaled Corticosteroid dose (BDP)		1113 (798-1469)	1225 (985-1520)	p = 0.60
FEV_1 (L)	Pre-steroids	1.7 (1.4-1.9)	1.9 (1.7-2.1)	p = 0.13
	Post-steroids	2.1 (1.8-2.4)	1.8 (1.6-2.1)	
FEV_1 (%)	Pre-steroids	56.0 (47.4-64.6)	61.3 (55.3-67.3)	p = 0.97
	Post-steroids	70.8 (62.6-79.0)	59.7 (52.8-66.5)	
Serum 25(OH)D (nmol/L)		38.8 (27.6-50.1)	36.9 (27.6-46.2)	p = 0.51
		n=12	n=23	

Table 4.1 Patient Characteristics

Data are shown as the mean and 95% confidence interval. Where applicable, data are shown as the mean with 95% confidence intervals or frequency (%). Where p values shown for non-categorical clinical parameters assessed by an unpaired t-test. Atopy was defined by skin prick testing with a panel of local aeroallergens. BDP: Beclometasone dipropionate; BMI: body mass index; 25(OH)D: 25-hydroxyvitaminD.

Compliance to therapy is of significant importance in placebo-controlled trials. Our patients were handed out diary cards to help them remember to take their treatments (see supplementary material).

Corticosteroids induce neutrophil leukocytosis. Studies looking into the mechanism behind this have shown decreased adhesions of marginated neutrophils and/or a reduced capacity of neutrophils to migrate from the vasculature. Additionally, the induction of granulocyte-colony stimulating (GCS) factor is thought to contribute to neutrophil

production and release into the circulation [265]. Furthermore, corticosteroid treatment leads to reduced eosinophil adherence and decreased chemotaxis, with both effects resulting in eosinopenia [266].

As a measure of compliance we assessed peripheral blood total and differential leukocyte counts before and after prednisolone therapy in the screening and treatment phases. In each phase, prednisolone therapy resulted in similar and significant increases in blood neutrophils and reductions in blood eosinophils (Figure 4.2 B and 4.2 C).

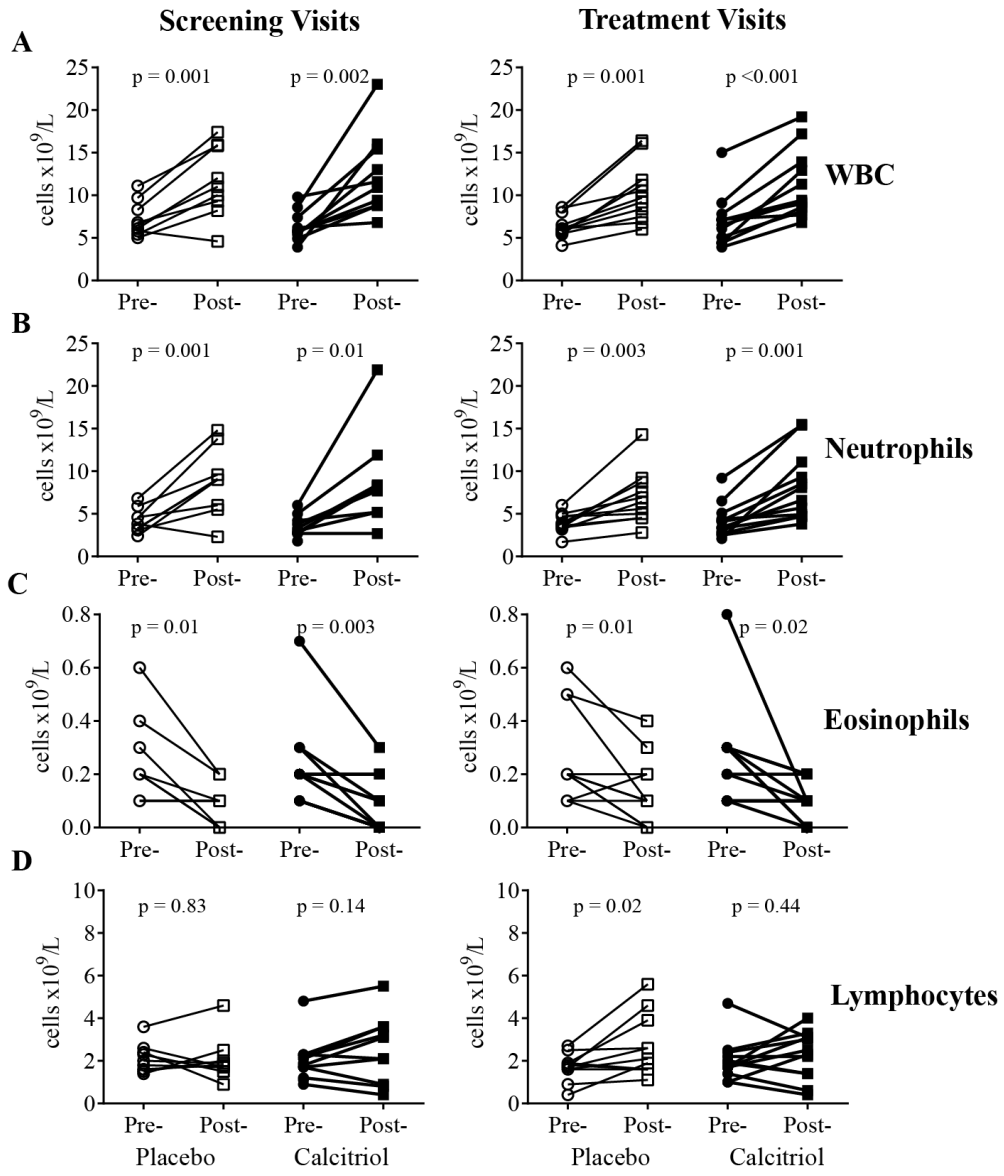


Figure 4.2 Prednisolone therapy-associated changes in blood total and differential leukocyte counts

A, Whole Blood Count (WBC), **B,** Neutrophils, **C,** Eosinophils, and **D,** Lymphocytes in placebo group (open symbols) and calcitriol (closed symbols) pre- and post-prednisolone during the Screening Visits (left) and Treatment Visits (right). **A-D,** analysed by paired t-test.

All patient were monitored for hypercalcaemia and hyperphosphataemia, well-described, potential side effects of calcitriol. Mean corrected serum calcium and phosphate concentrations were not significantly altered by active therapy (table 4.2).

		Screening Visits		Treatment Visits		
		Pre-steroids	**Post-steroids**	**Pre-treatment**	**Post-treatment Pre-steroids**	**Post-treatment Post-steroids**
Calcium (mmol/L)	Placebo	2.30 (2.23-2.37)	2.33 (2.27-2.40)	2.28 (2.16-2.40)	2.26 (2.17-2.34)	2.30 (2.20-2.41)
	Calcitriol	2.22 (2.15-2.28)	2.19 (2.08-2.30)	2.15 (2.01-2.29)	2.23 (2.13-2.34)	2.21 (2.15-2.27)
Phosphate (mmol/L	Placebo	0.95 (0.81-1.08)	1.01 (0.85-1.17)	1.09 (0.94-1.24)	0.97 (0.88-1.05)	1.09 (0.84-1.33)
	Calcitriol	1.07 (0.92-1.21)	1.24 (0.98-1.51)	1.09 (0.94-1.25)	1.02 (0.87-1.17)	1.21 (1.07-1.34)

Table 4.2 Corrected serum calcium and phosphate levels at each study visit
Data shown as mean (+95% confidence intervals).

Adverse events during the study are listed in Table 4.3. There were no serious adverse events, and all events were self-limiting. One patient reported constipation and back ache after 3 days into the treatment phase. She was asked to stop the treatment immediately and when reviewed on day 5 was symptom-free and clinical examination as well as blood tests (including calcium levels) were unremarkable. Upon unblinding at the end of the study it transpired that she was taking the active study drug and her symptoms, albeit mild, may have been attributable to calcitriol.

Adverse Event	***Study Phase***	***Study Drug***	***Related to study drug***
Mild indigestion	Screening	Prednisolone	Yes
Nausea	Screening	Prednisolone	Possible
Increased hunger	Screening	Prednisolone	Yes
Coryzal symptoms	Treatment	No - wash out	No
Diarrhoea	Treatment	Placebo	No
Coryzal symptoms	Treatment	Calcitriol	No
Coryzal symptoms	Treatment	Calcitriol	No
Coryzal symptoms	Treatment	Calcitriol	No
Constipation, Back pain	Treatment	Calcitriol	Possible
Bang to head	Treatment	Calcitriol	No

Table 4.3 Side effects documented throughout the trial
All effects were non severe and resolved spontaneously.

4. 2. 2 Primary Outcome

Based on the primary clinical outcome of comparing lung function at screening and at the end of treatment, we saw no significant difference in % predicted FEV_1 between the two groups (p=0.82): placebo start 59.9 (48.8-71.0) and final 65.9 (55.0-76.8); calcitriol start 62.5 (55.2-69.8) and final 68.1 (55.4-80.7). FEV_1 (%; mean +/- 95%CI) (table 4.4).

		Screening 1	*Screening 2*	*Treatment 2*	*Treatment 3*
FEV_1 (L)	Placebo	1.82 (1.44-2.21)	1.82 (1.79-2.44)	1.83 (1.42-2.23)	2.01 (1.61-2.41)
	Calcitriol	1.98 (1.67-2.29)	1.88 (1.52-2.23)	1.96 (1.58-2.34)	2.20 (1.64-2.75)
FEV_1 (% predicted	Placebo	59.9 (48.8-71.0)	59.9 (48.3-71.3)	60.6 (46.7-74.7)	65.9 (55.0-76.8)
	Calcitriol	62.5 (55.2-69.8)	59.5 (49.8-69.2)	61.6 (53.3-69.9)	68.1 (55.4-80.7)

Table 4.4 Primary endpoint

Change in FEV1 in absolute Litres (L) or % predicted: Data shown as mean (+ 95% confidence intervals).

Nevertheless, a within group comparison of mean absolute and predicted FEV_1 showed a modest but statistically significant improvement in response to prednisolone only in those receiving calcitriol (Figure 4.3 (i) p=0.03 and (ii) p=0.03 respectively).

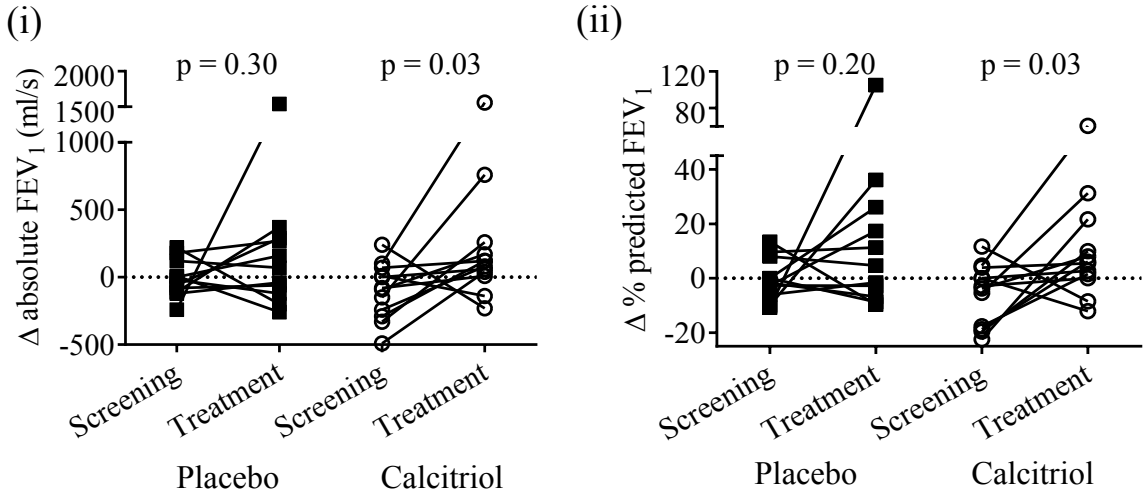


Figure 4.3 Effects of calcitriol versus placebo on the clinical response to oral prednisolone therapy

Comparison of changes in (i) absolute and (ii) % predicted FEV_1 in individual patients in response to 2 weeks of oral prednisolone in the screening and treatment phases of the study in patients randomised to receive calcitriol or placebo. Assessed by Student's paired t-test.

4. 2. 3 Secondary Outcomes:

A. ACQ (Asthma Control Questionnaire)

Patients rated their asthma control and quality of life through the Asthma Control Questionnaire (ACQ) where a lower score predicts better asthma control. The table below shows ACQ score before and after treatment; there was no significant difference between the two groups (p=0.46, table 4.5). However in sub analyses it was observed that those patients showing the greatest increase in predicted FEV_1 (after 2-weeks prednisolone; treatment phase) were those individuals who showed the greatest improvement in ACQ (Figure 4.4).

		pre-steroids	post - steroids
ACQ 7	**Placebo**	2.15 (1.14-3.17)	1.48 (0.97-1.98)
	Calcitriol	2.15 (1.38-2.94)	1.80 (1.04-2.55)

Table 4.5 ACQ 7 scores of patients prior to and after prednisolone treatment during the treatment phase

Data shown as the mean and 95% confidence interval.

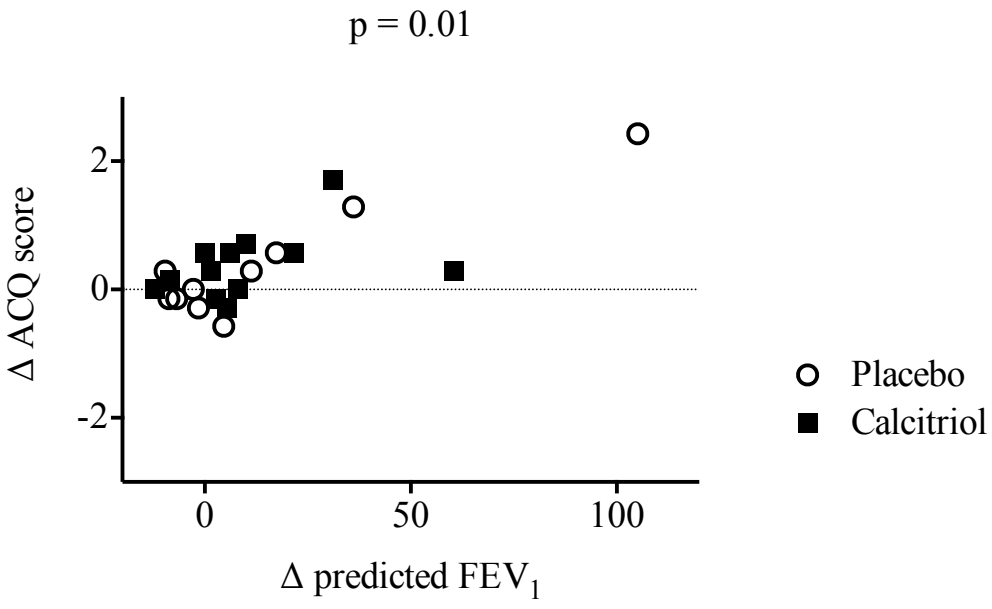


Figure 4. 4 Change in ACQ score post-steroids correlates with change in lung function

Correlation between absolute changes in lung function (% predicted) following oral prednisolone therapy in the treatment phase of the study and corresponding changes in ACQ 7 scores. ACQ >0 is improvement in asthma symptoms; ACQ <0 is a worsening of asthma symptoms. Open Circles – Placebo, Closed Squares – Calcitriol. Analysed by Spearman's rank correlation test, r = -0.518, p = 0.0113.

B. FeNo; Fractional exhaled nitric oxide

Nitric oxide (No) plays a key role in lung biology and has been implicated in the pathophysiology of asthma[267]. Patients with asthma have high levels of No in their exhaled breath. No originates in the airway epithelium as a result of inflammation [268]. Thus, exhaled No may be regarded as an indirect marker of airway inflammation.

Table 4.6 shows fractional exhaled nitric oxide (FeNo) values throughout the trial. We saw no statistical differences between treatment groups whether the patient was on placebo or calcitriol. Unexpectedly patients in the placebo group had much lower baseline values. A reason for the discrepancy is likely the small numbers recruited.

However it was very interesting to see that steroid sensitive patients had lower levels after prednisolone treatment: FeNO showed on average a greater than 40% reduction compared to baseline values. This response is, in accordance to the ATS guidelines, a minimally important decrease of 10ppb and indicative of treatment (steroid) response (see discussion of this chapter).

		Screening 1	***Screening 2***	***Treatment 2***	***Treatment 3***
FeNo	Placebo	19.4 (16.8-22.0)	23.1 (11.8-34.5)	21.9 (13.2-30.5)	19.7 (8.7-30.7)
FeNo	Calcitriol	34.1 (19.5-48.8)	30.7 (17.1-44.3)	34.9 (20.7-49.1)	32.4 (20.1-44.8)
FeNo	SS	33.75 (16.8-50.7)	19.75 (12-27.5)		

Table 4.6: Fractional exhaled nitric oxide throughout the trial

Data presented as mean values and 95% confidence intervals.

4. 2. 4 Serum (25(OH)D) levels

Patients enrolled into the study had their serum 25(OH) levels measured on the first screening visit. All but three patients were vitamin D insufficient with the majority being deficient (figure 4.5). The three highest levels were from patients having recently returned from a holiday in Australia, a keen gardener and an outdoor cyclist.

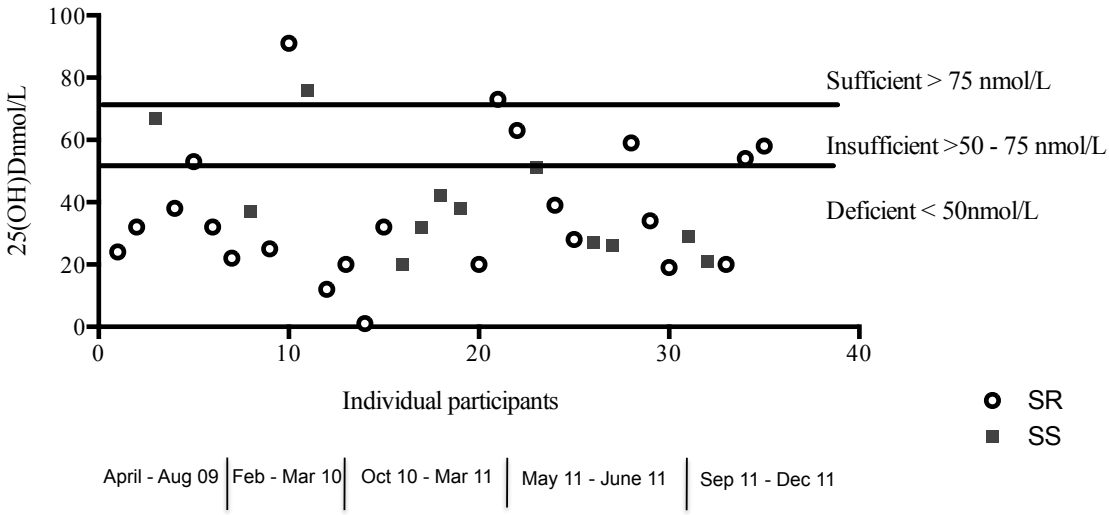


Figure 4.5: Serum 25(OH)D levels at baseline (Screening Visit 1):

Serum 25(OH)D levels of all patients who completed the screening period and were identified as steroid sensitive (SS closed squares) and of patients who successfully completed the treatment period (steroid resistant, SR, open circles) are shown. The figure also shows the month the patients were recruited into the study and suggest seasonal differences in levels. Serum was analyzed by 2-dimensional high performance liquid chromatography system – tandem mass spectrometry (2D LC-MS-MS): All but three patients were insufficient of deficient of 25(OH)D.

At baseline, we saw no difference between patients who were steroid resistant and those who were steroid sensitive (figure 4.6 A). Patients of African or Asian origin had significantly lower levels as compared to Caucasians (figure 4.6 B). 1,25(OH)D3 has a negative feed back effect on 25(OH). Serum levels of 25(OH were measure at the start and at the end of the study. There was no difference seen whether the patient was on placebo or calcitriol (figure 4.6 C).

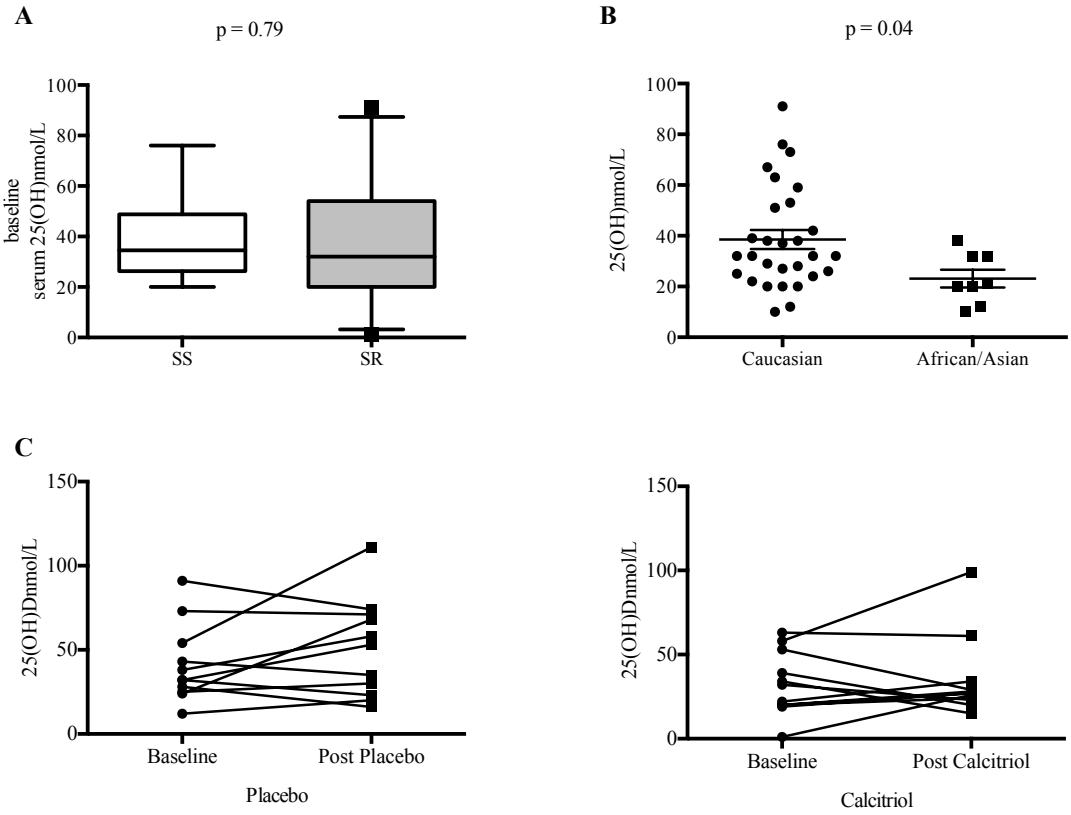


Figure 4.6 Serum 25(OH)D in SS *vs* SR patients, according to ethnic origin and before and after treatment with $1,25(OH)_2D$

A: No significant difference was seen in SS (n =12) versus SR (n=23) patients. Whiskers = 5-95th percentile. Assessed by unpaired t-test, p = 0.792. **B:** the African / Asian population had significantly lower serum 25(OH)nmol/L levels as assessed by unpaired t test: p = 0.04. **C:** Calcitriol had no effect on serum 25(OH) levels, analyzed by paired t-test, Placebo p = 0.204. Calcitriol: p = 0.635

4. 2. 5 Sub-analysis of the Placebo arm

In the placebo cohort four out of the 11 patients showed a >10% improvement in their lung function post-prednisolone in the treatment, as compared to the screening, phase. Retrospective analysis of serum concentrations of 25(OH)D of the 11 patients showed that six patients experienced elevation of their baseline serum 25(OH)D concentrations from start to final visit of study (Figure 4.7 A). It was interesting to see that there was a clear trend towards a positive correlation between serum 25(OH)D concentrations and change in predicted lung function following prednisolone ($r=0.56$, $p=0.08$; figure 4.7 B). Despite the very low numbers, a trend ($p=0.09$) towards a greater improvement in FEV_1 following prednisolone in those with relatively high (>50 nmol/L; n=6) as compared with low (<50 nmol/L; n=5) serum concentrations of 25(OH)D was seen (Figure 4.7 C).

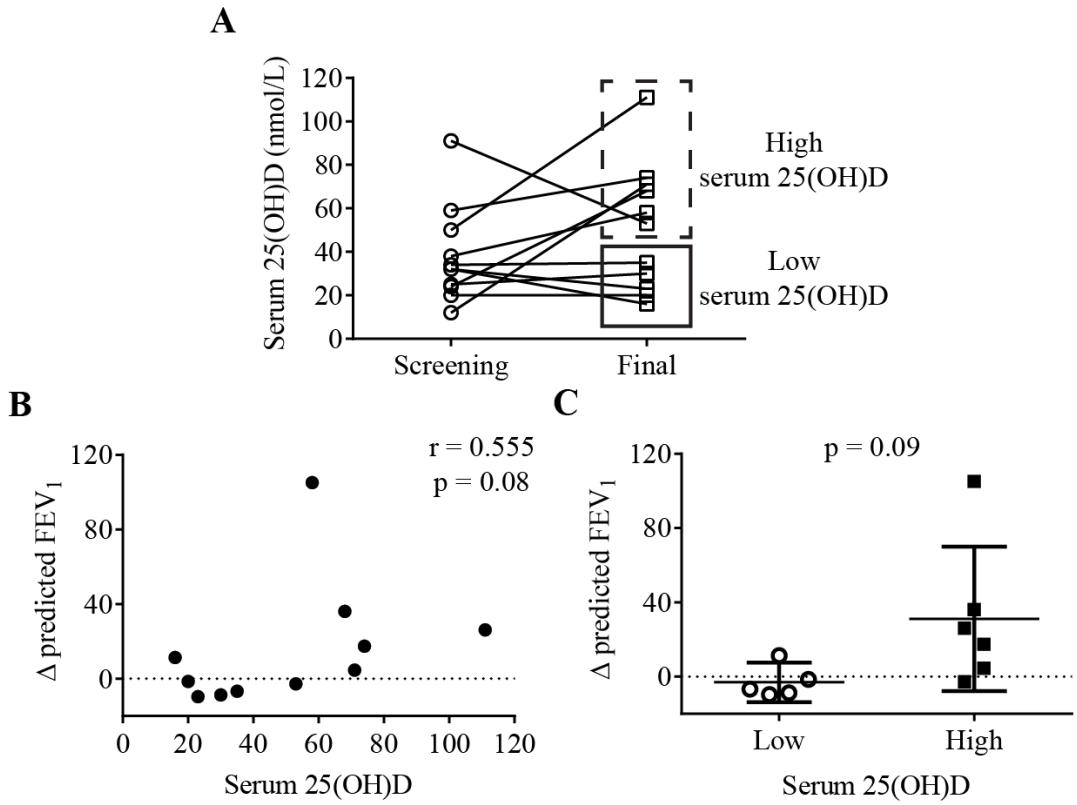


Figure 4.7 Association of final serum 25(OH)D concentration with clinical responsiveness to oral prednisolone in patients randomised to receive placebo

A: Serum 25(OH)D concentrations in placebo treated asthmatics at screening visit 1 as compared to the final visit (treatment visit 3). Boxes delineate "low" (black) and "high" (dotted) serum 25(OH)D concentration groups. **B:** correlation between final serum 25(OH)D concentrations and absolute changes in lung function (predicted %) following oral prednisolone in the treatment phase. **C:** comparison of change in lung function post-glucocorticoids during treatment phase (Treatment 3 compared to Treatment Visit 2) shown as means ±95% CI. B, analysed by Spearman's rank correlation C, analysed by paired t-test.

4. 3 Discussion

The results of this chapter discuss the clinical outcome data of 'The Calcitriol Study'. They are in support of the hypothesis that the clinical responsiveness of asthma patients to steroid therapy is subject to manipulation, at least in the short term. The most notable observation was that a four week course of calcitriol improved the clinical response to steroids in patients classified as clinically steroid resistant (SR) in a within group comparison of changes in FEV_1 (figure 4.3).

Yet, the *primary study outcome* of a between group difference was not significant.

Considering why the primary outcome was not met, a number of potential explanations may contribute to the observations made. We may have failed to detect a difference due to the small number of patients recruited and studied; the treatment period may have been relatively short for what is a chronic disease - many patients have suffered from asthma for many years and could likely have developed airway remodeling and possibly fixed obstructive airways disease to some extent. As a proof of concept and safety study the design of this trial was short term rather than over a lengthy treatment period.

The rationale for the present study was based on the laboratory's earlier immunological data demonstrating that steroid- induced T cell synthesis of the anti-inflammatory cytokine IL-10, impaired in adult SR asthmatics, was restored by calcitriol not only *in vitro*, but also *ex vivo* [163]. This has influenced the clinical approach, and the choice of calcitriol rather than cholecalciferol supplementation. The alternative supplementation with cholecalciferol may be more practical and attractive, offering a relatively cheap, and safe option. Also, long term treatment with calcitriol would almost certainly raise concerns about the risk of drug induced hypercalcaemia. However, the combination of calcitriol and a steroid, known to reduce circulating calcium levels and the expression of Cyp27B1, the catalytic enzyme that converts circulating 25(OH)D into active calcitriol ($1,25(OH)_2D3$), could potential reduce the risk sufficiently. An interesting *in vitro* study

of a murine model of colitis used a combination of the steroid dexamethasone and calcitriol and demonstrated a promotion of Treg cell functions with distinct increases of the regulatory set of IL-10, TGF- and FoxP3 and inhibition of entero-antigen specific T-cell differentiation and as such significantly reduced the clinical severity of this chronic inflammatory disease [269]. It is noteworthy however that the majority of these studies use doses of calcitriol that are 100-200 times greater than therapeutic doses in humans and the immuno-modulatory effects described in *in vitro* studies required local concentrations of calcitriol of 10^{-6} M – 10^{-10} M. Maintaining such concentrations *in vivo* would require supraphysiological dosing prescriptions and there remain uncertainties on how to achieve this, *via* supplementation of vitamin D or calcitriol, and indeed whether it is safe to do this.

The concept however extends others' and our own observations of the potentially promising additive effect of calcitriol and dexamethasone. The antimicrobial activity of calcitriol further makes it an attractive adjunct to steroids in view of the increased risk of infections that come with chronic steroid treatment.

Looking at our secondary endpoints, we did not see a significant difference in the ACQ score whether or not the patient was on placebo or calcitriol (table 4.5). This again may be because of the low numbers recruited or the relatively short treatment period. The fact that all patients were subject to a two-week course of prednisolone, a drug the majority of the patients were rather reluctant to take, might have influenced their quality of life adversely. We issued diary cards and we measured cortical levels as means to optimize and check compliance. Those patients who experienced the strongest improvement in FEV1 had the lowest ACQ score, strengthening the questionnaire's predictability of asthma control (figure 4.4).

There was no difference seen in FeNo values between the placebo and calcitriol group (table 4.6). However, it was interesting to find, that SS patients showed a significant

reduction in FeNo levels after prednisolone treatment whereas SR patients did not. According to the ATS guidelines a decrease of more than 10ppb (for baseline values lower than 50ppb, or > 20% for baseline values higher than 50ppb) is clinically important and indicative of steroid responsiveness [267]. Our data are in agreement with this and support the fact that measurements of FeNo could be an attractive adjunct to help identifying SS or SR patients. An independent, retrospective study in 295 patients with asthma found that a threshold of 42 ppb discriminates between eosinophilic and non-eosinophilic asthma [270]. *Pérez-de-Llano et al* aimed to evaluate the accuracy of baseline FeNO to recognize individuals with difficult-to-treat asthma who have the potential to achieve control with a guideline-based, stepwise strategy: a FeNO cut point greater than or equal to 30 ppb demonstrated a sensitivity of 88% and a specificity of 91% for the identification of steroid responsive individuals with asthma, and a value less than or equal to 30ppb had a negative predictive value for steroid response of 92% [271]. The difference in FeNo levels seen between SS and SR patients is striking and FeNo measurements are an attractive, non-invasive tool to identify steroid sensitivity amongst patients with difficult to control asthma. Randomized controlled trials have so far failed to show improvement in asthma control based on using regular FeNo measurements for adjusting the dose of inhaled corticosteroid therapy [272]. One of the reasons for this might be the fact that there are inconsistencies with regards to the dose–response relationship of the drugs used in relation to the outcomes measured, the fact that treatment adherence and non-adherence is poorly documented, and a lack of agreement on the selection of FeNo cut points/decision points. Whilst disease monitoring via FeNo measurements so far lacks evidence that it improves asthma control, measurements to better identify steroid responsiveness are a valuable option.

As pointed out above, The Calcitriol Study was not designed to assess impact of 25(OH)D status on asthma control, nevertheless it provided interesting data as all patients had serum 25(OH)D levels measured upon entering the study. All but one

participant was vitamin D insufficient (<75nmol/L) at the start of the trial (figure 4.5). This is in keeping with epidemiological studies assessing vitamin D status in the British population and in fact across the globe including countries exposed to sunshine all year round [200, 273,167].

The basis for the current adult vitamin D recommendation has been more than arbitrary and was substantiated on the basis of seven women with severe nutritional osteomalacia, whose bones showed a response when given 100IU/day [274]. Currently the Department of Health recommends '*that people not exposed to much sun should take a daily supplement containing 400IU*' but also advises us not to take more than 1000IU a day [275]. Food and Nutrition Board guidelines specify 2000IU/d as the highest vitamin D intake that healthy adults can consume without risking hypercalcemia, this is the upper limit, or what has been termed as the no adverse effect level (NOAEL). A prolonged intake of 95 μg vitamin D/d is said to be the lowest observed adverse effect level (LOAEL), a dosage that causes hypercalcemia in healthy adults. There are few data from which to establish vitamin D safety and toxicity limits. Some studies that might be considered relevant because they include data on serum calcium, urinary calcium, or both have major shortcomings, eg, ≤6 subjects, follow-up times ≤3 months, nonspecification of the form of vitamin D used (vitamin D_2 or vitamin D_3) or nonverification of the accuracy of the stated dose. 2000IU/d dosage of vitamin D_3 has been shown to effectively increase 25(OH)D levels to high-normal concentrations with serum 25(OH)D within the physiologic range and is therefore considered to be a safe intake.

The proposed benefits of normal – high serum vitamin D levels on the frequency of asthma exacerbations and respiratory infections, the most frequent overall precipitant of asthma exacerbation[276, 277], has prompted numerous initiatives to study the impact of vitamin D supplementation - with mixed outcomes [184, 277-281].

A subanalysis of our data showed that over a third of the patients randomised to placebo had a considerable improvement of their serum 25(OH) levels from the start of the study to the final visit (from insufficient/deficient to sufficient). Although the patient numbers were indeed very low, these patients demonstrated what was a near significant trend for improvement in lung function alongside changes in circulating vitamin D levels (figure 4.7). The increase in serum 25(OH)D levels was surprising and reasons for this may include exposure to sunlight, dietary intake or supplementation (despite the fact that all patients were asked not to take any vitamin supplements during the trial period).

The association between serum 25(OH)D concentrations in our placebo cohort and clinical steroid responsiveness was very interesting to find. Other investigators have found similar positive results. An independent study on asthma exacerbations as a clinical outcome showed that children who were treated with inhaled corticosteroids (ICS) plus cholecalciferol (500IU) had fewer exacerbations than children on ICS only [219]. It is noteworthy that the two groups didn't differ in their serum 25(OH) levels after treatment, which might be due to the relatively low supplementation dose or a possible lack of treatment adherence. Another recent placebo controlled supplementation study (the intervention group received 100 000 IU once, followed by 4000 IU/d for 28 weeks) found that although the primary outcome, time to first asthma treatment failure, was not significantly affected by vitamin D supplementation, patients in the treatment arm were more likely able to reduce their inhaled steroid dose ($p = 0.05$) [218]. The study was underpowered, with a lower than expected event rate in the placebo arm, which might explain the reasons the primary endpoint wasn't met. Furthermore, not all patients in the supplementation groups achieved sufficient serum 25(OH) levels. Although these data support our findings suggesting a possible steroid enhancing role for vitamin D, studies of larger sample size and longer duration are needed. Results from trials that are heterogeneous in baseline vitamin D status of the study populations and in dosing

regimens will inevitably be conflicting and there is a real need for standardized trial designs.

The findings presented here raise important questions about the design of future studies. The decision to study a well-characterized cohort of steroid resistant asthma patients produced significant challenges with recruitment and retention, but on the other hand may have facilitated to achieve significance despite the low numbers of asthmatics studied. I made attempts to increase participant numbers by extending the study to three different sites. We were fortunate to be able to employ a dedicated specialist nurse for patient recruitment after the first year. Future studies will need to expand recruitment to a wider cohort of asthma patients, and to consider study criteria, in particular treatment with prednisolone. Notably, it was reassuring that no significant adverse events were observed.

The results of this trial complement and expand previous studies that showed a positive association between serum vitamin D status and the use of anti-inflammatory medication in asthma[174, 204]. Steroids continue to be the most commonly prescribed drugs to treat asthma, and in the case of systemic steroids to combat exacerbations and to ameliorate disease in those suffering from the most severe form [130]. Maintenance oral steroid treatment has further been shown to be the key factor driving healthcare cost in asthma[282].

Trials with biologicals directed at targets such as IgE, IL-5, IL-4 and IL-13, and non-pharmacological therapies such as bronchial thermoplasty show some promise at least in subgroups of severe asthmatics and allow for a reduction in steroid dose [58, 283-285]. Unlike vitamin D, however, none of these strategies are likely to be universally effective, affordable or convenient.

Given the continuous increase in asthma prevalence across the globe, finding a steroid sparing drug that is a safe, cheap and is easy to administer could have a substantial

socioeconomic impact, not withstanding the benefit for asthma patients, whose lives are blighted by poor asthma control and numerous steroid induced side effects. The data discussed here are encouraging and warrant further investigations in larger, ideally clinically well-defined cohorts, including in paediatric asthma patients where epidemiological data are arguably strongest. Studies looking at supplementation during pregnancy or during early life will shed light on the important questions as to whether vitamin D can manipulate disease development or activity. If beneficial effects of vitamin D are confirmed future trials will need to address the optimal delivery, dosing and safety of vitamin D supplementation.

Chapter 5

Immunological and Clinical Differences between Steroid Resistant and Steroid Sensitive Asthma Patients

5. 1 Introduction

Asthma is characterized by clinical and immunological heterogeneity encompassing different phenotypes that require different treatment plans. Indeed, three decades ago *Schwartz* and colleagues described a subgroup of patients who demonstrated clinical responsiveness to beta-2-agonists but who had persistent airways hyper-reactivity despite intravenous administration of hydrocortisone [286,287]. *In vitro* studies followed showing that proliferation of PBMCs stimulated by PHA was not inhibited by steroids in vitro in patients with treatment refractory asthma[79].

Steroid resistant (SR) asthma has rapidly become an accepted entity and has since driven research into finding more effective treatments for this patient group [80, 287]. Several mechanisms accounting for decreased anti-inflammatory activity of steroids have been proposed and are discussed in chapter one. The exact mechanisms of steroid resistance are still unknown. In order to improve asthma control it is crucial to explore all possibilities driving therapy refractoriness and find ways to enhance treatment responses.

Early investigations of cytokine expression in studies with lymphocytes from SR patients in culture revealed that steroids failed to inhibit IL-2 and IFN-γ secretion in SR patients as compared to SS individuals [80]. Our laboratory has previously shown that SR asthmatics have an impaired expression of anti-inflammatory IL-10 by CD4+ T cells in response to steroids in culture, and in keeping with this our data collected from the earlier parts of 'The Calcitriol Study' showed the frequency of circulating $Foxp3^+$ $CD4^+$ T regulatory cells is significantly lower in SR than in SS asthmatic patients [288].

The active form of vitamin D, calcitriol, increases the production of the immuno-regulatory IL-10 both directly and through enhancing steroid-induced IL-10 responses from CD4+ T cells in vitro, as previously shown by our laboratory and published by *Xystrakis* [163]. Calcitriol has also been found to improve steroid responsiveness via inhibition of LPS-induced cell activation and IL-6 production in SR asthmatics [289].

'The Calcitriol Study' was designed to primarily test the effect of calcitriol on lung function in SS and SR asthmatics (as discussed in chapter four) and to that extent was a proof of concept study of *Xystrakis'* earlier findings [163]. Furthermore, it also offered the opportunity to investigate the cellular mechanisms driving this effect in a larger cohort of patients than studied before [163].

The aim of the experiments presented in this chapter was to identify specific cytokines associated with steroid responsiveness. All patients enrolled into the study had spirometry performed upon entering the trial and this allowed us to investigate the association between cytokine synthesis in culture, lung function and treatment response. We chose to investigate the production of Th2 cytokines IL-5 and IL-13, classically associated with steroid sensitive asthma, and Th1 cytokines IFNγ and TNFα, whose role in asthma to date is less clear but there has been an association with COPD, a chronic inflammatory lung disease mostly described as being steroid resistant [290]. Elevation of serum IL-17A concentrations appears to be both a marker [291] and an independent risk factor for severe asthma [129-132, 292]. Involvement of Th17 cells in severe steroid resistant asthma is proposed from murine and human studies and in addition to IL-17A, IL-22 is commonly produced by Th17 cells. [135, 293]. Studies were further performed looking at the anti-inflammatory IL-10 to complement our earlier observations.

We hypothesized that pro-inflammatory cytokines such as IL-17A, IL-22 and Th1 cytokines are associated with poor lung function and reduced response to steroid (prednisolone) treatment and that anti-inflammatory cytokines such as IL-10 positively correlate with improved treatment response.

We further hypothesized that treatment with calcitriol has the potential to manipulate cytokines towards an immuno-protective milieu in the peripheral blood.

To investigate this, CD8-depleted PBMCs, as used in our original studies [158], were isolated from patients recruited into the clinical trial as described in chapter two

(Materials and Methods). Intracellular cytokine production was assessed by flow cytometry and cytokine production in cell culture supernatants by Cytometric Bead Array (CBA) and following *in vivo* (oral prednisolone) and *in vitro* (dexamethasone in culture) treatment.

Each patient performed lung function tests as described in chapter two and absolute values (L) and % predicted values of FEV_1 were analysed and compared against cytokine synthesis in culture.

5. 2 Results

Patients were assessed for their corticosteroid responsiveness by means of FEV_1 prior to and after a two-week course of prednisolone at a dose of 40mg/1.73m^2 body surface area (BSA). Notably the two patient groups exhibited comparable disease severity, based on impairment of lung function, and were taking comparable doses of inhaled steroids (total dosages based on beclomethasone dipropionate (BDP) equivalence) (for patient's characteristics see table 3.1 in chapter three).

Figure 5.1 demonstrates how clearly different the two patient groups responded to treatment: SR patients showed no improvement in their FEV_1 (mean pre: 1.90L/s, post: 1.75L/s) whereas SS patients had on average an improvement of 14.8% in their FEV_1 (mean pre: 1.65L/s, post: 2.01L/s). Figure 5.1 B shows the change in lung function (FEV_1) in both groups.

Dr Emma Chambers and I performed experiments to determine whether any significant differences existed in the numbers of lymphocytes between SS and SR patients, prior to and after oral prednisolone treatment. Cell surface staining was performed on peripheral blood cells isolated from SS and SR asthmatics before and after treatment. There were no significant differences seen in the numbers of circulating total B cells, total T cells, CD4+ T cells and CD8+ T cells between SS and SR patients prior to oral prednisolone treatment (see Supplementary Figure 1 A). Following oral prednisolone, although there was no significant change in the total numbers of circulating lymphocytes in both the SS and SR asthmatic patients, the total number of circulating B cells significantly increased in both groups without significant change in the numbers of circulating total T cells, CD4+ T cells or CD8+ T cells (Supplementary Figure 1B). This resulted in a significant reduction of the median T:B cell ratios in both groups (Supplementary Figure 1C). Prednisolone therapy was also associated with a trend towards an increase in the number

of circulating Foxp3+ T regulatory cells in the SS, but not the SR asthmatics (Supplementary Figure 1D).

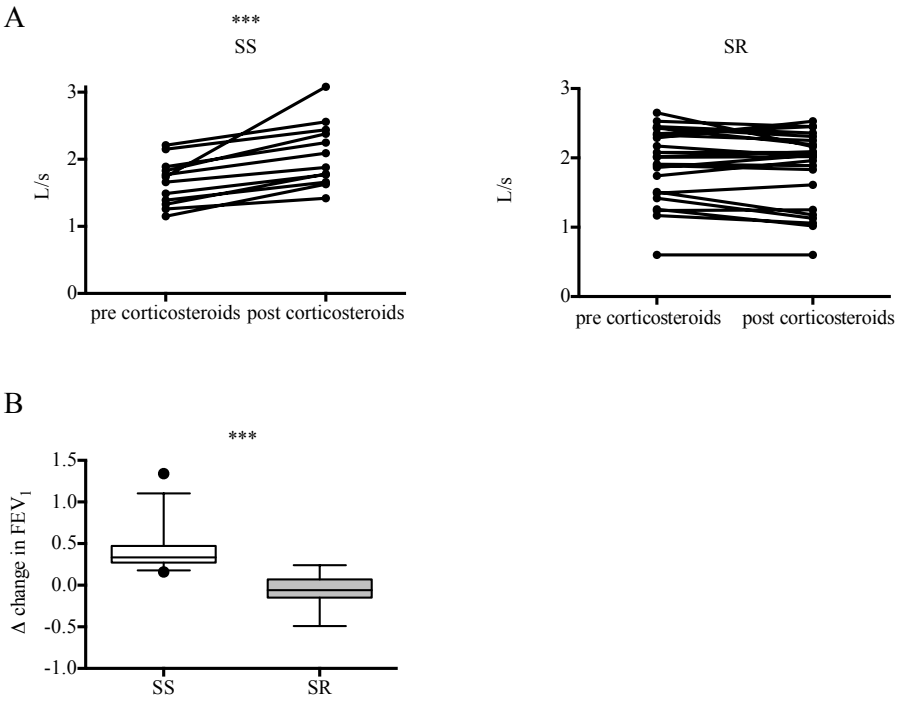


Figure 5.1 Predicted FEV_1 prior to and after a two week course of prednisolone at 40mg/1.73m^2. A: Steroid sensitive (SS) patients had a mean improvement of their FEV_1 of 14.8% ($p = 0.0006$, assessed by t test) whereas steroid resistant (SR) patients had a mean deterioration of their FEV_1 of -1.22%. B: The change in FEV_1 was significantly different between the two patient groups; $p < 0.0001$; Graph with 10-90 percentile, outliers staggered. Assessed by Mann Whitney test.

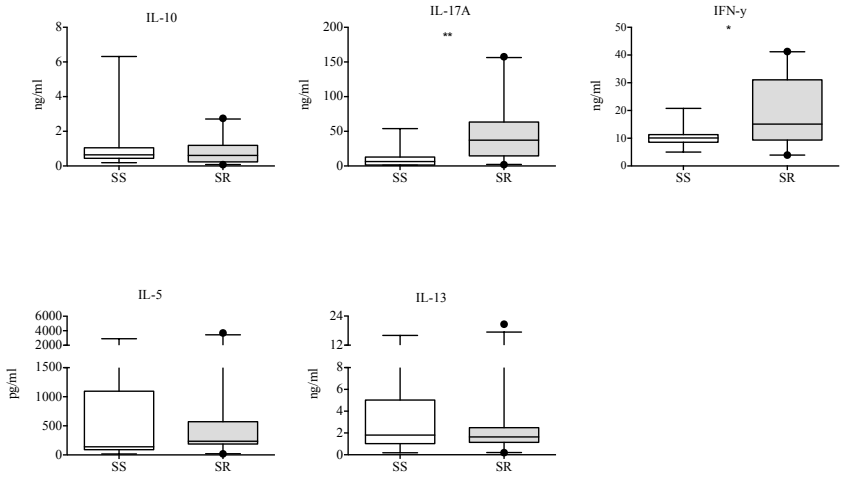
5. 2. 1 *In vitro* Cytokine Profiles at Baseline

5. 2. 1. 1 SR patients have significantly higher protein levels of IFNγ and IL-17A

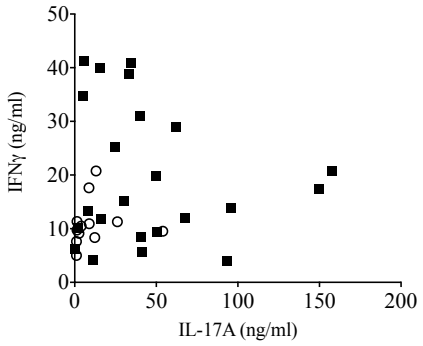
The capacity of PBMCs from SS and SR patients to synthesize the anti-inflammatory cytokine IL-10, the Th17 cytokine IL-17A, the Th1 cytokine IFNγ, and the Th2 cytokines IL-5 and IL-13 in culture was compared at baseline, i.e. peripheral blood was collected from patients at screening visit 1. CD8 T cells were depleted from PBMCs using magnetic beads as described in chapter two and efficacy of depletion was routinely assessed with typical purity 97%. Cells were cultured at a cell density of $1x10^6$/ml in RPMI containing 10% FCS for 7 days with anti-CD3 (1μg/ml) and IL-2 (50IU/ml). At day 7 phorbol 12-myristate 13-acetate (PMA; 5ng/ml) and Ionomycin (500ng/ml) was added for 4 hours, with the final 2-hours containing Monensin (2μM) to allow intracellular cytokine production to be assessed by flow cytometry. In parallel cultures cells were also recounted at day 7 and re-cultured at $1x10^6$/ml in the presence of anti-CD3 and IL-2 for a further 48-hours to account for differential cell loss. Culture supernatants were collected and cytokine production was assessed using Cytometric Bead Array (CBA). Comparisons were made between the two patient groups at baseline (screening visit 1). Culture supernatants from SR asthma patients contained significantly higher quantities of secreted IL-17A and IFNγ (IL-17A: p=0.006; IFNγ p=0.02). There was no significant difference seen in Th2 cytokines or IL-10 between the two groups (Figure 5.2 A). When comparing IL-17A and IFNγ, there was a non significant trend seen for patients with high IFNγ to have lower IL-17A and vice versa (Figure 5.2 B). Having demonstrated elevated culture levels for IFNγ and IL-17A in SR patients, the sensitivity and specificity of baseline IL-17A and IFNγ for glucocorticoid-responsiveness was next analysed. The combination of IL-17A and IFNγ production produced a significant predictability to test for glucocorticoid resistance with ROC

analysis showing p=0.0002, sensitivity of 86.4% and specificity of 75.0%, with a cutoff for IL-17A+IFNg of >27.2 ng/ml.

A



B



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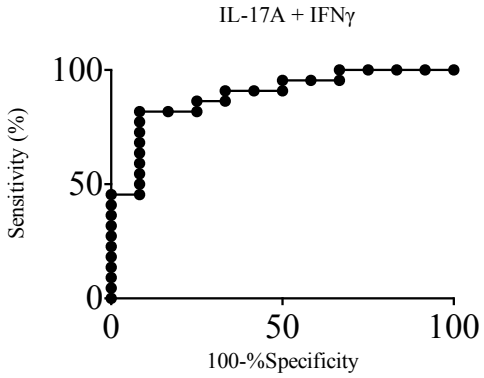
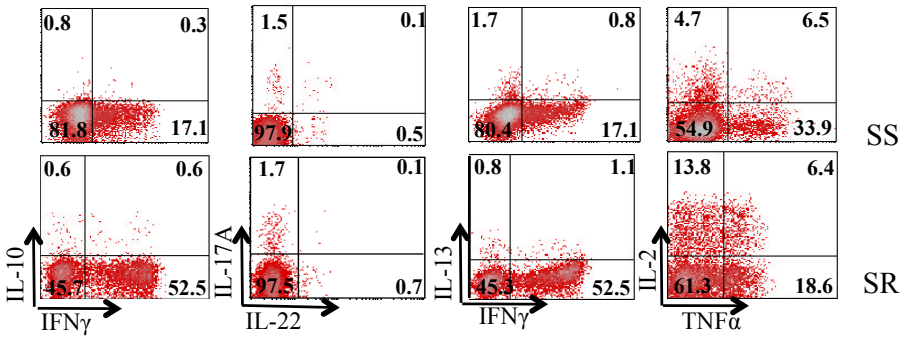


Figure 5.2 A, B and C Cytokine production in cell culture supernatants from SS and SR asthma patients at baseline. PBMCs depleted of CD8+ T cells were stimulated *in vitro* with anti-CD3 (1μg/ml) and IL-2 (50IU/ml) for seven days. At seven days cells were harvested and re-cultured at equal cell densities for 48h with anti-CD3 and IL-2 at which time cell culture supernatants were harvested for cytokine analysis using cytometric bead array (CBA). **A:** SR patients synthesized significantly higher levels of IL-17A and IFNγ. Graph with 10-90 percentile, outliers staggered, assessed by unpaired t test. IL-17A: p = 0.006; IFNγ: p = 0.02. SS: white, n = 12, SR: grey, n= 23. **B:** Comparison of culture levels of IL-17A and IFNγ, SS: open circles, SR: closed squares. **C**: ROC curve of IL-17A and IFNγ: ROC analysis p=0.0002, sensitivity 86.6% and specificity 75.0% with a cutoff for IL-17A+IFNγ of >27.2ng/ml.

Intracellular cytokine expression was assessed in eight SS and 14 SR patients. Once the trial was up and running it became clearer what type of experiments were feasible to perform given the time and staff we had available to care for the patients and therefore the extent of laboratory work performed on visit days. Experiments looking at intracellular protein were started some time into the trial, resulting in lower numbers as compared to the assessment of protein in supernatants. PBMCs depleted of CD8+ T cells were treated as stated above and were stained with fluorescently labeled monoclonal antibodies to the Th1 cytokines IFNγ, IL-2 and TNFα, the Th2 cytokine IL-13, for IL-10 and for the Th17 cytokines IL-17A and for IL-22. In contrast to the secreted cytokines data there was no significant difference seen between the two patient groups for the frequency of cells expressing any of the cytokines as shown in Figure 5.3 A and B. There was a significant variability in values between patients seen as can be seen by in the cumulative data presented in 5.3 B and one example of each a SS and SR patient presented in 5.3 A.

A



B

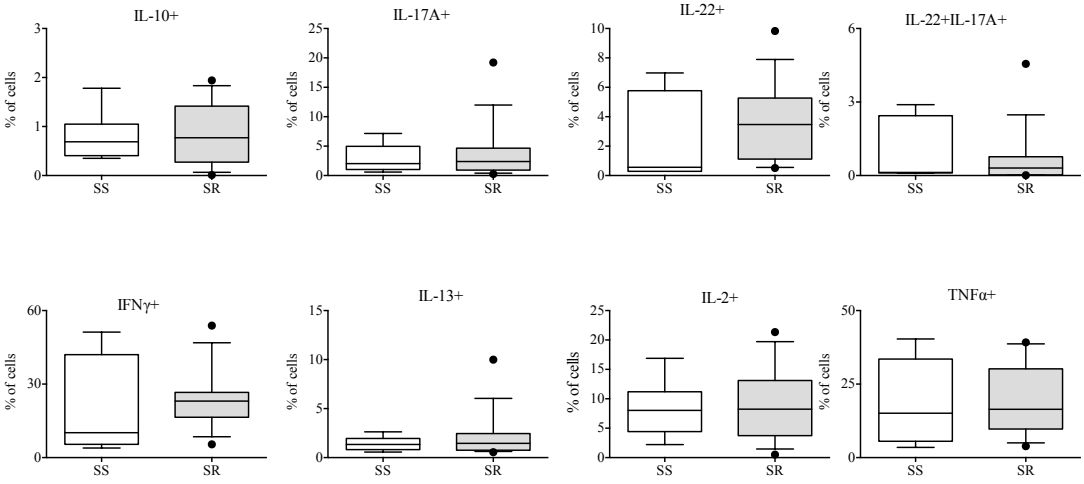


Figure 5.3 Intracellular protein expression of SS and SR patients at baseline

PBMCs depleted of CD8+ T cells were isolated from human peripheral blood and stimulated for seven days with anti CD3 and IL-2 as described in Figure 5.2. At day seven, cells were stimulated with 5ng/ml PMA and 500ng/ml ionomycin for four hours and 2μM Monensin for the final two hours and stained for the expression of intracellular IL-10, IL-17A, IL-22, IFNγ, IL-13, IL-2 and TNFα as fully described in Materials and Methods. **A:** representative dot plots of one SS patient and one SR patient. **B:** cumulative data of % cytokine positive cells within the lymphocyte gate (see Materials and methods Figure 2.3). No significant difference was seen between the two patient groups for any cytokine as assessed by unpaired t test. Graph with 10-90 percentile, outliers staggered. IFNγ: p=0.79, IL-13: p=0.70, IL-10: p=0.84, IL-22: p=0.26, TNFα: p=0.95, IL-2: p=0.97, IL-17A: p=0.31. SS: white, n = 8, SR: grey, n = 14.

5. 2. 2 The Effect of in vivo Treatment on Cytokine Profiles in Culture

5. 2. 2. 1 Cytokine expression in supernatant cultures of SS and SR patients

Following a two-week course of prednisolone (40mg/1.73m^2 BSA) the *in vitro* cytokine levels in culture supernatants of all SS and SR patients who successfully completed the screening visits 1 and 2 were measured, using the same protocol and methodology as used for the initial (visit 1) experiments.

There was no significant difference seen in absolute cytokine levels synthesized in culture between the two patient groups after prednisolone (visit 2; Table 5.1) in contrast to the data from the start of the trial (visit 1; Figure 5.2)) where statistically significantly increased secretion of IL-17A and IFNγ was observed in SR cultures as compared to SS ones. The median values of each cytokine (and range) are shown in Table 5.1.

However, by assessing *the change* from baseline versus post prednisolone treatment, a significant difference in IL-17A levels was seen (Figure 5.4). In comparison to SS patients, SR patients had a greater reduction of IL-17A following oral corticosteroid treatment ($p = 0.003$). A reason for this could be in vivo trafficking of the cells to the disease site, explaining lower levels in the peripheral blood. No significant difference was seen for all other cytokines studied (IFNγ, IL-10, or IL-13) between the two patient groups (Figure 5.4). Table 5.1 shows the median values of change in cytokine production after steroid treatment.

	SS		***SR***	
Cytokine	***Pre Steroids*** *ng/ml (range)*	***Post Steroids*** *ng/ml (range)*	***Pre Steroids*** *ng/ml (range)*	***Post Steroids*** *ng/ml (range)*
IFNγ	10.11 (5.01 – 20.75)	9.81 (2.65 – 27.85)	15.1 (3.92 – 41.27)	12.36 (3.72 – 40.85)
IL-10	0.64 (0.18 – 6.31)	0.46 (0.03 – 2.34)	0.61 (0.07 – 2.75)	0.58 (0.05 – 4.8)
IL-5	0.34 (0.02 – 1.75)	0.13 (0.01 – 2.89)	0.23 (0.01 – 3.69)	0.25 (0.02 – 1.02)
IL-13	1.8 (0.18 – 16.04)	1.64 (0.4 – 16.04)	1.63 (0.19 – 20.64)	1.64 (0.31 – 8.47)
IL-17A	6.37 (1.12 – 53.99)	13.59 (0.79 – 252.9)	37.28 (1.83 – 157.5)	16.6 (0.52 – 91.97)

Table 5.1 The effect of *in vivo* steroid treatment on *in vitro* cytokine secretion following culture in SS and SR patients

A comparison of median values (and range) of cytokines (ng/ml) synthesized in culture before and following a two-week course of systemic corticosteroid treatment (prednisolone). See Figure 5.4 for experimental details.

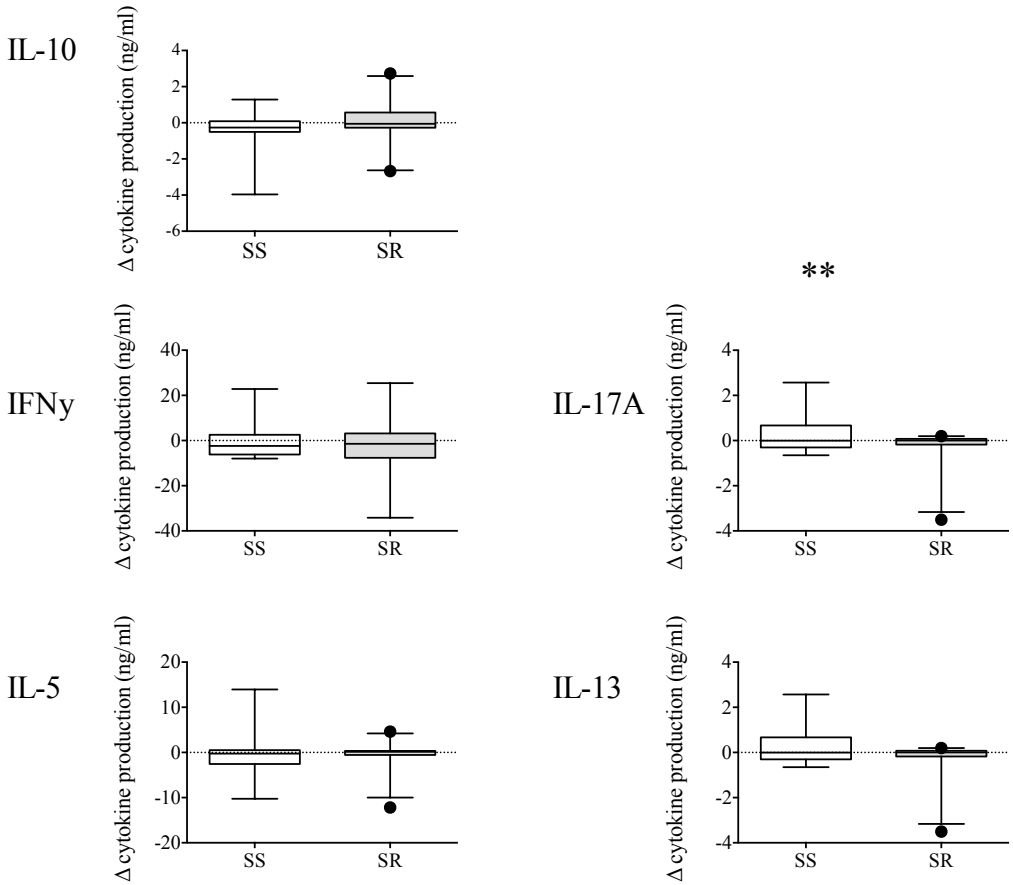


Figure 5.4 A comparison of the effect of *in vivo* steroid treatment on *in vitro* cytokine secretion in cell cultures of SS and SR patients expressed as change (delta) from baseline (visit 1) to after treatment with prednisolone (visit 2).

PBMCs depleted of CD8+ T cells were stimulated *in vitro* with anti-CD3 and IL-2 for seven days as described in Figure 5.2. At seven days cells were harvested and re-cultured at equal cell densities for 48h with anti-CD3 and IL-2 alone, when CBA was used to assess cytokine content of culture supernatants. Results are shown as change in cytokine production (Δ; ng/ml cytokine pre-prednisolone – ng/ml cytokine post prednisolone). In comparison to baseline the reduction in IL-17A levels observed after prednisolone was significantly greater in SR patients (n=23), as compared to SS (n=12) patients ($p = 0.003$). Graph with 10-90 percentile, outliers staggered. Assessed by Mann-Whitney test.

5. 2. 1. 2 Intracellular cytokine expression in SS and SR patients

The intracellular expression of IL10, IL-17A, IFN, IL-13, IL-2 and TNFα following culture was also assessed in SS and SR after prednisolone. This treatment did not result in a significant change of the frequency of cells expressing the above cytokines compared to baseline. Table 5.2 lists the median values (and range) of each cytokine. Figure 5.5 shows the change in % cells expressing cytokines.

	SS		***SR***	
Cytokine	***Pre Steroids*** *% cells (range)*	***Post Steroids*** *% cells (range)*	***Pre Steroids*** *% cells (range)*	***Post Steroids*** *% cells (range)*
IL-10+	0.68 (0.35 – 1.78)	0.62 (0.08 – 3.63)	0.77 (0.01 – 1.93)	0.76 (0.11 – 4.59)
IFNγ+	10.16 (3.93 – 51.21)	13.43 (4.84 – 47.53)	23.06 (5.41 – 53.88)	12.24 (1.82 – 49.79)
IL-17A+	2.03 (0.59 – 7.15)	1.58 (0.13 – 12.16)	2.39 (0.25 – 19.21)	1.35 (0.1 – 16.23)
IL-22+	0.56 (0.29 – 6.98)	0.97 (0.18 – 1.41)	3.47 (0.51 – 9.83)	1.46 (0.38 – 13.22)
IL-17A&IL-22+	0.13 (0.1 – 2.89)	0.21 (0.01 – 1.18)	0.35 (0.01 – 4.56)	0.17 (0.01 – 6.44)
IL-13+	1.34 (0.57 – 2.63)	0.98 (0.39 – 7.71)	1.46 (0.55 – 9.99)	1.34 (0.23 – 2.74)
IL-2+	8.03 (2.22 – 16.89)	8.49 (3.05 – 44.33)	8.24 (0.5 – 21.34)	9.8 (1.38 – 27.41)
TNFα	15.12 (3.47 – 40.37)	16.79 (6.2 – 66.56)	16.42 (3.9 – 39.17)	11.66 (0.88 – 66.3)

Table 5.2 The effect of *in vivo* steroid treatment on intracellular protein expression following culture in SS and SR patients

A comparison of median values (and range) of cytokines (% cytokine positive cells) before and following a two-week course of systemic corticosteroid treatment (prednisolone). See Figure 5.5 for experimental details.

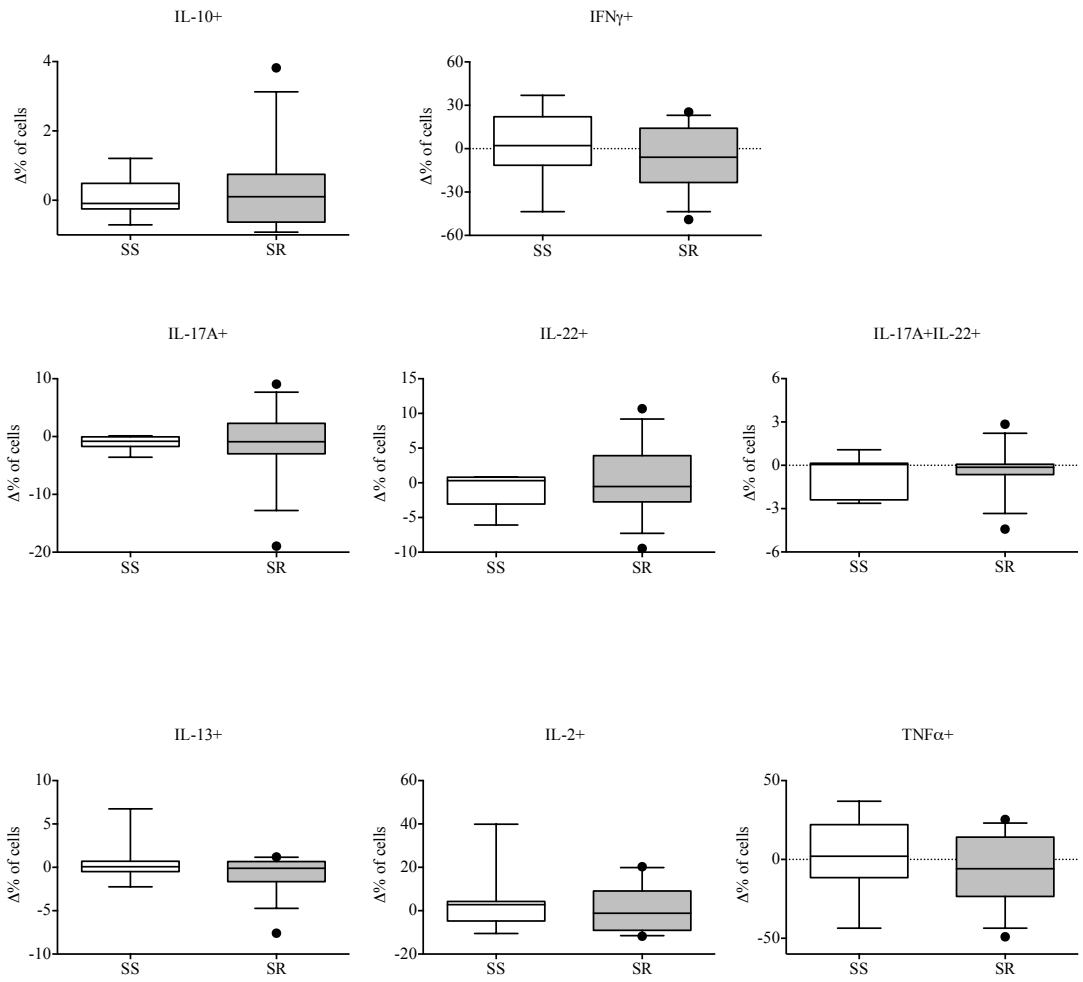


Figure 5.5 Change in intracellular protein expression following culture of PBMC from SS and SR patients following (*in vivo*) treatment with prednisolone

Before and after a two-week course of prednisolone PBMCs depleted of CD8+ T cells were isolated from peripheral blood and stimulated for seven days with anti CD3 and IL-2 as described before. At day seven, cells were stimulated with PMA and ionomycin for four hours, with monensin added for the final 2 hours, and stained for the expression of intracellular IL10, IFNγ, IL-17A, IL-22, IL-17&IL-22, IL-13, IL-2 and TNFα. Results shown as change (Δ) in % lymphocytes expressing cytokines. Graph with 10-90 percentile, outliers staggered. Assessed by Mann-Whitney test. SS: white, n = 8, SR: grey, n = 14.

5. 2. 3 The Effect of *in vitro* Treatment on Cytokine Profiles in Culture

5. 2. 3. 1 Cytokine expression in supernatant cultures of SS and SR patients

The effect of *in vitro* exposure to the synthetic steroid dexamethasone on cytokine synthesis by cultures of PBMC from SS and SR patients was studied next. PBMCs depleted of CD8+ T cells were stimulated *in vitro* with anti-CD3 and IL-2 as described before, in the absence or presence of dexamethasone at the dose indicated (10^{-9}M – 10^{-6}M), and with or without 10ng/ml IL-4 in culture (Figure 5.6).

The SR asthmatics show significantly greater IFNγ and IL-17A production in culture as compared to the SS across the concentration range of dexamethasone. No difference was seen in between patient groups for IL-10 and the Th2 cytokines IL-5 and IL-13.

The production of IFNγ was significantly reduced by dexamethasone in the presence or absence of IL-4; additionally IL-13 was inhibited by dexamethasone in the presence of IL-4. Interestingly the SR asthmatics showed a significant increase in the production of IL-17A in the presence of 10^{-7}M dexamethasone, an effect that was not observed in the SS asthmatics. There was a highly significant interaction between steroid sensitivity status and dexamethasone concentration on IL-10 production in IL-4 treated cultures, with significant dexamethasone-induced IL-10 only in the SS group, in line with our earlier observations (Figure 5.6). Historically the laboratory's experiments demonstrated highest induction of IL-10 and Th2 cytokines by CD4+ T cells following treatment with dexamethasone when IL-4 was added to the culture media and therefore the same protocol of addition of IL-4 was used in the current study. However, earlier experiments revealed that IL-17A was undetectable with IL-4 in culture and IL-17A was therefore only measured in culture media with no added IL-4.

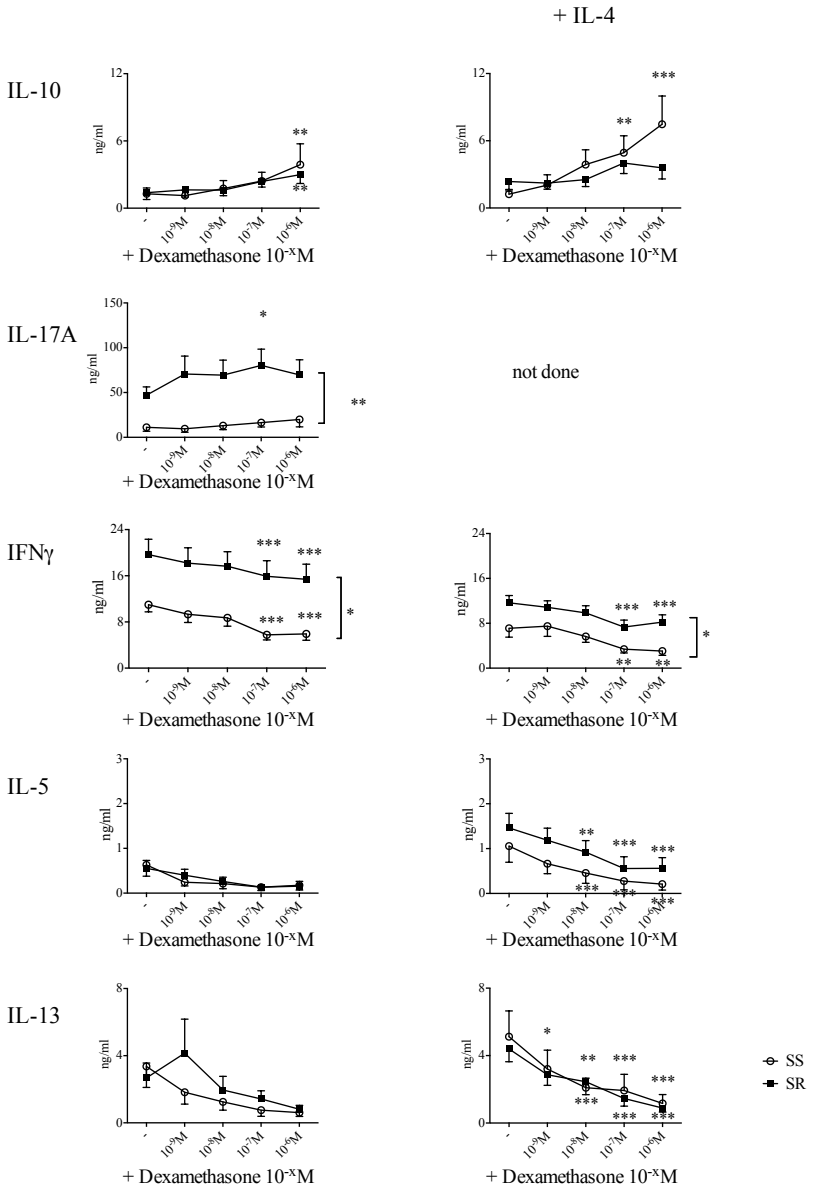


Figure 5.6 The effect of dexamethasone in culture on cytokine production of SS and SR asthma patients

PBMCs were stimulated with anti-CD3 + IL-2 and dexamethasone (10^{-x}M) as described before with (right column) or without (left column) the addition of 10ng/ml IL-4 in culture. Treatment with dexamethasone significantly increased IL-10 synthesis at 10^{-6}M and, whith IL-4 in culture, at 10^{-6}M and 10^{-7}M. IL-17A was significantly increased at 10^{-6}M. IFNγ, IL-5 and IL-13 were significantly inhibited by dexamethasone Data presented as mean with SEM. Assessed by two-way ANOVA with Dunnett's multiple comparison's test of cytokine production in presence of different concentrations of dexamethasone compared to no dexamethasone. *p = <0.05, **p = <0.01, ***p = <0.001 ****p = <0.0001. SS: open circles, SR: closed squares.

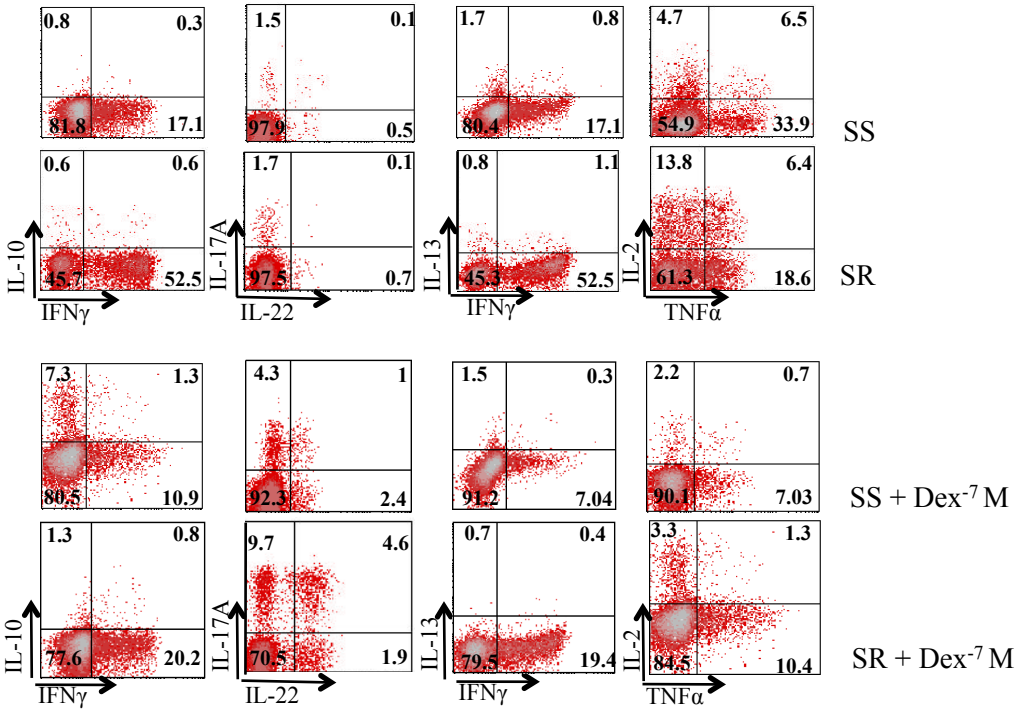
5. 2. 3. 2 Intracellular cytokine expression in SS and SR patients

Experiments were also performed to assess the change in frequency of lymphocytes expressing IL-10, IL-17A, IL-17A+IL-22 double positive cells, IL-22, IL-13, TNFα and IL-2 for both patient groups after treatment of cells in culture with dexamethasone 10^{-7}M. PBMCs were treated as described for Figure 5.3 and flow cytometry was performed. Figure 5.7 A shows a representatives FACS plots of cytokine expression by single representative SS and SR patients; Figure 5.7B shows cumulative data of % cytokine positive cells and Figure 5.7C p values for these data.

There were significant differences seen for cytokines IL-10, IFNγ, IL-17&IL-22, IL-13 and TNFα following in vitro treatment with dexamethasone. Both patient groups showed a significant increase in intracellular expression of IL-10 after treatment. However, only SR patients demonstrated a higher frequency of lymphocytes co-expressing IL-17A+ IL-22+ cells after dexamethasone treatment.

Dexamethasone treatment significantly reduced expression of IL-13 in both groups, which was more significant in SR patients likely due to the greater number of patients studied in this group. Dexamethasone treatment also reduced expression of IFNγ and TNFα, but only in SR patients.

A


0.8
0.3
81.8
17.1
1.5
0.1
97.9
0.5
1.7
0.8
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17.1
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6.5
54.9
33.9
SS
0.6
0.6
45.7
52.5
1.7
0.1
97.5
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0.8
1.1
45.3
52.5
13.8
6.4
61.3
18.6
SR
IL-10
IFNγ
IL-17A
IL-22
IL-13
IFNγ
IL-2
TNFα
7.3
1.3
80.5
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1
92.3
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0.3
91.2
7.04
2.2
0.7
90.1
7.03
SS + Dex-7 M
1.3
0.8
77.6
20.2
9.7
4.6
70.5
1.9
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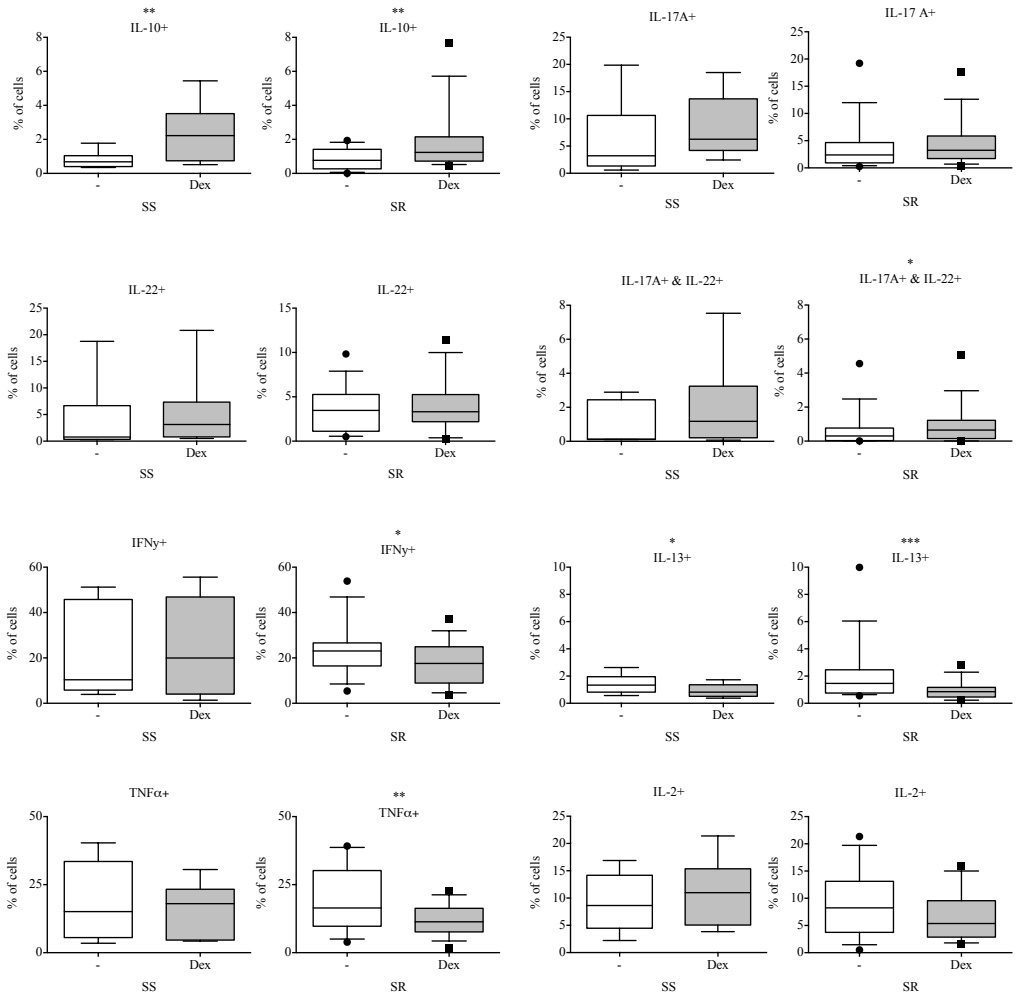
79.5
19.4
3.3
1.3
84.5
10.4
SR + Dex-7 M
IL-10
IFNγ
IL-17A
IL-22
IL-13
IFNγ
IL-2
TNFα

B


**
IL-10+
% of cells
8
6
4
2
0
-
Dex
SS
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IL-10+
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IL-17 A+
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SR
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IL-2+
% of cells
25
20
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0
-
Dex
SR

C

Cytokine:	*SS*	*SR*
IL-10	**p = 0.0078**	**p = 0.0034**
IL-17A	p = 0.109	p = 0.107
IL-22	p = 0.484	p = 0.454
IFNγ	p = 0.742	**p= 0.018**
IL-17A & IL-22	p = 0.375	**p = 0.025**
IL-13	**p = 0.031**	**p = 0.0002**
IL-2	p = 0.461	**p = 0.057**
TNFα.	p = 0.25	**p = 0.002**

Figure 5.7 A, B and C The effect of *in vitro* dexamethasone treatment on intracellular cytokine expression in SS and SR patients

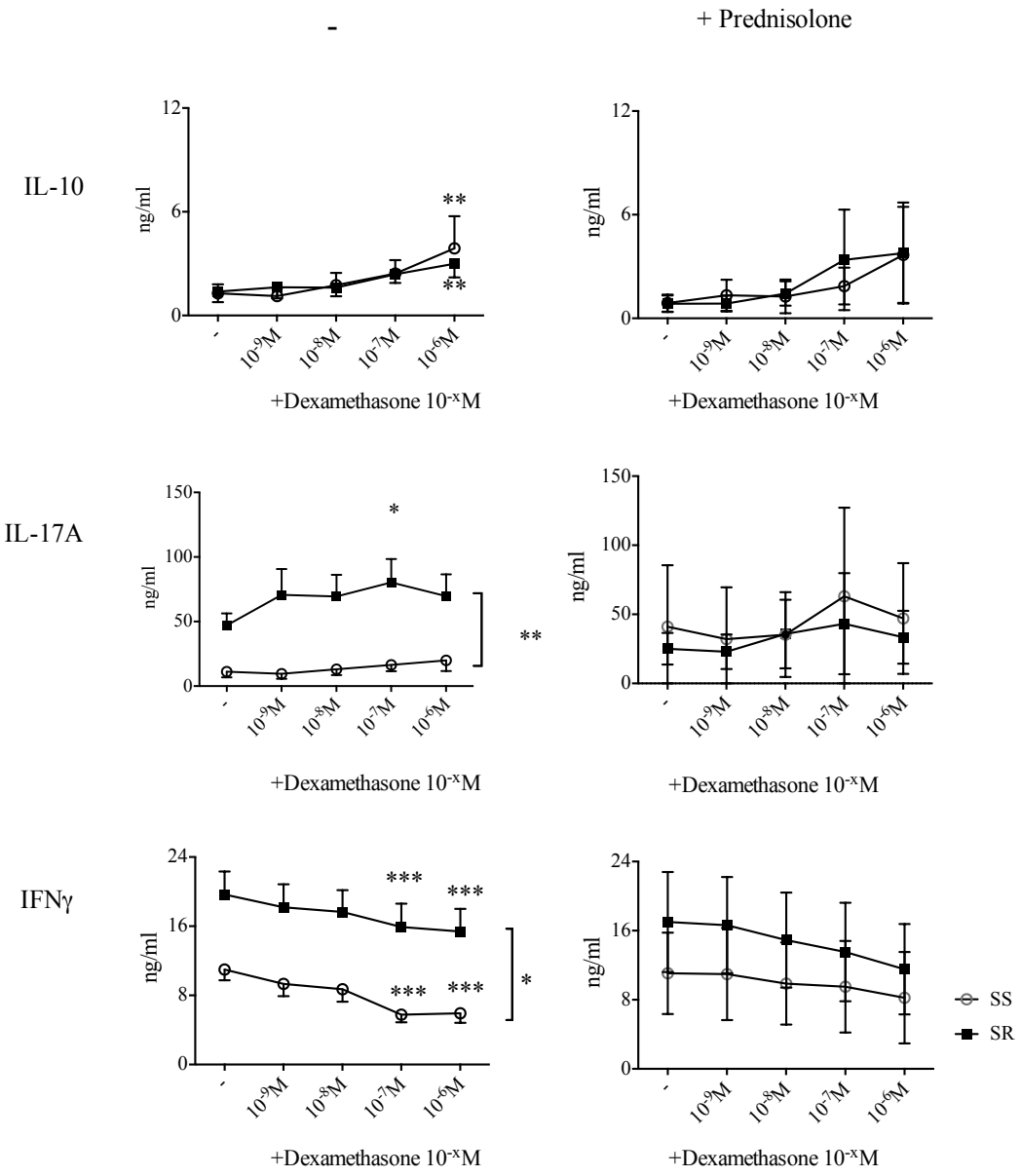
PBMCs depleted of CD8+ T cells were treated as before and stained for the expression of intracellular IL-10, IL-17A, IL-22, IFNγ, IL-13, IL-2 and TNFα. SS: white, n = 8, SR: grey, n = 14. **A:** representative dot plots of one SS patient and one SR patient. **B:** Graph: SS patients had a significant increase in IL-10+ and a significant decrease in IL-13, SR patients had a significant increase in IL-10, IL-17A&IL-22+, and a significant decrease in IFNγ, IL-13 and TNFα. Graph with 10-90 percentile, outliers staggered. **C:** Table showing all p values. Analysed by Wilcoxon t test.

5. 2. 4 The Effect of *in vivo* Steroid Treatment on *in vitro* Response to Dexamethasone

The effect of *in vivo* treatment with steroids (prednisolone 40mg/1.73m^2 BSA) on the *in vitro* response to dexamethasone was investigated next: Following a two-week course of prednisolone study participants attended for a trial visit. PBMCs depleted of CD8+ T cells were isolated as described before and stimulated *in vitro* with anti-CD3 and IL-2 and dexamethasone at the dose indicated (10^{-9}M – 10^{-6}M) with or without 10ng/ml IL-4 in culture. Cytokine levels were assessed by CBA.

Following prednisolone treatment, the previously significantly increased secretion of IL-10 after treatment with 10^{-6}M dexamethasone was not seen any longer although there remained a trend towards an increase in cytokine production.

Cytokine levels of IL-5 and IL-13 in culture were significantly reduced in both patient groups (Figure 5.8).


-
+ Prednisolone
IL-10
IL-17A
IFNγ
ng/ml
+Dexamethasone 10-xM
SS
SR

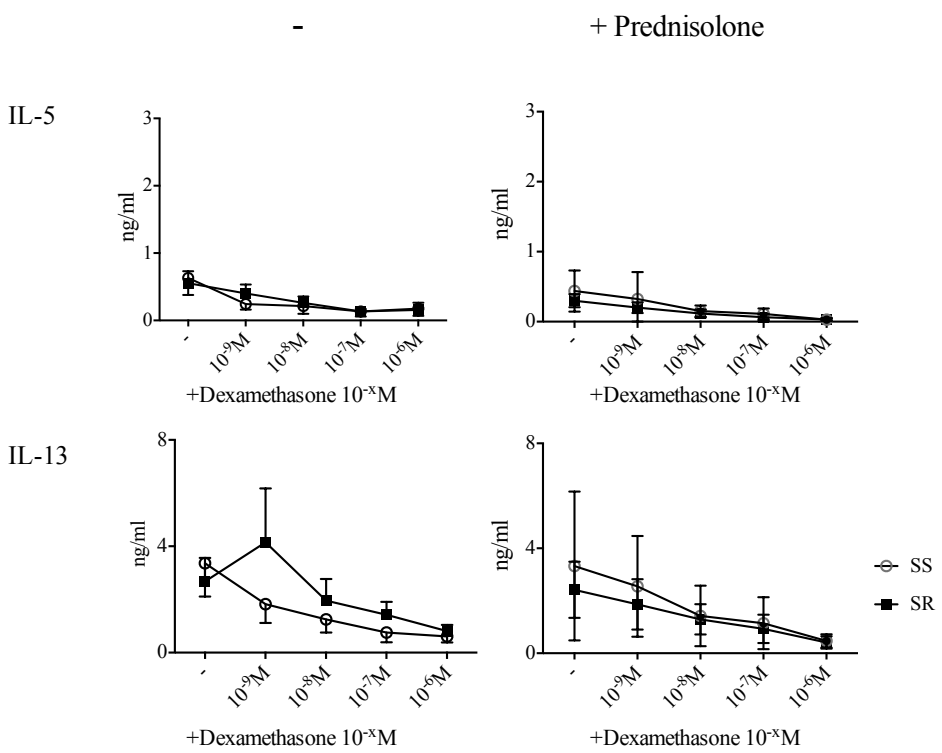


Figure 5.8 The effect of *in vivo* steroids (prednisolone) and *in vitro* dexamethasone in culture on *in vitro* cytokine production: differences between SS and SR

After a two–week course of oral prednisolone (40mg/1.73m^2 BSA) participants attended for blood tests and PBMCs were stimulated with anti-CD3 + IL-2 and dexamethasone (10^{-x}M) as described before. The previously significant difference in IL-17A and in IFNγ between the two patient groups (left column) was abolished following in vivo prednisolone treatment (right column). Data presented as mean with SEM. Assessed by two-way ANOVA with Dunnett's multiple comparison's test of cytokine production in presence of different concentrations of dexamethasone compared to no dexamethasone. *p = <0.05, **p = <0.01, ***p = <0.001; SS: open circles, SR: closed squares.

When IL-4 was added to the culture media SS patients had a significant induction for IL-10 synthesis in culture following stimulation with *in vitro* dexamethasone as compared to SR patients. This effect was not further augmented after *in vivo* treatment (Figure 5.9).

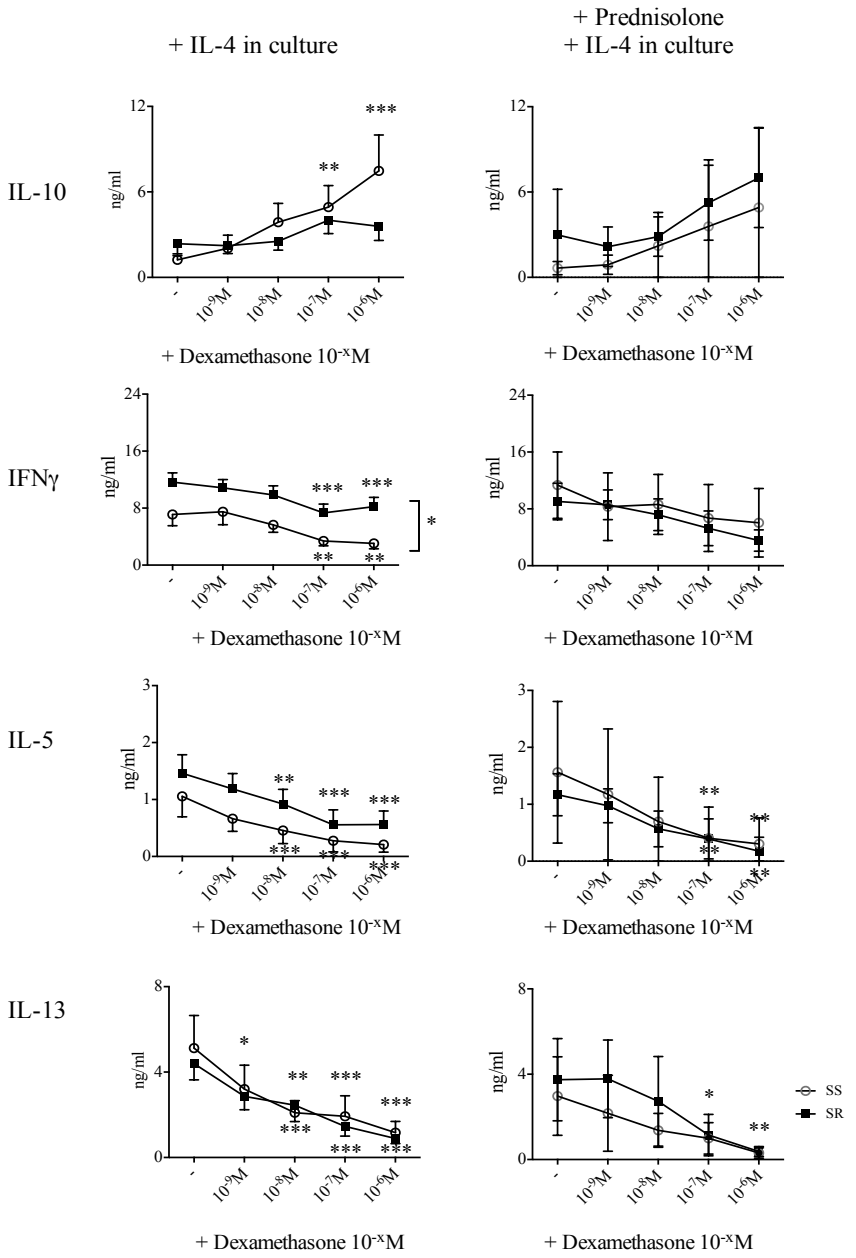


Figure 5.9 The effect of *in vivo* prednisolone treatment and *in vitro* dexamethasone on *in vitro* cytokine production in culture: results with IL-4 in culture media

PBMCs were stimulated with anti-CD3 + IL-2 and dexamethasone (10^{-x}M) with the addition of IL-4 in culture as described before. The difference in IL-10 synthesis in culture after in vitro treatment with 10^{6} M dexamethasone was no longer significant between SS and SR patients. Data presented as mean with SEM. Assessed by two-way ANOVA with Dunnett's multiple comparison's test of cytokine production in presence of different concentrations of dexamethasone compared to no dexamethasone. *p = <0.05, **p = <0.01, ***p = <0.001. Left column - before prednisolone treatment, right column - after prednisolone treatment. SS; open circles, SR; closed squares.

5. 2. 5 The Correlation between Lung Function and Cytokine Levels

SS and SR patients who fulfilled the study criteria performed spirometry at Screening Visit 1 using a PC based spirometer and software (WinspiroPRO) and as described in chapter two. Also, PBMCs from each patient were isolated and stimulated with anti-CD3 and IL-2 with or without IL-4 as described in Figure 5.2. Cytokine synthesis in culture supernatants was analysed by CBA.

There was no significant correlation seen when data of all patients (SS and SR) was analysed. SR patients alone showed a significant positive correlation between IL-10 levels in culture supernatants and FEV_1 (L) ($p = 0.037$, $r = 0.448$) at Screening Visit 1. There was a trend towards significance for SR patients with high levels of IFNγ being associated with worse lung function ($p = 0.072$) (Figure 5.10).

When IL-4 was added to the culture media SR patients showed a significantly positive correlation between IL-5 and FEV_1 values. Table 5.3 shows r and p values of the correlation between cytokine levels in culture and FEV_1 in absolute values (L) with and without IL-4 in culture media.

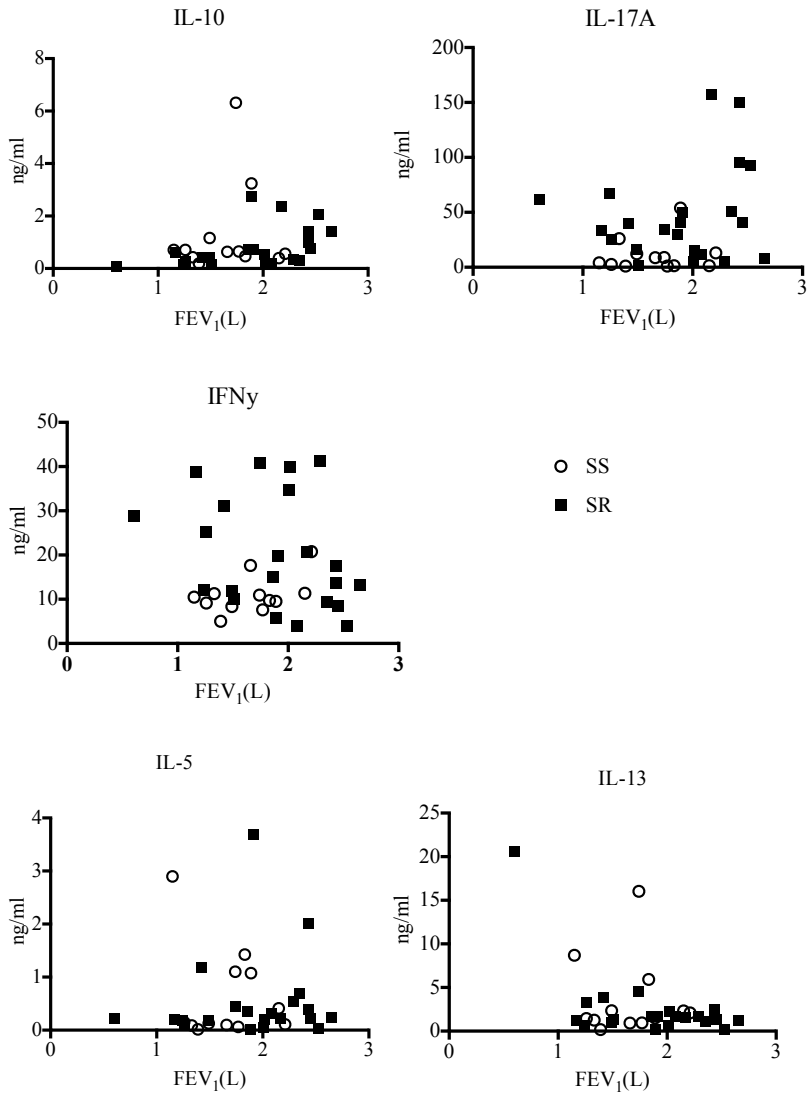


Figure 5.10 The correlation between lung function (FEV_1 in L) at baseline (Screening Visit 1) and cytokine synthesis in culture at Screening Visit 1 (graphs without IL-4 in culture)

PBMCs were stimulated with anti-CD3 and IL-2 as described before; cytokine levels in culture were measured by CBA. Lung function was measured at Screening Visit 1 using a PC based spirometer and software (WinspiroPRO). The graph shows FEV_1 in L (absolute values) and cytokines measured in supernatants. Correlation assessed by Spearman's rank statistical test. SS open circles, SR closed squares.

	-			with IL-4		
Cytokine	**SS**	**SR**	**All Severe**	**SS**	**SR**	**All Severe**
IL-10	r=0.141 p=0.661	**r=0.448** **p=0.037**	r=0.194 p=0.273	r=0.127 p=0.695	r=0.237 p=0.289	r=0.249 p=0.155
IL-17A	r=0.136 p=0.674	r=0.122 p=0.331	r=0.281 p=0.107	not done	not done	not done
IFNγ	r=0.350 p=0.266	r=-0.382 p=0.072	r=-0.117 p=0.502	r=-0.203 p=0.528	r=-0.107 p=0.635	r=-0.192 p=0.277
IL-5	r=0.063 p=0.852	r=0.215 p=0.336	r=0.175 p=0.323	**r**=0.546 p=0.088	**r=0.614** **p=0.002**	**r=0.549** **p<0.001**
IL-13	r=-0.119 p=0.716	r=-0.183 p=0.404	r=-0.107 p=0.541	r=0.188 p=0.607	r=-0.169 p=0.476	r=-0.040 p=0.838

Table 5.3 The correlation between cytokine synthesis in culture and FEV_1 (L) at Screening Visit 1 with or without IL-4 in culture

PBMCs were isolated and stimulated as described in Figure 5.2 and cytokine levels in culture were measured by CBA. Spirometry was performed using a PC based spirometer and software (WinspiroPRO). A significant correlation was seen between the production of IL-10 and absolute values for FEV_1 (L) in SR patients. When IL-4 was added to the culture media all patients together and SR patients as a group had a significant correlation between lung function (FEV1) and IL-5 in culture media. Assessed by Spearman's rank correlation statistical test.

Following two weeks of oral prednisolone participants attended for Screening Visit 2. PBMCs were cultured and stimulated as described before. Patients repeated spirometry using the same protocol as for Screening Visit 1. Cytokines in culture were assessed by CBA. Change in FEV_1 (L) was calculated as FEV_1 (L) Screening visit 2– FEV_1 (L) Screening Visit 1. Patients who showed the greatest improvement in lung function had significantly lower levels of IL-17A and higher levels of IL-10 in culture supernatants assessed using PBMCs from study Screening Visit 1 (Figure 5.11 and Table 5.4). SS patients had a positive correlation between IL-13 in culture and change in FEV_1 (L). When assessing for cytokines IL-17A and IFNγ, which were highest at baseline, a significant negative correlation was seen.

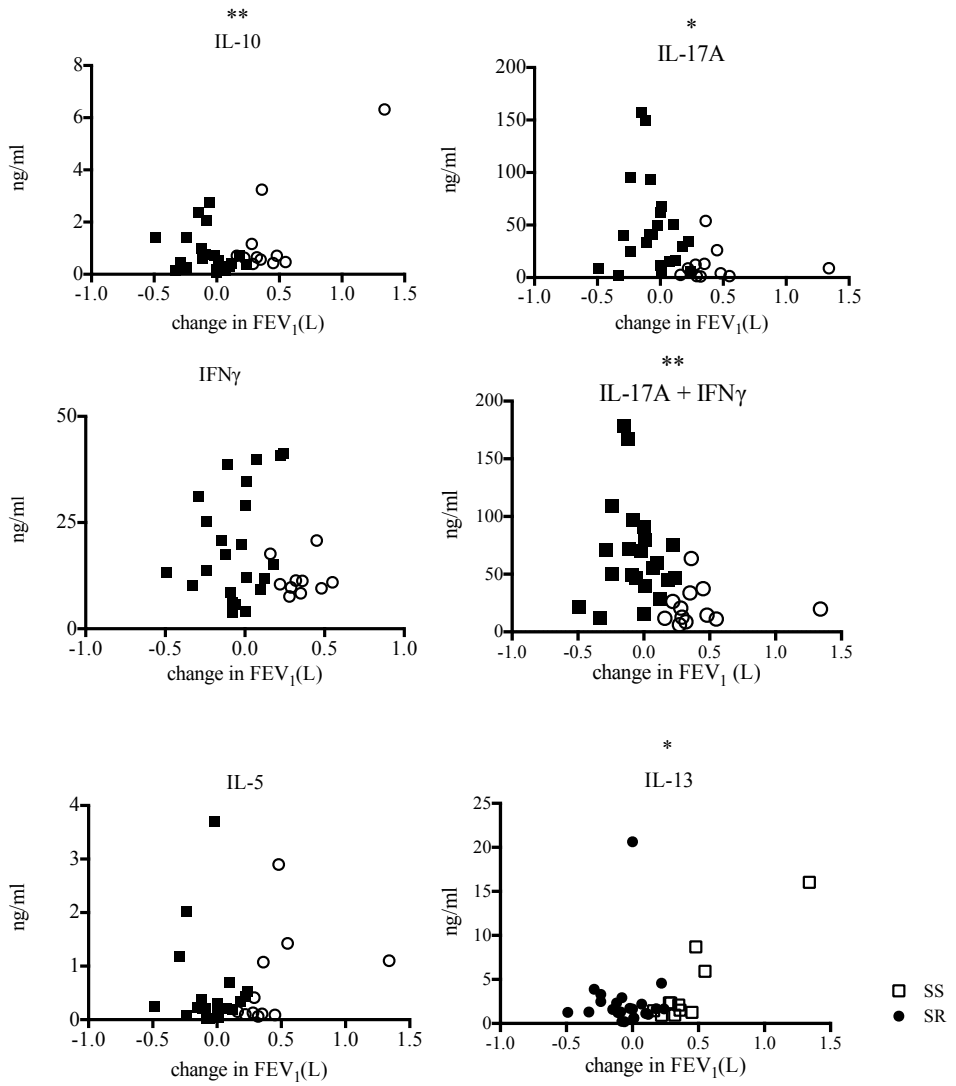


Figure 5.11 Correlation between change in lung function (FEV_1 in (L)) after prednisolone treatment and cytokine synthesis in culture at Screening Visit 1 (graphs without IL-4 in culture)

PBMCs were isolated and stimulated as before and cytokine levels in culture were measured by CBA (see Figure 5.2). Change in lung function was calculated as FEV_1 (L) Screening Visit 2 – FEV_1 (L) Screening Visit 1. Assessed by Spearman's rank correlation statistical test. * p = <0.05, ** p = < 0.01

	-			with IL-4		
Cytokine	**SS**	**SR**	**All Severe**	**SS**	**SR**	**All Severe**
IL-10	**r=0.830** **p=0.002**	r=-0.259 p=0.244	**r=0.488** **p=0.004**	**r=0.777** **p=0.005**	r=-0.151 p=0.504	r=-0.026 p=0.887
IL-17A	r=-0.132 p=0.558	r=-0.03 p=0.937	**r=-0.354** **p=0.044**	not done	not done	not done
IFNγ	r=0.118 p=0.735	r=0.196 p=0.369	r=-0.136 p=0.445	r=0.082 p=0.818	r=-0.102 p=0.651	r=-0.208 p=0.246
IL-5	r=0.527 p=0.100	r=-0.025 p=0.911	r=0.071 p=0.694	r=-0.224 p=0.537	r=-0.021 p=0.924	r=-0.136 p=0.460
IL-13	**r=0.618** **p=0.048**	r=-0.100 p=0.658	r=0.158 p=0.370	r=0.500 p=0.178	r=-0.157 p=0.508	r=0.023 p=0.905
IL-17A + IFNγ	r=0.003 p=0.991	r=-0.052 p=0.816	**r=-0.511** **p=0.002**			

Table 5.4 The correlation between cytokine synthesis in culture at screening visit 1 and change in lung function (FEV_1 (L)) following prednisolone 40mg/1.73m^2; FEV_1 (L) Screening visit 2 – FEV_1 (L) Screening Visit 1

Significant correlations were seen for the synthesis of IL-13 and IL-10 in culture in SS patients and IL-17A in all patients combined. When IL-4 was added to the culture media, IL-10 culture levels correlated significantly positive in SS patients with change in lung function. FEV_1 (L). Assessed by Spearman's rank correlation statistical test.

5. 2. 6 The correlation between serum 25(OH)D levels and cytokine levels

It has previously been reported that low levels of serum 25(OH)D is not only associated with poor asthma control (see chapter one) but in particular with reduced lung function [203, 294]. We have not seen a significant association between lung function and serum 25(OH)D levels in our cohort. This might be due to the fact that we have small patient numbers, a selected patient group of severe asthma with low FEV_1 and the fact that the majority of our patients were deficient of serum 25(OH)D.

Having demonstrated a significant correlation between cytokine levels in culture and change in lung function it was next investigated whether there was a correlation between serum 25(OH)D levels and patients' cytokine levels in culture supernatants at Screening Visit 1.

We hypothesised that cytokine levels are amenable to manipulation by vitamin D status in that higher levels of 25(OH)D correlate with anti-inflammatory cytokines such as IL-10.

SS and SR patients were assessed for their circulating serum 25(OH)D levels by isotope-dilution liquid chromatography–tandem mass spectrometry. CD8 depleted PBMCs were isolated and cultured with anti-CD3 and IL-2 with or without IL-4 as described before. Cytokines in culture supernatants were measured by CBA. Figure 5.12 demonstrates a positive correlation between IL-13 and 25(OH)D. Table 5.5 shows r and p values of the correlation between cytokines in culture with and without IL-4 in the culture media and serum 25(OH)D levels. Measured at baseline (Screening visit 1).

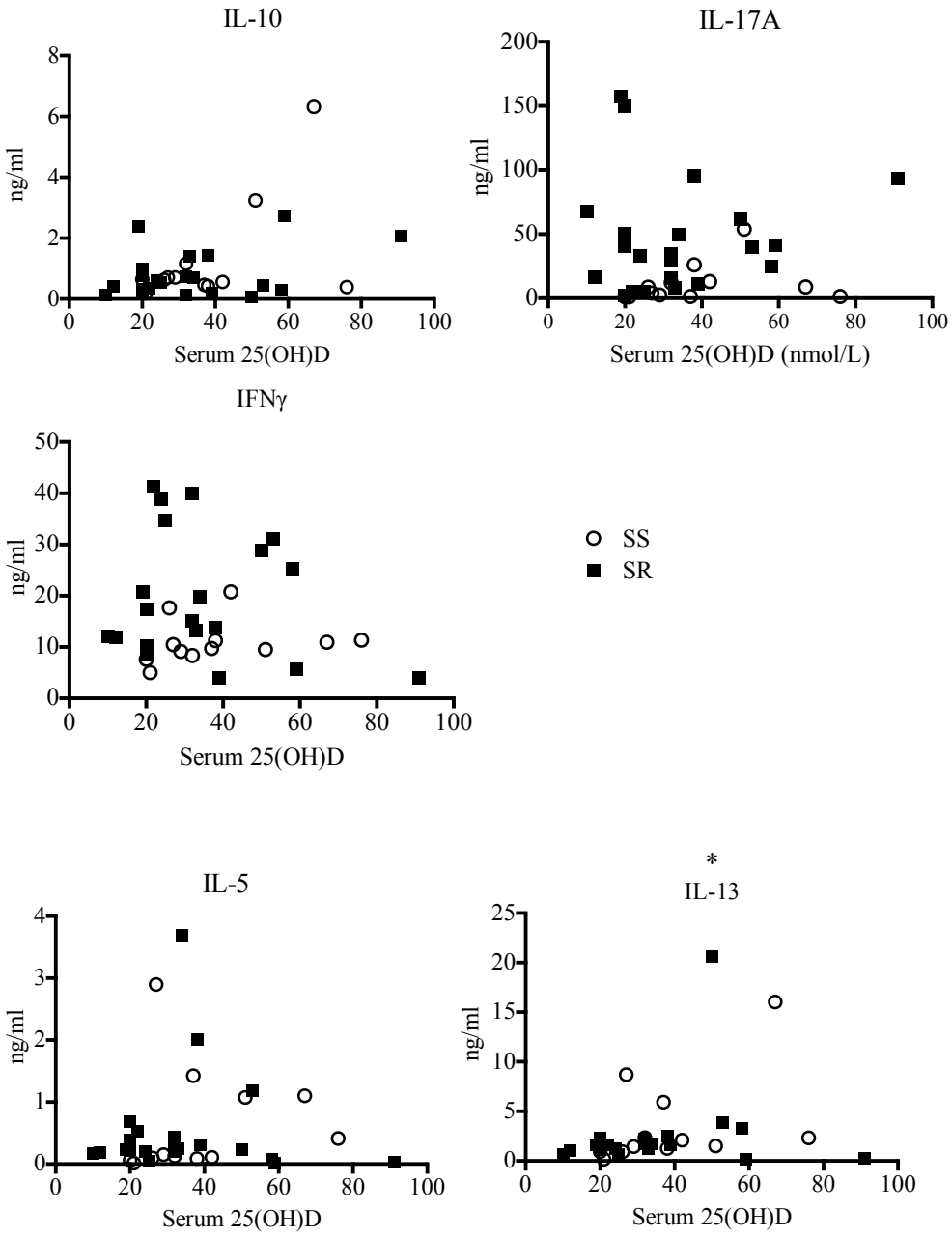


Figure 5.12 The correlation between serum 25(OH)D levels and cytokine levels

PBMCs were cultured with anti-CD3 and IL-2 as described before. The graphs shows cytokine from cultures without IL-4. Serum 25(OH)D was measured at screening visit 1 by isotope-dilution liquid chromatography–tandem mass spectrometry. There was a positive correlation seen between IL-13 and serum 25(OH) ($p = 0.024$). Assessed by Spearman's rank correlation statistical test.

	-			with IL-4		
Cytokine	**SS**	**SR**	**All Severe**	**SS**	**SR**	**All Severe**
IFNγ	r=0.532 p=0.079	r=-0.155 p=0.488	r=-0.120 p=0.500	r=0.183 p=0.569	r=0.205 p=0.373	r=0.13 p=0.46
IL-5	r=0.490 p=0.101	r=0.050 p=0.840	r=0.080 p=0.657	r=0.581 p=0.065	r=0.119 p=0.606	r=0.299 p=0.091
IL-13	r=0.559 p=0.063	r=0.254 p=0.266	**r=0.393** **p=0.024**	**r=0.697** **p=0.031**	**r=0.594** **p=0.005**	**r=0.55** **p=0.002**
IL-10	r=0.133 p=0.683	r=0.156 p=0.498	r=0.22 p=0.22	r=0.357 p=0.256	r=-0.136 p=0.558	r=-0.165 p=0.351
IL-17A	r=0.497 p=0.104	r=0.007 p=0.972	r=0.046 p=0.796	not done	not done	not done

Table 5.5 The correlation between cytokine production in culture and serum 25(OH)D levels assessed at Screening Visit 1

All (SS and SR) patients had a significant positive correlation between IL-13 in culture and 25(OH)D serum levels at Screening Visit 1. When IL-4 was added to the culture media significant correlations were seen in steroid sensnitive (SS) patients for IL-5 and IL-13, for steroid resistant (SR) patient for IL-13 and for all patients (SS and SR) for IL-13. Assessed by Spearman's rank correlation statistical test.

5. 2. 7 The Effect of Calcitriol on Steroid Resistant Asthma

The second part of this chapter discusses data from the treatment period of 'The Calcitriol Study'. Steroid resistant asthma is currently inadequately managed let alone understood. Based on the important findings of *Xystrakis et al* on the effect of calcitriol for the induction of IL-10 in culture and the clinical improvement in lung function associated with this, the aim was to investigate the effect of a four-week course of calcitriol (0.25µg twice a day) on in-vitro cytokine production.

Patients who successfully completed the screening period of a two week course of prednisolone and who failed to improve their lung function (FEV_1) by > than 10% were characterized as steroid resistant and were double-blinded and randomised to receive either calcitriol or a matching placebo. Eleven patients received calcitriol and 12 patients received placebo. After two weeks all patients started a two week course of prednisolone, at the same dose they had received during the screening period and which they took alongside the calcitriol / placebo until the end of the treatment period. Blood tests were performed at the beginning of the calcitriol / placebo treatment and before and after corticosteroid treatment. PBMCs depleted of CD8 cells were isolated and stimulated with anti-CD3 and IL-2 as described before. Supernatants were assessed for cytokine production by CBA as described before.

5. 2. 8 The Effect of Calcitriol on *in vitro* Cytokine Production

At the Final Visit, after a four-week treatment course with calcitriol / placebo and two weeks of prednisolone, there was no statistical difference seen on in-vitro cytokine production in culture as compared to the start of the study (Screening Visit 1) between the two treatment groups (Figure 5.13). However, there was a trend towards decreased IL-17A levels in the patient group receiving calcitriol ($p = 0.34$).

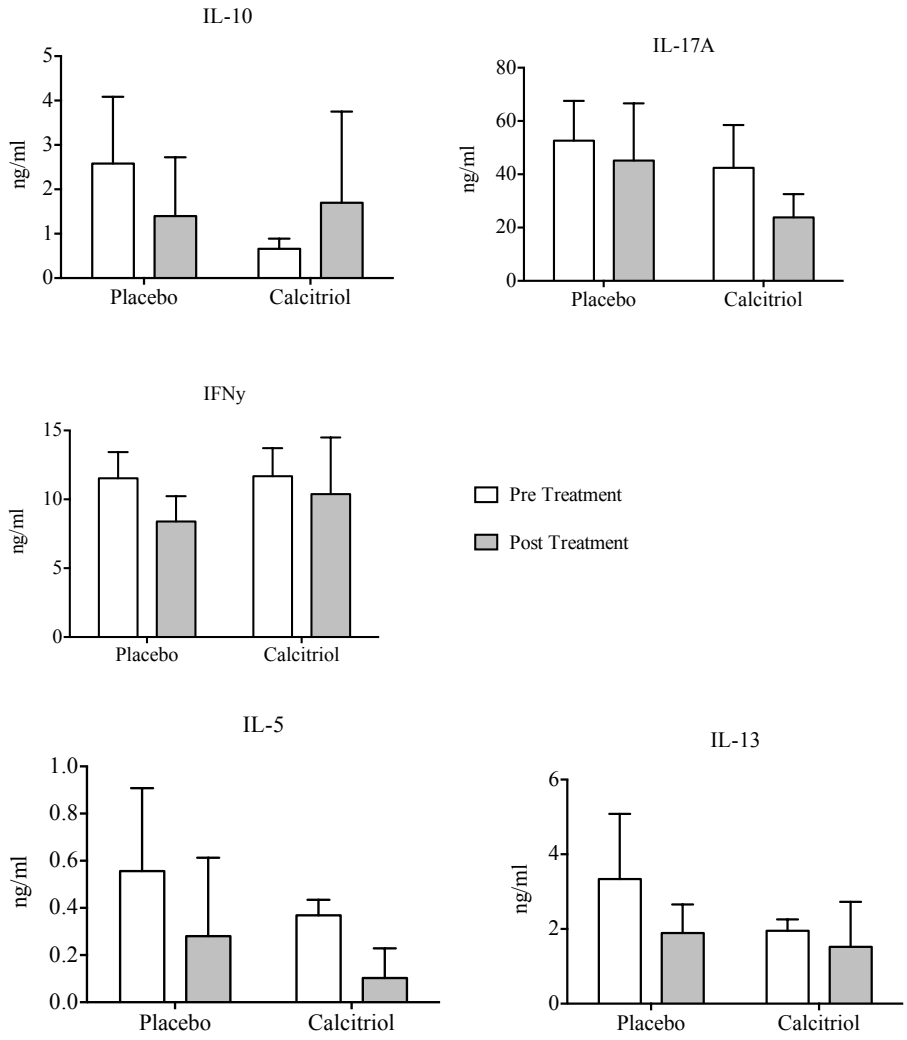


Figure 5.13 Cytokine production in culture by SR patients at baseline (Screening Visit 1) and after treatment with placebo/calcitriol and prednisolone (Final Visit)

PBMCs were isolated and treated as before. Cytokine production in cell culture supernatants was assessed by CBA. There was no statistical significance seen. Data presented as mean + SEM. Assessed by multiple t-test corrected for multiple comparisons using the Holm-Sidak method.

It was next investigated whether calcitriol treatment on its own without steroid treatment had an effect on cytokine synthesis in cultures. Figure 5.14 shows cell culture cytokine levels after two weeks of calcitriol / placebo but before prednisolone. There was no significant difference seen. There was a trend for reduced IL-17A levels after treatment ($p = 0.133$).

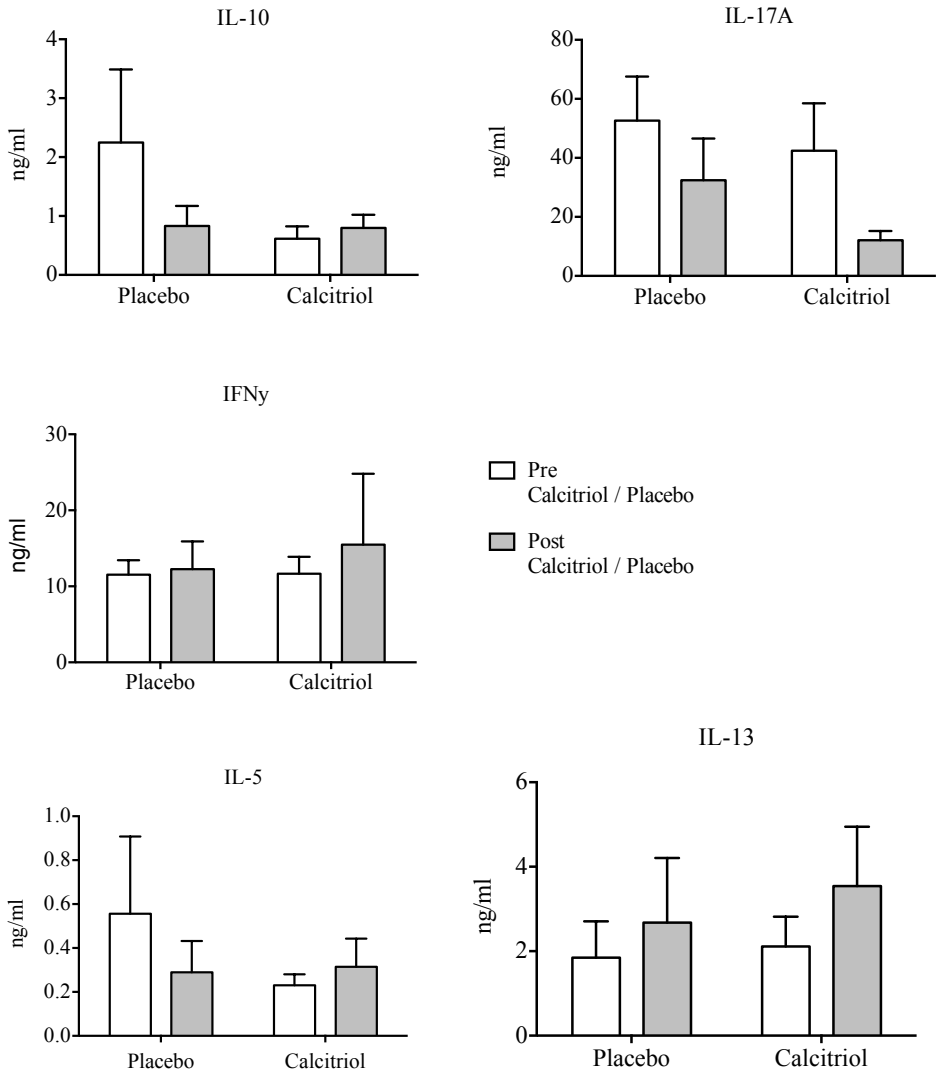


Figure 5.14 Cytokine production in culture by SR patients at baseline (Screening Visit 1) and after treatment with placebo/calcitriol (Treatment Visit 2)

PBMCs were isolated and treated as before. Cytokine production in cell culture supernatants was assessed by CBA. There was no statistical significance seen. Data presented as mean + SEM. Assessed by multiple t-test corrected for multiple comparisons using the Holm-Sidak method.

5. 2. 9 The effect of Calcitriol on the *in vitro* Response to Dexamethasone

The final data presented here discusses the in vitro production of cytokines in response to in vitro dexamethasone following in vivo treatment with calcitriol. Our understandig of cytokines involved in the pathogenesis of asthma has greatly evolved in the last years and it is now well accepted that different T helper cell subsets are driving the disease. Our aim was to investigate the effect of calcitriol on the in vitro response to dexamethasone treatment in culture. We hypothesised that calcitriol enhanced the anti-inflammatory properties of dexamethasone by increasing IL-10 and inhibiting pro-inflammatory cytokines such as Th2 and Th17 cytokines IL-17A, IL-5 and IL-13.

CD8+ depleted PBMC of all SR patients were collected and stimulated with anti-CD3 and IL-2 and co-cultured with dexamethasone at the dose indicated (10^{-9}M – 10^{-6} M), (as described in Figure 5.2), after in vivo treatemnt with calcitriol / placebo for four weeks including oral prednisolone for the last two weeks.

Figure 5.15 shows that after four weeks calcitriol or placebo and a repeat two week course of prednisolone, there was significant induction of IL-17A by dexamethasone in the placebo control group but not in the calcitriol treatment group. Furthermore, calcitriol treated SR asthmatics showed a significant increase in dexamethasone-induced IL-10 (when IL-4 was added to the culture media only), an effect that was not observed in the placebo group. There was no significantly difference seen between the two patient groups for the induction of IL-13 or IL-5. Similarly, there was no significant effect of calcitriol treatment on IFNγ production.

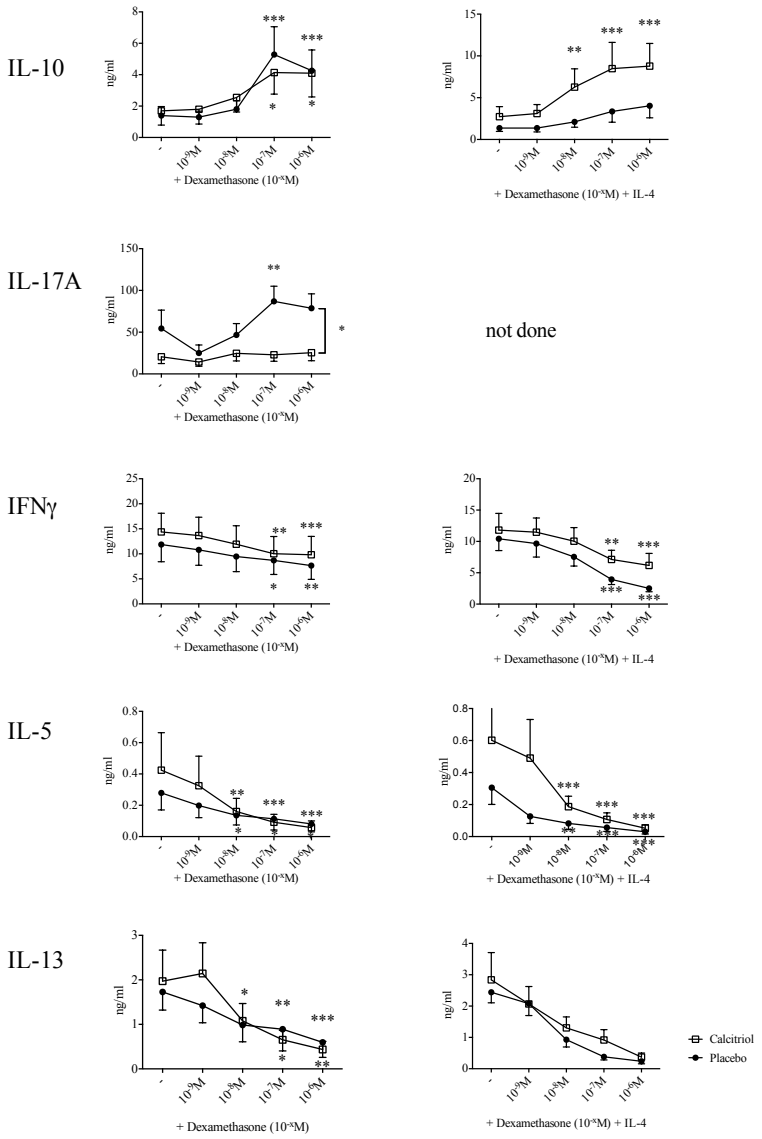


Figure 5.15 The effect of Calcitriol / Placebo treatment on the in vitro response to dexamethasone

CD-8 depleted PBMCs were stimulated as described before with dexamethasone at the dose indicated (10^{-6}M – 10^{-9}M) and without (left column) or with (right columns) IL-4 added to the culture media. Cytokines in culture supernatants were assessed by CBA. Calcitriol significantly increased the synthesis of IL-10 in cultures stimulated with dexamethasone 10^{-7} M and 10^{-6}M. When IL-4 was added to the culture media the effect was significant at 10^{-8}M, 10^{-7} M and 10^{-6}M for patients on calcitriol treatment. There was no significant difference in between patiet groups seen for IFNγ, IL-5 and IL-13. Data presented as mean with SEM and assessed by two-way ANOVA with Dunnett's multiple comparisons tests. p = <0.05, **p = <0.01, ***p = <0.001; Calcitriol open squares, placebo closed circles.

5. 3. Discussion

There is currently significant interest in better defining asthma phenotypes, both clinically and mechanistically, in order to improve treatment outcomes and hence asthma control. Indeed recent clinical trials using biologics have reported successful outcomes when treatments were targeted at specific subgroups [59, 285]. The detection of differences in cytokine production is a valuable approach to obtain information about the distinct nature of a chronic inflammatory process. The focus of this work was to assess the cytokine profiles of clinically characterised steroid sensitive (SS) and steroid resistant (SR) asthma patients and to identify whether distinct profiles could be defined, that were biomarkers of steroid responsiveness.

The results presented here demonstrate significant differences between the two patient groups with the most striking data centering around anti-inflammatory IL-10 and pro-inflammatory IL-17A synthesis in culture and the correlation of those cytokines with the patient's clinical response to steroids. The data further highlights a potential role for IL-17A and IFNγ to predict steroid resistance in vitro.

The dichotomy in IL-10 and IL-17A synthesis observed between SS and SR patient' cultures is novel and exciting. Notably, whilst there was no correlation seen between cytokine production and lung function at baseline, patients who had the biggest improvement in lung function after a course of oral prednisolone were found to have the highest IL-10. In contrast, patients whose lung function failed to improve, and in some in whom it deteriorated, had significantly greater production of IL-17A, suggestive of a potentially deleterious effect of steroids in those patients (Figure 5.11).

In SR patients higher synthesis was not only seen for IL-17A but also for IFNγ (Figure 5.2). A difference in the *in vitro* synthesis of IFNγ between SS and SR patient in PBMC cultures was first described by *Corrigan* in 1991 [295], thus the present data confirm and

extend those initial findings. Higher frequencies of IFNγ producing cells have also been well described in patients with chronic obstructive pulmonary disease (COPD) [290, 296].

Severe, and in particular, SR asthma has many features similar to COPD, such as airway neutrophilia, a poor response to steroids, and also decreased IL-10 synthesis. SR asthma may to that extent represent a phenotype more closely related to COPD as opposed to the classical Th2 high atopic asthma. The asthma-COPD overlap syndrome, postulated over 50 years ago by the 'Dutch hypothesis', suggested that asthma and COPD have genetic and environmental risk factors such as allergens, infections, air pollution and smoking in common [297]. It is believed that up to a fifth of patients with chronic airways disease may have overlap syndrome; characteristics include increased airway neutrophilia, accelerated decline in lung function and more frequent and severe exacerbations [298]. Patients with overlap syndrome classically have less bronchial reversibility and are smokers or ex-smokers of less than 5 years.

There is also a link between higher IFNγ levels, recurrent infections and severe asthma. The role of respiratory viral infections in the development and particularly progression of asthma is well described. IFNγ is a central factor involved in antiviral and antibacterial responses and detected clinically in association with many respiratory infections.

It was interesting, and unexpected, to find that whilst both IFNγ and IL-17A cytokine synthesis in culture were elevated in SR patients as compared to SS individuals, those patients with high IFNγ expression did not have high IL-17A and vice versa (Figure 5.2 B), defining the SR subgroup further. ROC analysis of IL-17A and IFNγ synthesis in culture at baseline revealed a sensitivity of 86.4% and specificity of 75% to predict SR, rendering these cytokine valuable biomarkers to identify patients in whom steroid treatment on its own could have deleterious effects (Figure 5.2 C). This would certainly be worth exploring in more depth in the future. It has been suggested that T cells that co-

express IL-17A and IFNγ may represent more pathogenic cells than single cytokine positive cells [120, 299]. Unlike for IL-17A, there was no evidence of steroid mediated enhancement of the IFNγ response. In fact, the response was inhibited by dexamethasone in culture in either patient group. This suggests that there are SR patients who produce high levels of IFNγ in culture, that is amenable to *in vitro* steroid inhibition, whereas SR patients with IL-17A^{high} are clinically and immunologically SR.

There was no difference seen at baseline for IL-10 or for the Th2 cytokines IL-5 and IL-13 between SS and SR patients (Figure 5.2 and 5.3). This is in contrast to other studies that have found higher levels of Th2 cytokines in SS patients [300]. However, unlike our data, these results represent cytokine expression in the airways as opposed to peripheral blood and in mild asthma, whereas our patients, SS and SR, suffered from moderate to severe asthma. Importantly Th2 cytokines were strongly inhibited in both patient groups following in vitro treatment with dexamethasone. On the other hand, a significant steroid-induced increase in IL-10 synthesis in culture was observed in SS patients only. In agreement with our previous findings this was significant with IL-4 in the culture media only. Steroid treatment resulted in significant reduction of the Th2 cytokines IL-5 and IL-13 (Figure 5.6).

We have previously shown an association between steroid sensitivity and IL-10 synthesis in culture and a strong inhibitory effect of *in vitro* steroids on Th2 cytokine such as IL-13 [301]. I have briefly mentioned the landmark study by *Woodruff* in chapter one before. They identified periostin (*POSTN*), chloride channel regulator 1 (*CLCA1*), and serpin peptidase inhibitor, clade B, member 2 (*SERPINB2*) as epithelial genes that were specifically induced in asthma and directly regulated by IL-13 *in vitro*[44]. The group further identified patients with distinctly higher levels of IL-13, termed Th2-high asthma. This was in contrast to patients with cytokine expression including the Th1 IFNγ, who was significantly lower in the Th2-high group. Of particular interest was

their observation of corticosteroid responsiveness: lung function of Th2-low asthmatics failed to improve following treatment with inhaled corticosteroids, in fact patients' FEV_1 deteriorated suggesting a detrimental effect of corticosteroids in Th2-low asthma[44]. IL-13, a key driver of airway hyper-responsiveness is increased in sputum and bronchial biopsy specimens of patients with severe asthma, which makes it an attractive target for new asthma treatments[59, 302, 303]. Recent clinical trials have seen a therapeutic success of anti-IL-13 antibodies in selected patients only, namely patients with high levels of FeNo, eosinophils, IgE and serum periostin levels [59]. Each of these biomarkers has been associated with Th2 inflammation in the airway and steroid responsiveness. Collectively these data highlight that it is the Th2-low asthma cohort that remain clinically challenging and for whom new and improved treatments are needed.

The balance between Th1 / Th17 *versus* regulatory cytokines is likely to be important in the attenuation of airway inflammation in asthma. Whilst IL-17A is associated with more severe disease and poor response to treatment, IL-10 has been found to limit inflammation as a result of bacterial and viral infections, one of the most common causes for asthma exacerbations and a known factor associated with the progressive decline in lung function seen in severe asthma [304]. Patients with asthma have been found to have lower levels of IL-10 mRNA and protein as compared to healthy controls [151, 155] and there is a clear association with disease severity and lower levels of IL-10 [152, 305]. IL-10 down-regulates IL-17A production from Th17 cells [306, 307] and dampens T cell activation *via* antigen-presenting cells [61, 62, 308]. Promoting an anti-inflammatory environment by boosting IL-10 production is an attractive concept and has been successful in for example allergen immunotherapy [309, 310].

There is epidemiological evidence linking low serum 25(OH) levels with poor respiratory health, asthma development and treatment response [192]. Our previous data has shown that *in vitro* calcitriol improved steroid sensitivity via increasing IL-10 synthesis in culture. We have also shown that the steroid dexamethasone failed to

suppress the pro inflammatory IL-17A in culture. In this study, *in vivo* treatment with calcitriol, but not with placebo, resulted in diminished culture levels for IL-17A after stimulation with *in vitro* dexamethasone (Figure 5.15). Conversely, there was a trend in the absence, but a significant effect in the presence of IL-4 in culture of enhanced steroid-induced IL-10 synthesis after ingestion of calcitriol - reproducing the original *Xystrakis* observations [163] (Figure 5.15 A & B). This finding is exciting as it suggests a steroid-sensitizing effect of calcitriol by attenuating the pro-inflammatory activity of Th17 cells *via* up-regulation of IL-10 secreting cells.

Historically, our laboratory has found that under all but optimal conditions of stimulation, namely priming with both IL-4 and dexamethasone, CD4+ T cells synthesized the greatest amount of IL-10, implying that endogenously produced IL-4 synthesis can regulate T cell phenotype development.

My results showed a positive correlation between serum 25(OH)D levels and synthesis of the Th2 cytokine IL-13 (Figure 5.12). A large body of conflicting scientific literature exists describing the capacity of vitamin D to variously inhibit or enhance Th2 responses in culture and in animal models (reviewed in [166, 192, 311]). Importantly IL-13, and Th2 cytokines in general, are highly sensitive to steroid inhibition (as seen in Figure 5.10) further implying the capacity of vitamin D to skew towards a state of steroid responsiveness. The recently published paper by *Castro* [218] has been discussed in the previous chapter; together with our own data on the capacity of calcitriol to improve the clinical response to prednisolone. Castro et al showed that supplementation with vitamin D in asthma patients led to a small but significant reduction in the overall dose of inhaled steroids required to maintain asthma control. In addition a large body of epidemiological evidence demonstrate an association between low vitamin D status and response to treatment [192]. Our data offer immunological support to Castro's and other findings and pave the way for more, well-conducted clinical trials addressing the role of vitamin D as an adjunct to current treatments.

Limitations to the study design exist. The Calcitriol study only assessed peripheral blood cytokines. It would have been beyond our available resources to assess for BAL cytokines, and certainly more difficult recruitment processes but this would have been interesting to look into. The data presented in this chapter represents data from severe asthmatics only and not from healthy controls. I will discuss the role of IL-17A in severe asthma further in chapter six, where I investigated its expression in the healthy population and in patients. It would undoubtedly be interesting to compare some of the above data with data from healthy individuals.

There are other limitations in our experimental approach; the study was designed to investigate the effect of calcitriol in SR patients only. SS patients were excluded after the second screening visit and by targeting severe steroid resistant patients specifically during recruitment; the number of SS patients is much lower (12 versus 23 SR patients). This carries the risk for some of the data being skewed. In hindsight one would want to recruit equal numbers throughout the trial.

Cell cultures use for this proof of concept study were based on the laboratories earlier experiments with CD4+ T cells and APCs. It is well known that IL-17A is also secreted by CD8 cells, γδ T cells and others [96] and in future experiments it would be good to look at cytokine expression in different cell types. Other cytokines involved in steroid resistance have emerged recently, namely IL-6, which is involved in the differentiation and maintenance of Th17 cells, or IL-8, a key neutrophil chemoattractant, that has previously been shown to be sensitive to vitamin D treatment [312] and it would have been interesting to study the effect of calcitriol on more cytokines with more time and funding available. Interpretation of data after *in vivo* and *in vitro* stimulation with steroids is difficult potentially due to inter individual differences in absorption and metabolism of prednisolone and calcitriol and its effect on cell signaling *in vitro*. The

focus of our results centered on cytokine levels in culture at baseline and following either *in vivo* or *in vitro* treatment instead.

In conclusion, the data discussed here show that SR patient were distinct in expressing high culture levels for IL-17A and IFNγ, providing a possibility for *in vitro* testing for steroid resistance. Patients with high levels of IL-17A, that is less responsive to steroid treatment, could be in a chronic pro-inflammatory state with IL-17A potentially sustaining higher IFNγ levels hence poor asthma control. This is certainly reflected in the clinical response to steroids in terms of lung function, where best outcomes are seen in patients with low levels of IL-17A and high levels of the immuno-modulatory cytokine IL-10.

Calcitriol has the potential to enhance steroid activity through promotion of an anti-inflammatory environment with IL-10^{high} and IL-$17A^{low}$. Although our numbers are small, the data also showed that patients with high serum 25(OH)D had higher levels of the strongly steroid responsive IL-13 giving weight to the concept that 25(OH)D could lead to a shift in Th -cell balance by altering the immunophenotype away from a steroid resistant Th17 phenotype towards a more responsive Th2 immunophenotype. Thus, while calcitriol does not improve lung function in asthma directly it does improve responsiveness to steroid therapy.

Supplementary Data

Figure 1:

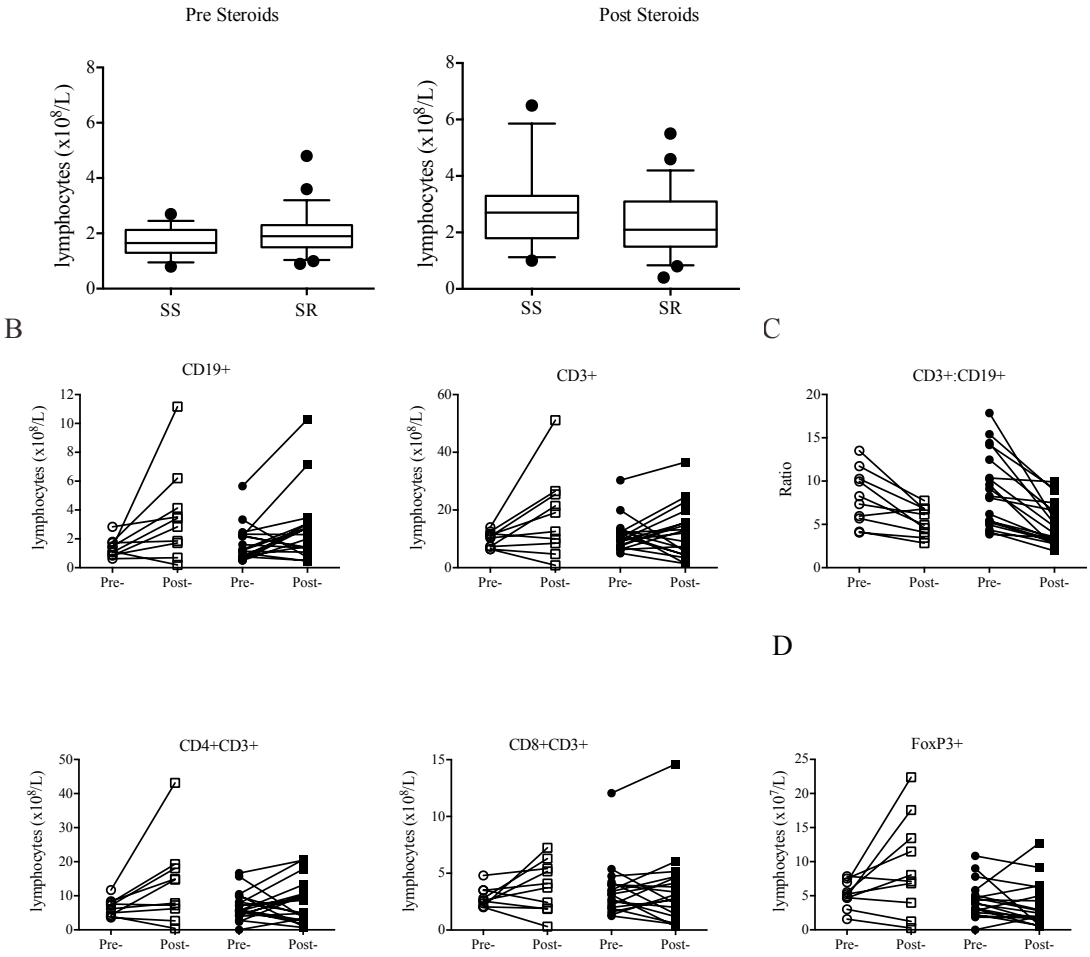


Figure S1 Absolute lymphocyte counts in the peripheral blood of steroid sensitive (SS) and steroid resistant (SR) asthma patients before and after a 2 week course of oral prednisolone.

A, total lymphocytes B, total B cells (CD19+), T cells (CD3+), CD4+ and CD8+ T cells C, the ratio of CD3+:CD19+ cell ratios and D, total Foxp3+ CD4+ T cells in the peripheral blood of steroids sensitive (SS) and resistant (SR) severe asthmatics. Data assessed by Wilcoxon matched-pairs test *=$p<0.05$, ** $p<0.01$ ***= $p<0.001$.

Chapter 6

Enhanced production of IL-17A in Severe Asthma is Inhibited by Calcitriol in a Steroid-Independent Fashion

6. 1 Introduction

Studies of recent years demonstrated, and indeed the results of the previous chapter are in support of this, that the immune mechanisms involved in asthma cannot be explained by a Th1/Th2 imbalance only, but that a complex interplay exists between different innate and adaptive immune cells. Depending on the cytokine milieu present at the time of antigen presentation and T cell activation, T cells differentiate into effector T cell subsets, which secrete various cytokines, such as IFNγ (Th1 cells), IL-10 (iTreg cells), IL-22 (Th22 cells) and IL-17A, IL-17F, IL-22 (Th17 cells). Understanding the role of the many different cytokines involved in asthma pathogenesis is important for the development of new therapies.

The previous chapter has highlighted that steroid resistant (SR) asthma patients recruited into 'The Calcitriol Study' were found to have significantly higher culture levels of the pro inflammatory cytokine IL-17A than steroid sensitive (SS) patients. These data are in agreement to previous reports, showing that increased IL-17A cytokine concentrations are both an indicator and a risk factor for severe asthma [131, 132, 291, 292, 313]. Furthermore, Th17 cells and IL-17A were implicated in steroid non-responsiveness in a murine model of allergic asthma [135]. *In vitro* and *in vivo* experiments have shown that transfer of Th17 or Th2 cells could both initiate airway hyperresponsiveness, but only Th2 cells were inhibited by steroid treatment [135].

Similarly, the cytokine IL-22 has been demonstrated to be important in sustaining inflammation in autoimmune disease and, as well as being elevated in patients with severe asthma [145], has been described to augment the pro-inflammatory activity of IL-17A [144, 147, 314].

Monoclonal antibodies against IL-17A have been trialled in asthma but not all were able to improve disease control. Furthermore there is a concern that blocking IL-17A could lead to an increase in infection [58, 72]. Genetic deficiency in IL-17RA is associated with

recurrent or persistent mucocutaneous infections caused by Candida albicans and, to a lesser extent, by Staphylococcus aureus [315]. Treatments with anti-IL-17A antibodies in psoriasis and arthritis were generally well tolerated in the reported phase 2 studies but the duration of these studies was relatively short and the probable side effects associated with anti-IL-17A treatments need to be evaluated further in studies to come and the aim should be to find treatments directed at controlling inflammation whilst maintaining host defense.

Previous studies have shown that the active from of vitamin D, $1,25(OH)_2D3$ (hereafter calcitriol) represses mRNA and transcription of several pro-inflammatory cytokines including IL-17A [249, 316, 317] while others have highlighted the capacity of calcitriol to inhibit Th17 responses[247, 318, 319].

Vitamin D is also known to have antimicrobial activity [320-323]. There is an interest in the potential of vitamin D to regulate autoimmune diseases and to reduce the amount of anti-inflammatory (=steroid) medication required for disease control without affecting host defense mechanisms negatively. Studies have highlighted the capacity of calcitriol to inhibit Th17 proliferation and function in mouse models of autoimmunity and also in human keratinocytes and T cells in vitro [247, 249, 318, 319].

Therefore, expanding on above studies and following our observations of higher levels of IL-17A in cultures of severe asthma patients this chapter discusses IL-17A and IL-22 cytokine levels in culture between healthy control subjects and patients and the role dexamethasone and calcitriol might have in controlling the synthesis of these cytokines. To address this IL-17A and IL-22 cytokine production from PBMCs of healthy controls and asthma patients were stimulated in culture in the absence and presence of dexamethasone and calcitriol.

The experiments in this chapter were performed whilst the clinical trial was ongoing and patients' *in vitro* data started to emerge. The results complement and expand data

discussed earlier. Time and funding restrictions limited the number of *in vitro* experiments that were performed on patient's and healthy control subject's material.

6. 2 Results

6. 2. 1 The Expression of IL-17A and IL-22 in Healthy Controls and Asthma Patients

The results of chapter five demonstrated that PBMCs from SR asthma patients synthesized significantly higher levels of IL-17A in culture than those of SS patients (figure 5.2) and that this effect was not abrogated by in vitro steroid treatment (figure 5.6) suggesting a role for IL-17A in steroid resistant asthma. IL-17A levels in culture also inversely correlated with a change in lung function after *in vivo* steroid treatment (figure 5.11).

To assess the *in vitro* production of IL-17A in healthy controls and severe asthma patients CD8-depleted PBMCs were isolated from the peripheral blood and stimulated with anti-CD3 and IL-2 for seven days. Cells were cultured at a cell density $1x10^6$/ml in RPMI containing 10% FCS with anti-CD3 (1μg/ml) and IL-2 (50IU/ml). At day 7 phorbol 12-myristate 13-acetate (PMA) (5ng/ml) and Ionomycin (500ng/ml) was added for 4 hours, with the final 2-hours containing Monensin (2μM) to allow intracellular cytokine production to be assessed by flow cytometry. In parallel cultures cells were also recounted at day 7 and re-cultured at $1x10^6$/ml in the presence of anti-CD3 and IL-2 for a further 48-hours to account for differential cell loss. Culture supernatants were collected and IL-17A cytokine production was assessed using Cytometric Bead Array (CBA), IL-22 cytokine production was assessed by ELISA.

Initial experiments investigated whether there was a difference in cytokine production in culture between healthy controls and patients enrolled into 'The Calcitriol Study' (SS and SR patients). Figure 6.1demonstrates significantly greater percentages of IL-17A+ ..control subjects. When patients were divided according to their response to prednisolone treatment after the initial screening phase in the trial, only SR patients showed a significant difference in IL-17A and IL-22 immuno-reactivity (IL-17A: SR p

= 0.003; SS p = 0.142; IL-22: SR p = 0.037; SS p = 0.701), compared to control subjects (Figure 6.1 B).

Cells co-expressing IL-17A and IL-22 are proposed to represent the more pathogenic population, and it was interesting to find they were significantly increased in all asthmatics and SR asthmatics as compared to healthy control subjects (all asthmatics p=0.029; SR p=0.009). Culture supernatants from asthma patients contained significantly higher quantities of secreted IL-17A and IL-22 as compared to healthy controls (Figure 6.1 C). Production of IL-17A and IL-22 was significantly increased in all severe asthmatics as compared to controls (IL-17A: p=0.009; IL-22: p=0.036). Supernatants from SR had significantly more IL-17A as compared to SS (p=0.016), whereas culture supernatants from SS and SR patients contained comparable levels of IL-22.

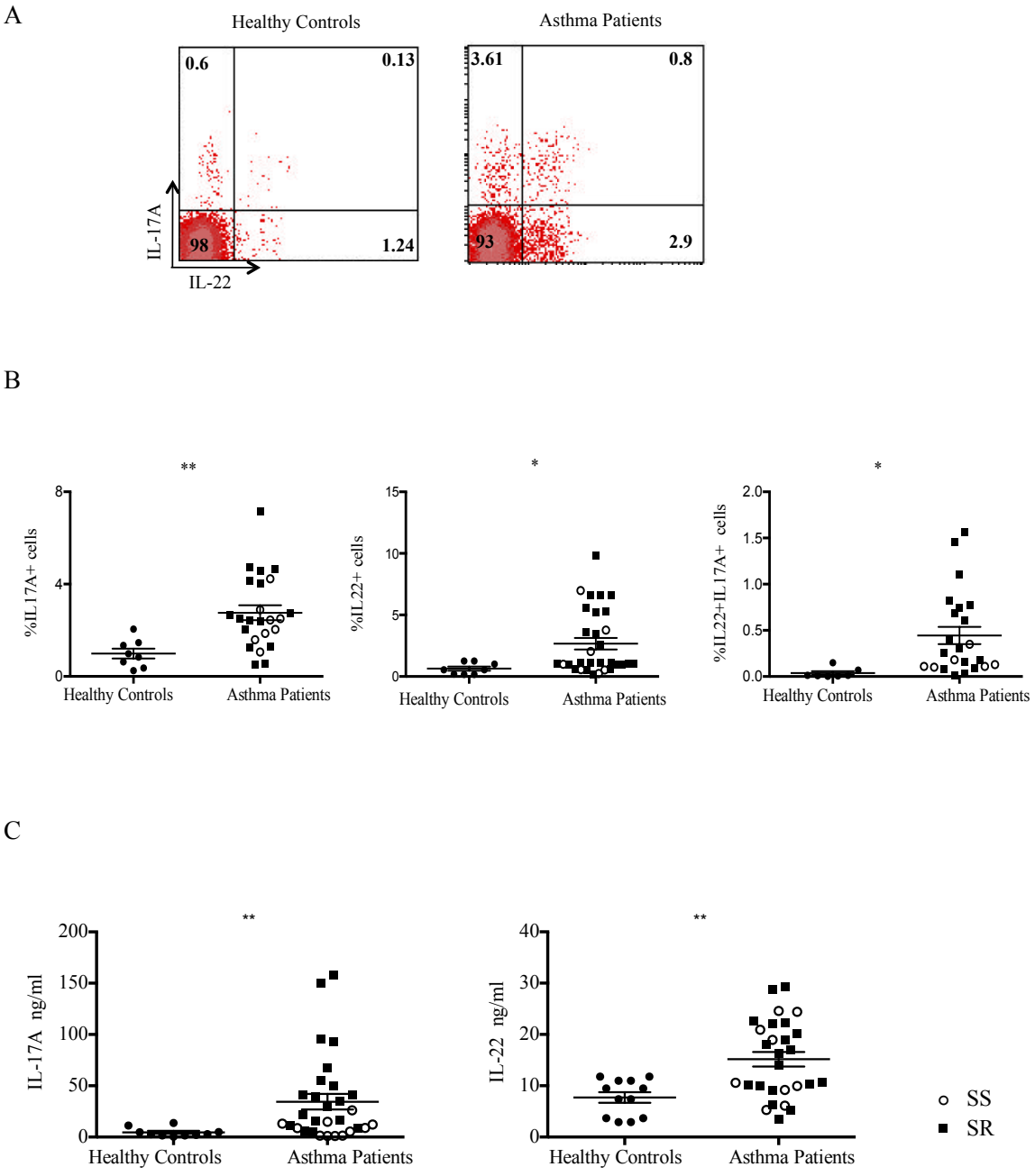


Figure 6.1 Severe asthma patients express higher levels of IL-17A and IL-22

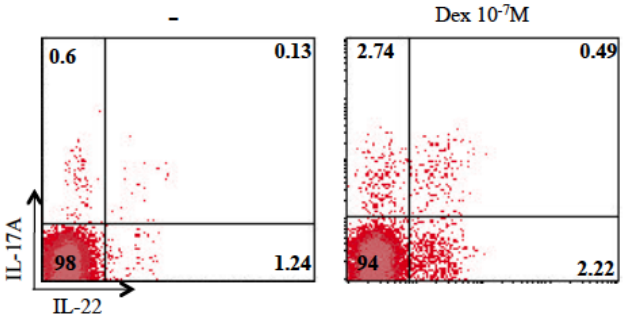
CD8-depleted PBMCs were stimulated for 7-days with anti-CD3 and IL-2. Control and asthmatic cultures were assessed for IL-17A and IL-22 production by intracellular flow cytometry and cytometric bead array (CBA) and ELISA respectively. A, Representative dot plots of one healthy control and one asthma patient. B, Cumulative data of cytokine positive cells (healthy n=8; SS n=8 and SR n=14) and C, secreted cytokines (healthy n=10; SS n=10 and SR n=14). Analysis by unpaired t-test, * $p<0.05$; ** $p<0.001$. SS: open circles; SR: closed squares; healthy controls: closed circles.

6. 2. 2 Dexamethasone Increases IL-17A and IL-22 Synthesis in Culture of Healthy Controls

Steroids are the most widely used therapy for asthma. Having found a significantly higher production of IL-17A in cultures of asthma patients as compared to healthy controls, the effect of *in vitro* dexamethasone treatment on IL-17A synthesis in culture was next investigated. PBMCs of healthy donors and asthma patients were isolated and stimulated as before with the addition of dexamethasone in cultures at the dose indicated (10^{-9} – 10^{-6}M). IL-17A and IL-22 cytokine production was assessed by flow cytometry where dexamethasone was used at a concentration of 10^{-7}M only due to a limited amount of blood available for experiments relating to the clinical trial and based on earlier observations in our laboratory for an optimum induction of IL-17A and other cytokines [301]. IL-17A cytokine production in culture supernatants was assessed by cytometric bead array (CBA) and IL-22 was assessed by ELISA.

Figure 6.2 shows that dexamethasone failed to inhibit IL-17A and IL-22. In fact, in healthy controls, there was a significant increase in the frequency of IL-17+ cells in cultures containing 10^{-7}M dexamethasone (Figure 6.2 B). IL-17A production in culture supernatants was significantly increased in a dose dependent manner (Figure 6.2 C). Cytokine levels in asthma patients were around 10-fold higher but did not further increase following treatment. Dexamethasone had no significant effect on the frequency of IL-22+ cells in culture or IL-22 in culture supernatants.

A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Dex 10-7M
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0.13
2.74
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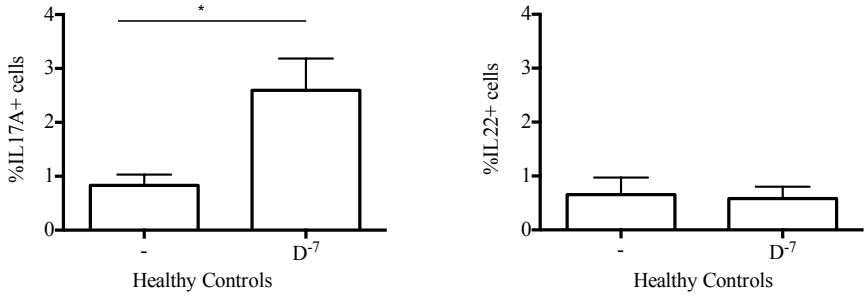
IL-17A
98
1.24
94
2.22
IL-22

B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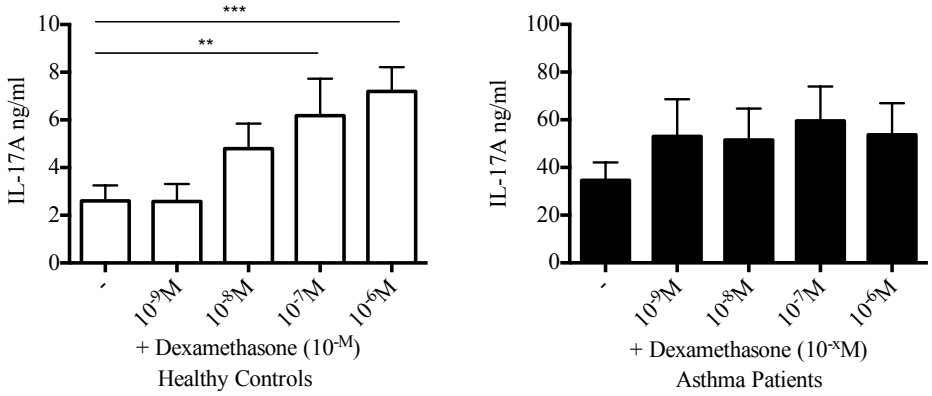
IL-17A ng/ml
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-
10-9M
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D

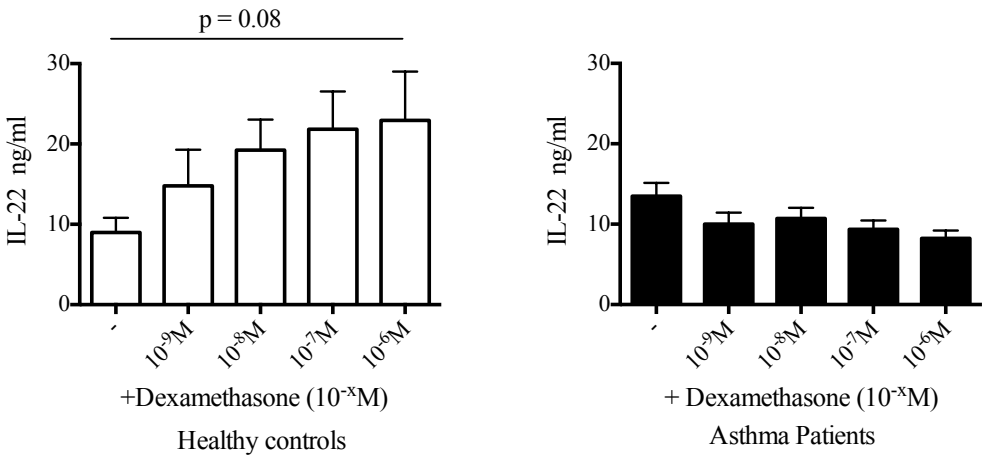


Figure 6.2 In vitro dexamethasone treatment increases IL-17A in cultures of healthy controls and has no effect in asthma patients.

CD8 depleted PBMCs from healthy controls and asthma patients were assessed for IL-17A and IL-22 production following stimulation with anti-CD3 and IL-2 as described before and dexamethasone at the indicated concentration. Intracellular flow cytometry and CBA for IL-17 and ELISA for IL-22 were performed following cell cultures as they are described in figure 1. **A,** Representative dot plots of healthy control. **B,** Cumulative cytokine positive IL-17A (n = 8) and IL-22 (n = 8) cells in healthy controls **C,** secreted IL-17A (healthy controls n = 10, asthma patients n = 20); **D**, IL-22 (healthy controls n = 5, asthma patients n = 20). Analysis by one way ANOVA with Tukey's multiple comparison post-test, * $p<0.05$; ** $p<0.001$, *** $p<0.0001$

6. 2. 3 The Effect of Inhaled Steroids on IL-17 Cytokine Levels

In vitro steroid treatment significantly increased IL-17A levels in cultures from healthy control subjects whereas in asthma patients the difference was small. One explanation could be that cytokine levels in patients were already significantly higher with limited scope to increase further. Another factor to consider was that all patients were on high doses of inhaled corticosteroid treatment.

To explore this further, a retrospective analysis of the relationship between inhaled steroid dose (equivalent to beclomethasone dipropionate) and IL-17A levels in culture supernatants was performed. This proved to be significantly positive (Figure 6.3) and raises the question of a possibly deleterious effect of steroids in certain patient groups.

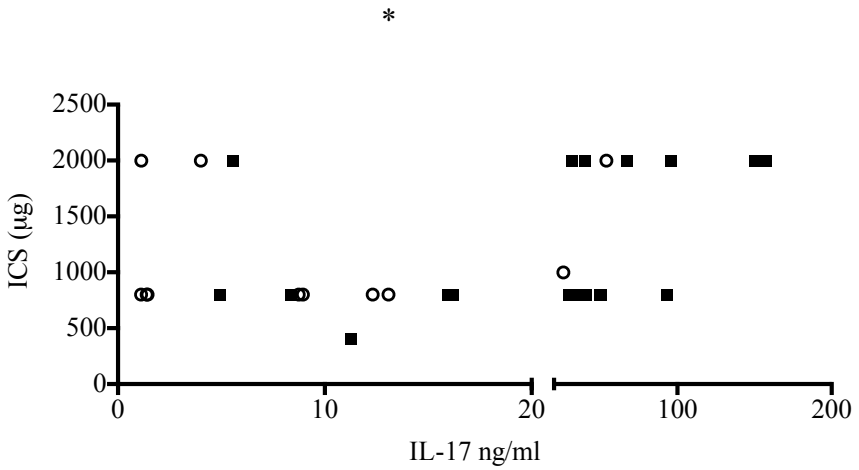


Figure 6.3 The correlation between inhaled corticosteroid (ICS) dose and IL-17A cytokine levels

SR and SS patients' inhaled corticosteroid dose was calculated to the equivalent of beclomethasone dipropionate (BDP). IL-17A was measured with cytokine bead array as described for figure 1. SS: open circles, SR: closed squares. Pearson Correlation r = 0.437, p = 0.016.

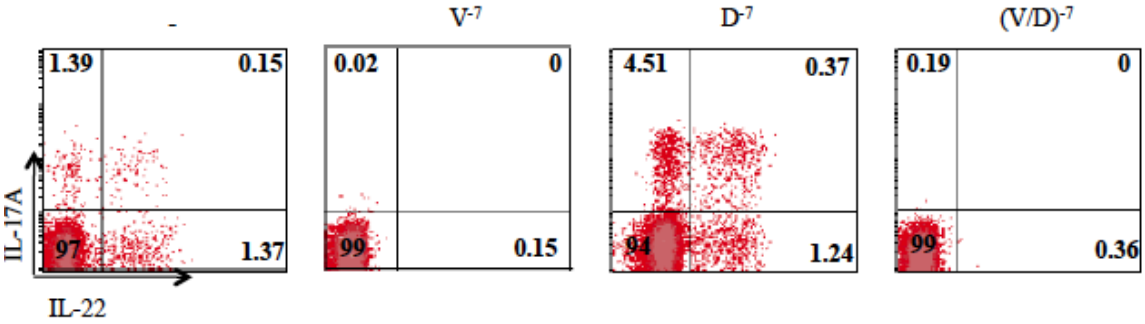
6. 2. 4 Calcitriol Inhibits IL-17A and IL-22 Expression in Severe Asthma Patients

Data in chapter five demonstrated that in patients treated with calcitriol *in vivo,* the stimulatory effect of dexamethasone on IL-17A cytokine levels in SR cell cultures was abolished, an effect that was not seen in patients on placebo. Hence the impact of calcitriol and dexamethasone *in vitro* was assessed in CD8-depleted PBMCs isolated from the peripheral blood of healthy donors and asthma patients to investigate for Th17-associated cytokine production. Cells were cultured as described before and intracellular cytokine production was assessed by flow cytometry and cytokine production in culture supernatants was assessed by Cytometric Bead Array (CBA) for IL-17A and ELISA for IL-22.

The data presented in Figure 6.4 demonstrates that not only did *in vitro* treatment with calcitriol significantly reduce IL-17A synthesis in culture levels, but it could overcome the stimulatory effect induced by dexamethasone in healthy controls and asthma patients.

There was a significant increase in the frequency of IL-17A+ cells in cultures containing 10^{-7}M dexamethasone and there is a similar trend observed in IL-22+ cells (Figure 6.4 A and B). When 10^{-7}M calcitriol is present in the cultures there was a significant decrease in the frequency of IL-17A+ and IL-22+ cells as compared to dexamethasone. Inhibition of IL-17A by calcitriol occurred independently of whether dexamethasone was present or not. There was a trend towards increase in the amount of IL-17A secreted in culture in the presence of dexamethasone ($p = 0.12$) which was significantly decreased with the presence of calcitriol. This effect was also seen for IL-22 (Figure 6.4 C).

A


-
V-7
D-7
(V/D)-7
1.39
0.15
97
1.37
0.02
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IL-17A
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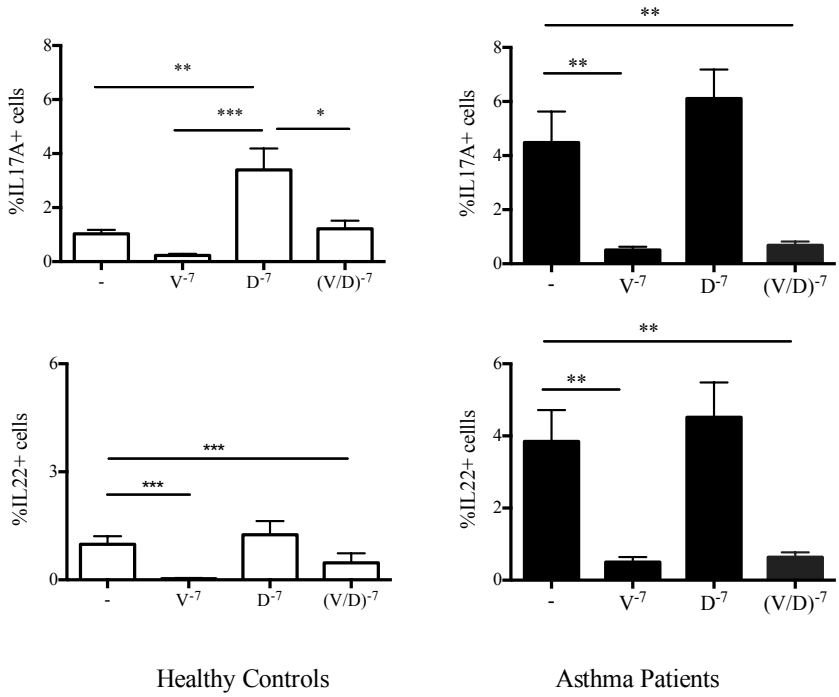
B


%IL17A+ cells
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Healthy Controls
Asthma Patients

C

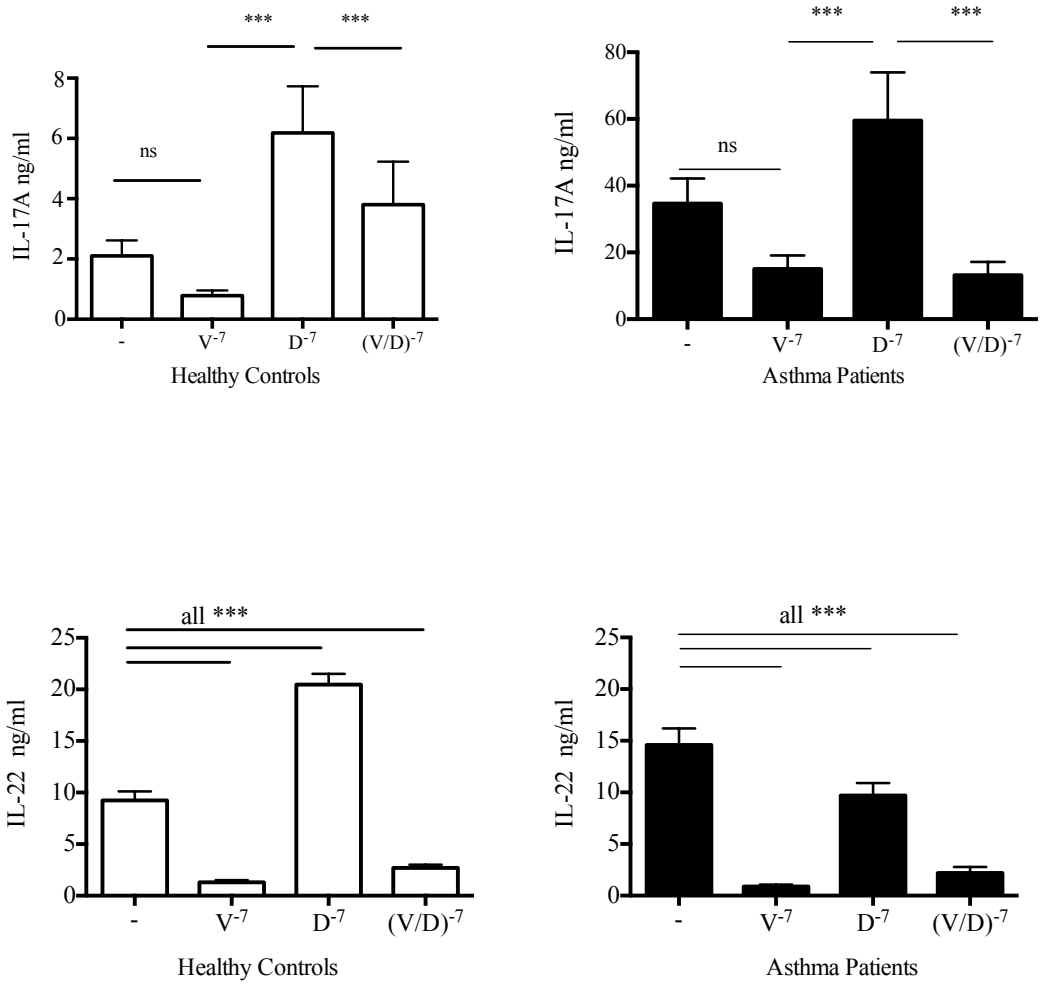

Figure 6.4 Dexamethsone fails to inhibit whereas calcitriol significantly decreases IL-17A production

CD8-depleted PBMCs stimulated for 7-days with anti-CD3 and IL-2 (no drugs: -) or additionally with the indicated concentration of calcitriol (V^{-7} ; 10^{-7}M) and/or dexamethasone (D^{-7} ; 10^{-7}M), assessed for IL-17A and IL-22 production by intracellular flow cytometry and CBA for IL-17A and ELISA for IL-22. A, representative dot plots of a healthy control subject. B, cumulative data of intracellular staining of cytokines. Healthy Controls: IL-17A n=5 and IL-22 n=4. Asthma Patients IL-17 n = 24; IL-22 n = 21. C, cumulative cytokine secretion data from CD8-depleted cells Healthy Controls: n=5. Asthma Patients: IL-17A: n=30, IL-22: n=28. B, and C, assessed by repeated-measures one-way ANOVA with Tukey's multiple comparison post-test * $p \leq 0.05$, **$p \leq 0.01$ ***$p \leq 0.001$.

6. 2. 5 Mechanisms of IL-17 Regulation

6. 2. 5. 1 The role of calcitriol on transcription factors

In order to investigate the mechanisms that could potentially be involved in the calcitriol induced inhibition of IL-17A, transcription factors were studied by PCR amplification.

PMBCs were isolated and stimulated as described above. At day seven, cells were harvested. RNA was isolated and 250ng of RNA was reverse-transcribed into cDNA as described in chapter two. Transcriptional expression of RORC, STAT3, IRF4 and BATF mRNA transcripts were determined by PCR amplification and qRT-PCR analysis performed.

STAT3 has a direct role in Th17 specification: the major cytokines that induce IL-17 (and to that extent, also IL-6, IL-23 and IL-21) all activate STAT3. It is also involved in signaling upstream and downstream of IL-22 expression. Patients with Hyper IgE syndrome (HIES), a primary immunodeficiency syndrome, suffer from a dominant negative mutation of STAT3 and have severely impaired ability to produce Th17 cells and have lower IL-22 production [324,325] resulting in overwhelming bacterial and fungal infections. RORγt (hereafter RORC) is a master regulator of Th17 cell differentiation and STAT2 and RORC each regulate the other gene's transcription and both induce IL-17 expression [326]. BATF, a member of the AP-1 transcription family, has been shown to be essential in Th17 differentiation and is increased in response to viral infection or IL-6 stimulation. BATF synergises with RORC to induce IL-17 expression through interaction with the IL-17 gene promoter [327]. IRF4 regulates IL-17 and IL-21 production and there is a strong association between IRF4 and STAT3 [326]. Figure 6.5 gives an abbreviated overview of the complex transcriptional regulation of IL-17A (adapted from Hwang E, Transcriptional regulation of T helper 17 cell differentiation 2010: Transcription factors regulating Th17 cell differentiation [328]):

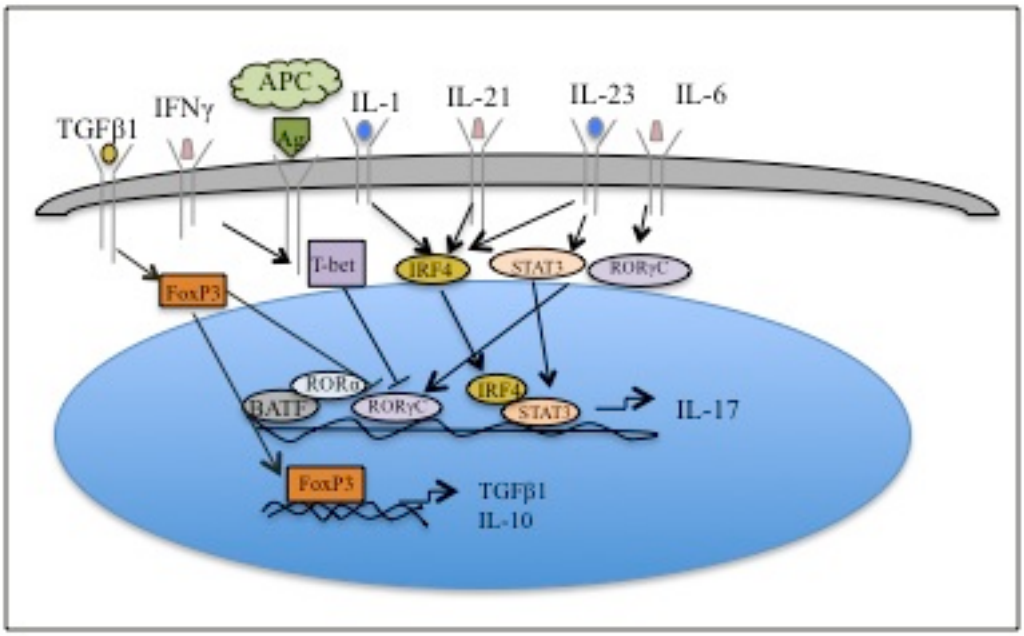

Figure 6.5 adapted from Hwang E, Transcriptional regulation of T helper 17 cell differentiation 2010: Transcription factors regulating Th17 cell differentiation [328]

T cell receptor stimulation activates BATF and stimulates IL-17 gene transcription. IL-6 or IL-21 activates STAT3 and induces IL-21 as well as autocrine regulation of Th17 cell differentiation. IL-21 also induces IRF4 which in turn induces IL-17 gene transcription. RORγC is strongly induced by IL-6 or IL-21 in the presence of low amounts of TGF-β. The induction of RORγC is dependent on STAT3, which is preferentially activated by IL-6, IL-21, and IL-23 and plays an important role in the regulation of IL-17 production in T cells. T-bet antagonizes RORγC activity and suppresses Th17 development. TGF-β stimulation induced both FoxP3 and RORγC: high TGFb increases FoxP3 whereas the presence of IL-6 and IL-21 activates STATs and hence IL-17.

I therefore investigated the effects of calcitriol on RORγt, STAT3, BATF and IRF4 gene expression. CD8+ depleted PBMC cells were isolated from the peripheral blood of healthy donors and cultured for 7-days with anti-CD3 and IL-2 in the presence or absence of drugs as indicated; cell pellets were harvested at day seven and PCR performed after isolation of RNA and reverse transcription. RORC was significantly reduced following treatment with a combination of calcitriol at 10^{-7}M and dexamethasone 10^{-7}M in culture. There was no effect seen on any of the other transcription factors (Figure 6.6).

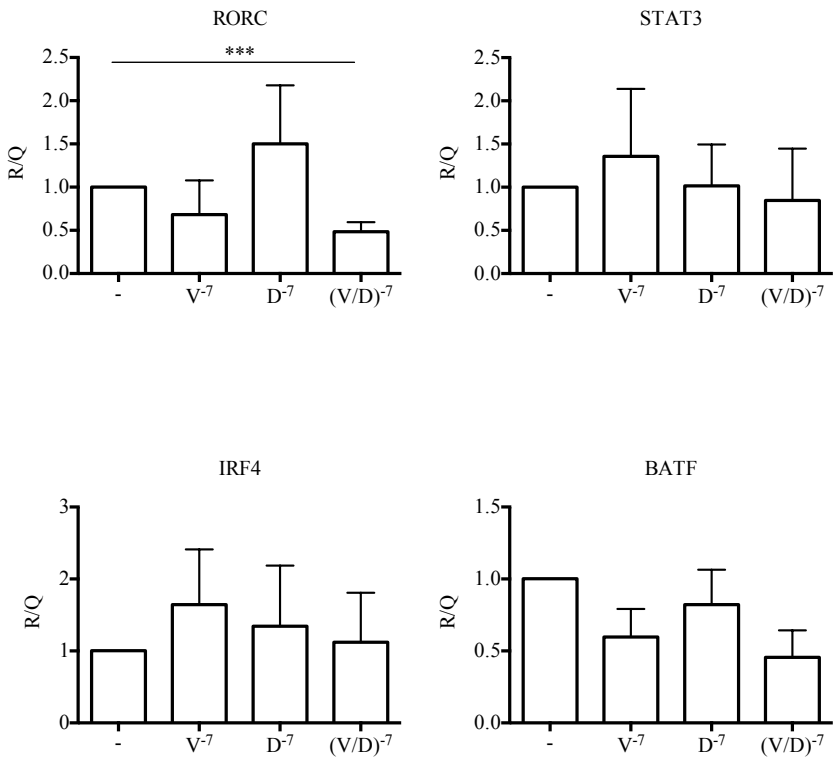

Figure 6.6 Gene expression following stimulation with calcitriol and / or dexamethasone in culture:

CD8 depleted PBMC cells from healthy volunteers were cultured for seven days with anti-CD3 and IL-2 as described before and +/- drugs as indicated: - = no drug; V^{-7} = calcitriol 10^{-7}M, D^{-7} = dexamethasone 10^{-7}M and $(V/D)^{-7}$ = calcitriol & dexamethasone 10^{-7}M. Relative mRNA expression was significantly reduced for RORC following treatment $(V/D)^{-7}$: $p < 0.0001$; treatment had no significant effect on STAT3, IRF4 or BATF; assessed by one-way ANOVA. n = 5.

6. 2. 5. 2 The role of IL-10 in calcitriol induced inhibition of IL-17A

The effect of calcitriol on IL-10 has been described in the past and has been published by our laboratory. To investigate whether the inhibitory effect on IL-17A is dependant on IL-10 I used an anti-IL-10 antibody during culture of PBMCs prior to intracellular staining for IL-17A: The inhibitory effect of calcitriol was not abrogated by anti-IL-10 and this implies that this effect is IL-10 independent (Figure 6.7).

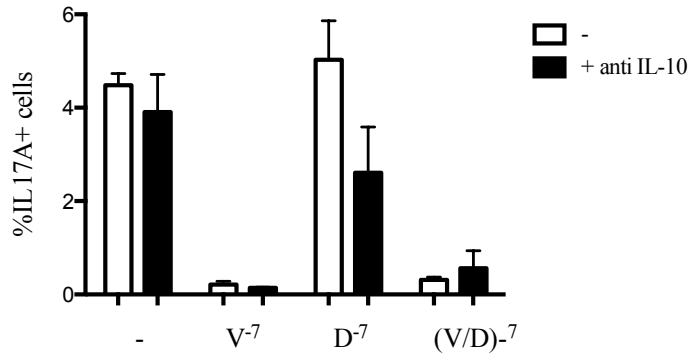

Figure 6.7 The inhibitory effect of calcitriol is not mediated via IL-10:

CD8 depleted PBMC cells from healthy volunteers were cultured for seven days with anti-CD3 and IL-2 +/- drugs as indicated: - = no drug; V^{-7} = calcitriol treatment 10^{-7}M, D 10^{-7} = dexamethasone 10^{-7} M and $(V/D)^{-7}$ = calcitriol & dexamethasone 10^{-7}M +/- the addition of 500μg/mL of anti-IL-10 in culture media. After seven days cells were harvested and assessed for IL-17A production by intracellular flow cytometry. There was no significant difference seen with or without anti-IL-10 in culture as assessed by paired t test, n = 3.

6. 3 Discussion

The data presented in this chapter demonstrate that the production of IL-17A and IL-22 by human PBMCs is elevated in severe asthma patients as compared to healthy subjects and that treatment with calcitriol significantly reduced cytokine synthesis in culture.

Dexamethasone failed to inhibit IL-17A or IL-22 production in asthmatic patients *in vitro*, but significantly enhanced IL-17 synthesis in healthy control cultures and, to a lesser extent, in asthma patients (Figure 6.2). Calcitriol not only significantly reduced cytokine level synthesis compared to no treatment, but also overcame the stimulatory effect of dexamethasone.

IL-17A was approximately 10-fold greater in asthmatics as compared to healthy controls (Figure 6.1). These data extend earlier observations showing an association between elevated IL-17A and IL-22 production and chronic lung disease [145]. The results are novel in terms of our ability to further characterise asthma patients as being either SS or SR. Strikingly, while blood T cells from all asthmatics demonstrated elevated IL-17A and IL-22 synthesis, the greatest differences were observed for SR patients whereas comparison of cytokine production between SS patients and healthy controls demonstrated a non-significant trend only for enhanced IL-17A and IL-22 production in SS.

Assessing SS versus SR asthmatics, the most notable differences were in the frequency of the levels of secreted IL-17A+ IL-22+ double positive cells (Figure 6.1 B). Double positive conventional T cells are proposed to represent the more pathogenic population with studies showing that IL-22 enhances the pro-inflammatory properties of IL-17A [145, 319]. In animal models IL-22 has been shown to act in synergy with IL-17A to promote pathological airway inflammation. Likewise, deficiency in IL-17A rendered IL-22 unable to promote airway inflammation. Instead, in the absence of IL-17A, IL-22 promoted essential tissue repair and homeostasis of the airway epithelium.

The association between IL-17A and steroid responsiveness has been discussed in previous chapters. IL-17A has been proposed to affect global corticosteroid responsiveness through effects on the glucocorticoid receptor (GR). GR-α is ubiquitously expressed and is responsible for the induction and repression of target genes, while GR-ß is expressed at much lower levels and is a dominant negative inhibitor of GR-α. Increased expression of GR-ß has been linked with severe asthma in some studies [333, 334], and a recent study suggested that IL-17A and IL-17F increase the expression of GR-ß, an effect that was more prominent in asthmatics than in healthy controls [293]. With hindsight it would have been interesting to investigate the expression of GRα/β in our patient cohort and healthy control subjects and assess whether there is a correlation with cytokine response.

Independent studies have suggested that Th17 cytokine production plays a mechanistic role in reducing corticosteroid sensitivity and, as a result of this, increasing asthma severity [135]. The data presented here is broadly complementary of studies in animal models, demonstrating that IL-17A production was not inhibited by dexamethasone in culture (see introduction to this chapter) [129]. It is difficult to fully exclude the possibility that elevated IL-17A production could be associated with inhaled corticosteroid administration. Certainly, culture of blood PBMCs from control subjects with dexamethasone resulted in significantly increased IL-17A production (and a trend seen for higher IL-22) from a very low baseline as compared to baseline levels in patients. On the other hand, dexamethasone did not further enhance PBMC Th17 cytokine production significantly in patients in whom it was already greatly elevated. In a post hoc analysis of the dose of inhaled corticosteroids the study patients were on, a significant association between inhaled corticosteroid dosage (beclomethasone equivalent prior to the trial of oral prednisolone therapy) and the amounts of blood PBMC IL-17A released *in vitro* (Pearson correlation r=0.459; p=0.014) was observed (Figure 6.3), suggesting that some of the IL-17A produced by PBMCs in asthmatics may

result from corticosteroid exposure. Interestingly a study in *steroid naïve* patients with mild asthma and relatively normal spirometric parameters found considerable heterogeneity in induced sputum cell counts with isolated neutrophilic inflammation and an impaired response to a treatment trial with steroids [335]. Neutrophilic asthma has also been associated with bacterial colonisation in the airways resulting in increased amount of oxidative stress and steroid insensitivity in some patients [336]. An unbiased cluster analysis done by the US National Institutes of Health Severe Asthma Research Program found that amongst patients with the most severe form of the disease more than a third had a mixed granulocytic inflammatory sputum profile. This implies that neutrophils might be an important biomarker for severe asthma, but also that concomitant persistence of airway eosinophilia might be a fundamental finding in participants with therapy refractory asthma.

RORC plays an important role in the production of IL-17A and is known to be co-operating with other transcription factors such as STAT3, BATF and IRF4; reviewed in [337] and illustrated in Figure 6.5. RORC expression was inhibited by the combination of calcitriol and dexamethasone in culture. However no effect was seen for the expression of STAT3, IRF4 and BATF. This suggests that the calcitriol-mediated effects on IL-17A are not predominantly genetically orchestrated.

We and others have shown that both calcitriol and dexamethasone increased the expression of the anti-inflammatory cytokine IL-10 *in vitro*, and IL-10 has been shown to inhibit IL17A production [338]. This implies a possible IL-10 dependent mechanism for the inhibition of IL-17A seen in our results. However, Figure 6.7 demonstrates that the use of an anti IL-10 antibody did not significantly alter the inhibitory effect of calcitriol on IL-17A expression.

Dr Chambers, the principal scientist working alongside me on 'The Calcitriol Study", investigated a possible mechanism for the inhibition of T cell IL-17A synthesis by

calcitriol and the ectonucleotidase CD39, which is expressed by memory T cells as well as Foxp3+ regulatory T cell and which has previously been reported to inhibit IL-17A synthesis [332, 339, 340]. Figure 6.7 shows that treatment with calcitriol increased the mean intensity of CD39 expression by human CD4+ T cells, while the CD39 antagonist POM-1 partially abrogated calcitriol -mediated inhibition of the IL-17A production, supporting a mechanistic role for this pathway but not excluding others. In contrast, dexamethasone tended to reduce CD39 expression.

I have mentioned Dr Chamber's data here to complement my own data and demonstrate a possible mechanism involved in the results shown here. This certainly needs to be explored further and in greater detail and unfortunately we were unable to do this due to time restrictions.

Together our data are consistent with the hypothesis that calcitriol, through inhibition of IL-17A, has the potential to improve disease control in asthmatics independently of their clinical steroid responsiveness.

Trials at blocking IL-17A cytokines in patients with asthma have to date shown mixed results as discussed in chapter one. IL-17A is important in host defense and blocking this cytokine might come with an increased risk of infections. The relationship between IL-17A and calcitriol is of interest. It has been shown in patients with atopic dermatitis that oral supplementation with cholecalciferol resulted in lower frequencies of IL-17-producing memory T cells [341]. Further animal and human studies have shown an inhibitory effect of calcitriol on IL-17A cytokine production in experimental autoimmune uveitis [247], Behçet's disease [342] or Vogt-Koyanagi-Harada disease [343].

This chapter discusses results that emerged whilst data for the calcitriol study were being collected. It is a small chapter and mechanistically incomplete. Once more patients entered and went through the clinical trial, producing a wealth of material, the focus of our laboratory work fully shifted onto the study and experiments with blood

from healthy controls were postponed. The findings of this chapter support a role for calcitriol in down regulating pro inflammatory cytokines such as IL-17A and IL-22 but with taking a smaller risk of infection related side effects than seen with IL-17A blocking antibodies and future studies are warranted to verify these results in an *in vivo* setting.

Chapter 7

General Discussion

Asthma is one of the most common chronic diseases worldwide, affecting up to 10% of adults and 30% of children in developed countries; the number of people affected by asthma in the UK is among the highest in the world [344]. Whilst many people with asthma have well controlled disease that seldom requires unscheduled visits to their GP, or indeed hospital admission, some patients suffer from daily symptoms ranging from breathlessness, cough and chest tightness to numerous and uncontrolled exacerbations; this might be because their asthma is difficult to control, complicated by co-morbidities, or because it is at the severe end of the disease spectrum - defined by a poor response to medication, in particular corticosteroids (steroids). All these patients have a greater risk of dying of their disease and their lives are often blighted by their condition, notwithstanding the disproportional amount of healthcare resources spent.

A recent national review analysed data from 192 asthma deaths in 2012-2013 [20]. The report shed light on some alarming facts. In 59% of patients who died, asthma treatment guidelines were not fully adhered to and many of those who died were undertreated. One reason for this may be a lack of awareness among doctors and patients on the severity of their disease. Suboptimal treatment occurs for several reasons including, doubt about the benefit, patient and physician reticence in prescribing long-term steroid therapy or poor adherence. The report underpins the fact that patients with severe asthma remain a substantial unmet clinical need.

Over the past years research has focused on the immune-mechanisms that drive asthma and significant effort has been directed at defining its endotypes or phenotypes in order to improve asthma management [345]. A phenotype is defined as the apparent characteristics of an organism resulting from its interactions of the environment, its genetic make up and distinct immune pathological mechanisms leading to clinically variable responses to treatment [18].

Vitamin D and Asthma

The reason to consider vitamin D and asthma came from a large number of epidemiological studies that highlighted the associated between low serum 25(OH) levels and the prevalence and/or severity of asthma [200, 204, 346]. Also numerous *in vitro* models have provided compelling supporting evidence and potential mechanisms for how vitamin D may influence asthma pathogenesis, as reviewed in [192]. If the association between low levels of serum 25(OH) and asthma severity could be causation, could it be reverse causation in that patients with severe asthma may be less likely to spend time outdoors in sunshine? Not according to a study by *Brehm* which found that even after adjusting for confounders such as time spent outdoors, race and atopy, there was still a strong relationship between vitamin D insufficiency and asthma exacerbations [200, 347].

To investigate these epidemiological associations and *in vitro* studies, only a few randomised controlled trials of vitamin D supplementation have so far been conducted (see table 1.3, chapter 1). Of particular interest in terms of overcoming steroid resistance is the previously mentioned VIDA study, which showed that treatment modestly decreased the overall dose of steroids used [218]. In line with that is a paediatric study by Yadav who found that supplementation with 60,000 U per month for six months allowed for a reduction in the dose of ICS in children with moderate to severe asthma [348].

The question of dose, formulation and duration will likely be important, and studies investigating the effect of vitamin D supplementation have to date used different drugs, concentrations, dosing regimens and varying periods of treatment time. In several studies many patients were not, or were borderline, vitamin D deficient, which may impact on the efficacy of vitamin D treatment. Based on findings to date, and when placed in the context of experimental data, beneficial effects of vitamin D supplementation are likely to be observed in individuals who are vitamin D deficient. Within VIDA, an exploratory analysis of patients showed that those who responded to vitamin D therapy with a rise in serum 25(OH) levels to > 30ng/ml had an overall lower

rate of treatment failure and exacerbations. This provides support to the hypothesis that flexible regimes of D replacement guided by serum 25(OH) D levels may have more therapeutic promise.

Another potential confounding matter is the observation that there may be racial differences amongst patient groups, a recent study has highlighted significant variations in vitamin D binding protein levels (to which most serum vitamin D is bound) between white and black North Americans, resulting in similar levels of bioavailable free 25(OH) D despite blacks having lower total 25(OH) D serum levels [347]. Hence, these data may also need to be taken into account when conclusions are being made with regards to serum 25(OH) levels and supplement requirements; however with an eye to clinical practicality, assays for total 25(OH) D are robust, cheap and available, this is not the case with free assays.

Calcitriol and Asthma – This Thesis

This thesis presents data of the clinical trial termed 'The Calcitriol Study', which I helped design, I obtained ethical approval for and of which I was the main clinical investigator. The study tested the efficacy of calcitriol, or active vitamin D, to improve a patient's response to steroid treatment and offered the opportunity to investigate the immunological characteristics of steroid resistant, severe asthma. The laboratory's earlier in vitro studies with calcitriol influenced the clinical approach and the choice of calcitriol rather than cholecalciferol supplementation. The alternative supplementation with cholecalciferol may be more practical and attractive, offering a relatively cheap, and safe option. Also, long term treatment with calcitriol would almost certainly raise concerns about the risk of drug induced hypercalcaemia. However, the combination of calcitriol and a steroid, known to reduce circulating calcium levels and the expression of Cyp27B1, the catalytic enzyme that converts circulating 25(OH)D into active calcitriol ($1,25(OH)_2D3$), could potential reduce the risk sufficiently. Calcitriol is a well tolerated

drug, that is easy to administer and cheap compared to many novel biological drugs; calcitriol is comparable in cost to oral prednisolone - a two-week course of 40mg prednisolone daily and calcitriol 250ng twice daily are £5.32 and £5.41 respectively. This trial has shown that treatment with calcitriol increased steroid sensitivity (see Chapter 4). The proposed steroid enhancing capacity of calcitriol, allowing for an overall reduction of prescribed oral steroids, would not only have significant effects on healthcare spending but most importantly spare patients of considerable side effects due to long term and often high dose steroid treatment frequently leading to complications such as osteoporosis, diabetes mellitus, hypertension, and adrenal insufficiency.

This study failed to show an improvement in lung function or asthma quality of life following treatment with calcitriol. More well designed clinical trials are needed to determine whether vitamin D supplementation improves asthma control and thus allows for a reduction in standard treatments such as inhaled or oral steroids. Alongside improving asthma control there are studies considering whether vitamin D may influence the *development* of the disease. The role of vitamin D in foetal lung development is well described [349, 350] and studies have shown that lower maternal intake of vitamin D is associated with increased risk of wheeze/asthma in the offspring during childhood, both in individual studies and in meta-analyses [193-195, 197].

The ongoing VDAART (Vitamin D Antenatal Asthma Reduction Trial) trial is supplementing pregnant women to either high (4400 IU daily) or low (400 IU daily) vitamin D and the results of this study will provide more information as to whether vitamin D has a preventative role in asthma.

The Importance of Defining the Immunophenotype

A Th2-high signature with elevated IL-4, IL-5 ad IL-13 cytokine levels and steroid responsiveness in patients (as opposed to Th2-low asthmatics, who failed to improve following treatment with inhaled corticosteroids) was first described by *Woodruff* [44]. In our study, cells from those asthmatics with the greatest response to prednisolone therapy

produced a significantly elevated median quantity of IL-13 at baseline, in line with *Woodruff's* data.

Mepolizumab and reslizumab - monoclonal antibodies directed against the cytokine IL-5 - have shown significant benefit in patients with severe asthma, having identified and selected those with refractory airway eosinophilia [54]. The same applied to antibodies directed against IL-13; in trials with the humanized IgG4 antibody lebrikizumab, patients selected for their high periostin levels experienced greatest treatment success [59]. After the initial lack of clinical efficacy in earlier trials with biological treatments, it has been shown that success of cytokine-targeted treatments is closely linked to the ability to define a patient's disease characteristics or endotype.

Patients with a Th2-low signature have so far remained a more complicated challenge. Th17 cytokines, such as IL-17A, have been associated with severe asthma [291]. A number of trials using antibodies directed against IL-17A in autoimmune diseases have been published with some success seen, for example in psoriasis [138], but studies in asthma have to date not only been disappointing but complicated by an increase in infections [72].

The 'Calcitriol Study' offered the unique opportunity to further identify the immunological phenotype of a clinically well-recognized and relevant population of steroid treatment refractory patients on the basis of their *in vitro* cytokine expression and found steroid resistance to be associated with an IL-17A^{high} phenotype. The results also imply that steroids might potentially be detrimental for these patients, but that treatment with calcitriol abrogated the IL-17A^{high} profile in a steroid-independent manner.

Treatment of steroid resistant asthma with calcitriol is a novel therapeutic approach without taking the risk of an increase in infection rates as seen with anti-IL-17A [72]. The data supports the concept that calcitriol improves responsiveness to steroids at least partly by altering the immune phenotype away from a steroid resistant Th17 phenotype towards a more responsive Th2 immune phenotype.

There are several possible ways in which the results of the 'Calcitriol Study' could be taken further. It would be interesting to test whether treatment with calcitriol *and* prednisolone during an asthma exacerbation leads to a better clinical response allowing for a reduced steroid dose and/or treatment period. It would also be interesting to see whether asthma patients, in particularly those with SR asthma have improved control of their disease after vitamin D supplementation to high-normal 25(OH) serum levels.

Further research is anticipated to reveal a more feasible way to test for biomarkers of IL-17^{high} SR asthma similarly to the development of periostin as a biomarker of $Th2^{high}$ asthma. With the substantial investment and development in immunotherapy and novel biological treatments against specific cytokines, there is acute focus on both accuracy and precision on the individual patient's endotype. Large, randomised controlled trials with clearly defined outcomes will provide new insights into the pathogenic role of various Th cell subsets and their cytokines and on the development and maintenance of the inflammatory condition that is asthma, its response to therapy and even its modulation.

With the global burden of asthma rising, the prospect of a therapeutic agent that will benefit the most severely affected patients has immense clinical and financial implications. To that extent is is worth exploring further whether vitamin D, in its active form or as readily available, easily tolerated and affordable cholecalciferol has a role in the management, or indeed prevention of asthma.

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Appendices

Clinical Trial Supporting Material

Publications

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STUDY PROTOCOL

A randomised control trial to test whether active vitamin D can improve the clinical response to steroids in asthmatic patients.

Protocol Number: VITD1/08

REC Ref Number: 08/H0804/84

EudraCT Number: 2008-002244-42

Investigational Product: Calcitriol

Indication: Asthma

Date of Protocol: 03-Aug-2011

Protocol Version No: 12

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Chief Investigator's Statement

I have read and understood the contents of this protocol and the Calcitriol Summary of Product Characteristics. I agree to the conduct of this study in compliance with the protocol, Good Clinical Practice, and other applicable regulatory requirements.

Chief/Principal Investigator Name: Professor Chris Corrigan

_____ _____

Chief/Principal Investigator Signature Date

CONTENTS

1 Background & Rationale

Glucocorticosteroids represent the cornerstone of asthma therapy and are safe and effective in most patients. However, 10-20% of the 5.2million UK asthma patients are characterised as glucocorticosteroid refractory, either failing to gain any clinical benefit from glucocorticoid therapy or requiring prolonged systemic treatment and are most at risk of their asthma[1].

The anti-inflammatory actions of IL-10 have been extensively documented[2,3]. IL-10 inhibits many functions relevant to asthma, it is acting on macrophages and dendritic cells to inhibit pro-inflammatory cytokine production and the capacity to activate T cells, including Th2 cells. IL-10 inhibits mast cells and eosinophil function and favourably modulates IgE to IgG_4 ratios. IL-10 is proposed to play a role in maintaining immune homeostasis in the lung (reviewed in [4]). Studies of IL-10 production in the lung and of polymorphisms in the IL-10 gene promoter suggest that individuals who make low IL-10 have more severe asthma[4-6]. Inverse correlations between IL-10 and disease severity are also observed in allergic patients[7-9]. These data have generated considerable interest in the therapeutic potential of IL-10 secreting T regulatory cells (IL-10-Treg) in allergy and asthma[4,10,11]. We believe that the principal mechanism of the anti-asthma effect of glucocorticoids is through the induction of IL-10.

The role of vitamin D and its metabolites in bone and calcium metabolism is well established, but there is increasing awareness of its importance in immune regulation [12-14]. 1-α-hydroxilase is expressed in leukocytes at site of inflammation, allowing local synthesis of calcitriol (subject to availability of 25{OH}D substrate), which can modulate immune responses in a paracrine fashion[15].
Vitamin D deficiency is very common[16] and predisposes to chronic inflammatory conditions, poor lung function and asthma[17-19]. Bioavailability of vitamin D in the body is greatly influenced by exposure to sunlight. Hypponen et al. [16] reported in their study that half of the British population that low levels of vitamin D during the winter months. This is not surprising given Great Britain's latitude, a cloudy climate and lifestyle, which involves working indoors or the use of protective clothing and sun screen. In a complementary body of work, we have demonstrated very high rates of vitamin D deficiency among individuals of South Asian origin living in London[19-21], who experience high rates of hospital admission for asthma[22]. Administration of a single dose of 2.5mg (100'000IU) vitamin D elevated 25(OH)D levels above 27.5nmol/ [23], and increased IL-10 production in whole blood stimulated ex-vivo (unpublished data).

Our published findings to date provide the rationale for this current study. We have shown that:

(i) Glucocorticosteroids induce the synthesis of the anti-inflammatory cytokine IL-10 by human CD4+ T cells[24];
(ii) T cells from steroid insensitive asthma patients fail to respond to glucocorticoids for induction of IL-10 synthesis, implying that induction of IL-10 contributes to the clinical efficacy of glucocorticoids[25];
(iii) Combining steroids with calcitriol greatly boosts IL-10 synthesis by T-cells (IL-10-Treg) from healthy donors, inducing a regulatory phenotype in these cells[26];
(iv) Dexamethasone and calcitriol induced IL-10-Treg inhibit proliferation and cytokine production by autologous T cells, including allergen specific Th2 cell lines[27];

(v) The treatment of T cells from steroid insensitive asthma patients with glucocorticoids plus calcitriol restores induction of IL-10 synthesis to levels observed in steroid sensitive patients treated with glucocorticoids alone[27];

(vi) Calcitriol prevents ligand induced down regulation of the glucocorticoid receptor[27], which may contribute to its efficacy in restoring steroid induced IL-10 synthesis in insensitive asthmatic patients;

(vii) A small pilot study has demonstrated that ingestion of calcitriol by steroid insensitive asthma patients greatly enhances the subsequent capacity of their T cells to respond to dexamethasone for induction of IL-10 synthesis[28]. This response appeared maximal by 3 days, in agreement with the known kinetics of calcitriol[28].

2 Trial Objectives, Design and Statistics

Calcitriol (1-α-25-dihydroxyvitamin D3, the active form of vitamin D3), enhances clinical responsiveness to systemic glucocorticoid therapy in asthma patients who are clinically insensitive to steroids. Induction of IL-10 secreting T-cells through the combined effects of glucocorticoid and 1-α-25-dihydroxyvitamin D3 is implicated in mediating this effect.

2.1 Trial Objectives

1. Our primary aim is to perform a clinical trial to determine whether 1-α-25 dihydroxyvitamin D3 (calcitriol at recommended pharmacological dosage) enhances the clinical response to systemic glucocorticoids in glucocorticoid refractory asthma patients.

2. We will monitor changes in IL-10, Th2, pro-inflammatory cytokines and other immunological markers during the course of the study to test their value in predicting or confirming drug action and efficacy for use in future clinical trials and to further implicate IL-10-Treg induction in the anti-asthma mechanism of glucocorticoids.

3. Identification of specific markers for drug-induced IL-10-Treg will facilitate monitoring of steroid responsive status, identification of non-responsive individuals, and tracking of drug-induced IL-10-Treg cells in vivo. We are using Affymetrix gene profiling, quantitative (q)RT-PCR and flow cytometry to analyse genes specifically expressed in human drug induced CD4+ IL-10-Treg cells, Th1 and Th2 cells. Markers of interest include Toll-like receptor (TLR)9, which is increased in vitro and in vivo by calcitriol and TLR2 which is upregulated by glucocorticoids. We also intend to store blood cells for further transcript analyses of biomarkers associated with vitamin sufficient and insufficient patients pre- and post supplementation of vitamin D.

2.2.1 Trial Design

Screen Visit 1
Informed consent will be obtained before screening starts. Suitable subjects will undergo full assessment of their medical history (including a complete smoking history) and laboratory evaluation for safety haematology and biochemistry profile, as well as baseline cortisol. Up to 120ml of venous blood will also be drawn for ex-vivo experiments. Spirometry will be performed and the FEV_1 and FVC obtained. Reversibility will be assessed by giving the subject a short acting beta agonist and measuring the FEV_1 pre- and 15-30mins post-bronchodilator. We will measure FeNO using an Aerocrine NIOXMINO monitor, following the ATS/ERS guidelines. A peak flow meter and a diary card will be given to the subject to record morning and evening peak flows throughout the study.
All these procedures are done in accordance to departmental Standard Operating Procedures.

Eligible subjects will be entered into the second part of the screening process. These Subjects will receive prednisolone 40mg/1.73m2/day (where m2 = Body Surface Area, BSA). The dose will be calculated as (patient's BSA (m2)/1.73)x 40mg and rounded down to the nearest 5 mg, to be taken at home.

Screen Visit 2
The subject's medical history and adverse events will be reviewed at the start of the screening visit 2. Spirometry will be performed and the FEV_1 and FVC obtained. Subjects who demonstrate an increase of > 10% in the FEV_1 when compared to baseline (screen visit 1) will be excluded. Subjects who are still eligible will be entered into the main part of the trial. We will repeat FeNO in exhaled air. Up to 120ml of venous blood will be taken for safety haematology and biochemistry, repeat cortisol and prednisolone levels to confirm compliance, and for ex-vivo experiments.

Treatment Visit Day 1
Following a 4 week washout period (from screen visit 2) participants will attend Treatment Visit Day 1. Under special circumstances Christmas breaks, summer holidays, for example) the wash out period can be extended for a maximum of 4 weeks (20 working days). The subject's medical history and adverse events will be reviewed at the start of this visit. Up to 100ml of venous blood will be taken for safety haematology and biochemistry and for ex-vivo experiments.
Participants will be randomised to receive either calcitriol or placebo. The first dose will be done under supervision. Subjects will be discharged following first dosing and will take the subsequent doses at home.

Treatment Visit Day 15
On day 15 of treatment subjects will attend Treatment Visit Day 15. Subjects will be asked to withhold their Day 15 treatment dose prior to attending. The subject's medical history and adverse events will be reviewed at the start of this visit Prior to dosing up to 120ml of venous blood will be taken for safety haematology, biochemistry and cortisol, and for ex-vivo experiments.
Subject will also undergo spirometry and fraction of nitric oxide in exhaled air (FeNO) measurement pre-dose. These will be done in accordance to departmental standard operating procedures. A standard Asthma Control Questionnaire (ACQ)[32] will also be completed by the subject pre-dose.

FeNo measurements are highly correlated with eosinophilic airway inflammation; eosinophilic inflammation is correlated with response to steroid treatment[33,34] . We will measure FeNO using an Aerocrine NIOXMINO monitor, following the ATS/ERS guidelines.

Subject's who are still eligible to continue in the study will be commenced on prednisolone 40mg/1.73m^2/day as a single dose for 14 days. The first dose of prednisolone and Day 15 dose of treatment article will be done under supervision. Subjects will be discharged following dosing and will take subsequent doses of treatment and prednisolone at home.

Treatment Visit Day 28
On day 28 of treatment subjects will attend Treatment Visit Day 28. Subjects will have completed a 28 day dosing period of treatment article and a 14 day dosing period of prednisolone. The subject's medical history and adverse events will be reviewed at the start of this visit. Following this up to 120ml of venous blood will be taken for safety haematology, biochemistry, cortisol and prednisolone, and for ex-vivo experiments. The subject will perform spirometry, and fraction of nitric oxide in exhaled air (FeNO). A standard Asthma Control Questionnaire (ACQ)[32] will also be completed by the subject. A physical examination will also be performed.

Follow up Day 56
After 1 month subjects will be interviewed either during a routine visit to the asthma clinic or via telephone call to check medical history and adverse events. Following all these procedures subjects will be discharged from the study.

Immunological studies
A central aim of this work is to further underpin the role of IL-10 in mediating the immunomodulatory effects of steroids and vitamin D. Our recent study clearly demonstrates a correlation between expression of IL-10 by T cells and their inhibitory functions relevant to asthma[27]. We aim to identify immunological responses to steroids and 1-α-25-dihydroxyvitamin D3, in the asthma patients under study

i. Cell analysis: all bloods will be analyzed for cellular content by antibody staining and FACS analysis to determine any effect of prednisolone +/- 1-α-dihydroxyvitamin D3 on relevant cell populations (i.e. T -, B-, NK-cells, monocytes, dendritic cells, eosinophils and neutrophils) and subsets [e.g. T naïve, memory, effector, central memory and regulatory cells] - 5mls blood required
ii. Serum will be prepared for analysis of vitamin D levels (P Timms, Homerton Hospital) and concentrations of cytokines of interest (including IL-17, Th1, Th2, IL-10), measured using a highly sensitive (1-5pg sensitivity) multiplex system (MSD) at indicated sampling points– 5ml blood required.
iii. Ex vivo cytokine analysis: we will collect cell pellets and prepare cDNA for direct ex vivo analysis of cytokine gene expression in total blood and purified CD3+CD4+ T cell preparations using qRT-PCR[27]. This will identify the overall biological response to the drugs versus the T cell contribution to any observed changes. Cytokines of interest include the anti-inflammatory mediators IL-10 and TGFβ, as well as the effector

cytokines IL-17 and Th1 and Th2-associated cytokines. Gene expression will be compared relative to 18S, using our established methodology[27].– 15mls blood required.

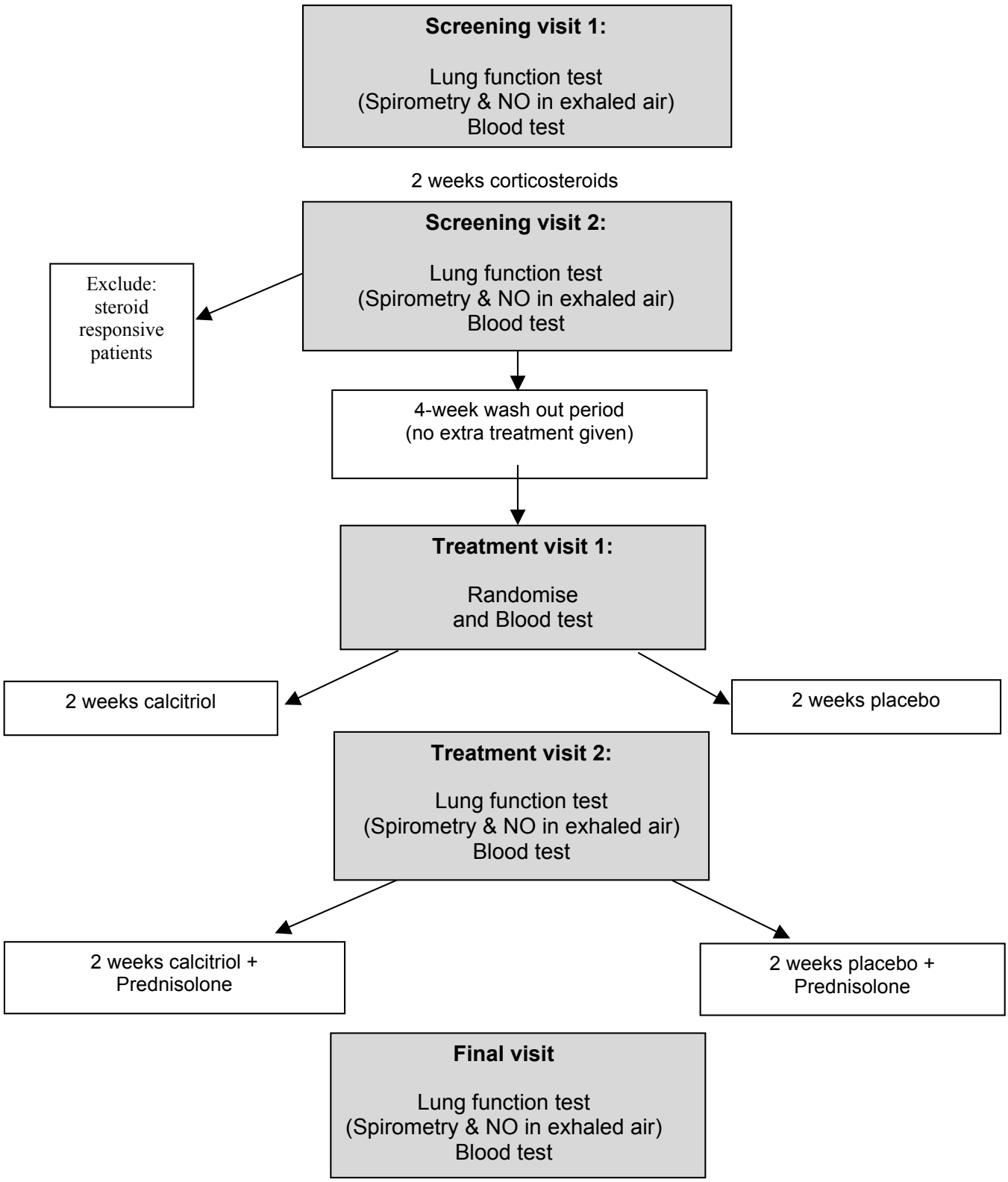
iv. Does drug administration correlate with the induction of biomarkers associated with IL-10 secreting T regulatory cells? We will analyse directly ex vivo by qRT-PCR and where feasible flow cytometry, changes in the expression of markers associated with naturally occurring CD3+CD4+25Treg (e.g. Foxp3), IL-10-Treg (e.g. TLR9, IL-10, CD38), Th1 (e.g. T-bet) and Th2 (e.g. GATA-3) lineages at the 5 time points to be sampled. Both whole blood and CD3+CD4+ T cells will be studied directly ex vivo or following a 4 hour activation protocol with PMA and ionomycin. In addition effects on GRα and GRβ will be monitored[27] – 20ml blood required.

v. Gene array profiling analyses (e.g. Affymetrix): whole blood will be stimulated with control stimuli or allergen for 20 hours and then prepared for gene array analysis – 15mls blood required.

vi. Analysis of changes in steroid sensitivity: We will compare changes in response to steroid (10^{-7} to 10^{-10}M) +/- 1-α-25-dihydroxyvitamin D3 (10^{-7}M) on cytokine gene induction and repression in cultures of total PBMC and purified CD+4 T cells stimulated with anti-CD3, using our published methodology[27], at all time points sampled. Cytokines of interest, as listed above, will be assessed using ELISA/MSD, qRT-PCR and FACS as appropriate – 40ml blood required.

2.2.2 Flowchart

	Screen Visit 1	Screen Visit 2	Treatment Visit 1	Treatment Visit 2	Final Visit
Patient information and informed consent	x				
Lung function					
- Spirometry	x	x		x	x
- NO	x	x		x	x
Blood test	x	x	x	x	x
ACQ Questionnaire				x	x
Treatment					
- Calcitriol			x	x	
- Prednisolone	x			x	


Screening visit 1:
Lung function test
(Spirometry & NO in exhaled air)
Blood test
2 weeks corticosteroids
Screening visit 2:
Lung function test
(Spirometry & NO in exhaled air)
Blood test
Exclude:
steroid
responsive
patients
4-week wash out period
(no extra treatment given)
Treatment visit 1:
Randomise
and Blood test
2 weeks calcitriol
2 weeks placebo
Treatment visit 2:
Lung function test
(Spirometry & NO in exhaled air)
Blood test
2 weeks calcitriol +
Prednisolone
2 weeks placebo +
Prednisolone
Final visit
Lung function test
(Spirometry & NO in exhaled air)
Blood test

2.3 Trial Statistics

2.3.1 Sample Size calculation

Although we cannot anticipate the actual magnitude of enhancement of the FEV_1 response to prednisolone produced by concurrent vitamin D therapy in this study, we can readily estimate our power to detect theoretical enhancements based on our resent study in which 23 moderate to severe asthmatics underwent a similar prednisolone trial[31] . Mean (SD) percent improvement in FEV_1 was 15.0 (32.5%). With >/= 40 subjects the proposed study will have 90% power at α=0.05 to detect a 20% enhancement of the FEV_1 response to prednisolone with concomitant vitamin D therapy. The powering is therefore conservative to detect even a relatively small improvement in the FEV_1 response.

2.3.2 Randomisation

A statistician/clinician not involved in the trial will generate a randomised list with the help of a computerised randomisation plan generator, which will be kept in Guy's pharmacy and treatment allocated accordingly. Allocation concealment will be maintained, so that investigators and patients will be blinded to the nature of treatment. Eligible participants will be randomised in a 1:1 ratio to receive either placebo or calcitriol.

Guy's and St Thomas' NHS Foundation Trust and a statistician/clinician not involved in the trial will keep a copy of the randomisation code. The investigator or treating physician may unblind a participant's treatment assignment only in the case of an emergency, when knowledge of the study treatment is essential for the appropriate clinical management or welfare of the subject.

2.3.3 Statistical Methodology

Our dual aims are to provide information for a larger study and seek evidence of a clinically important effect on lung function. Our analyses will centre on descriptive statistics such as means or medians and appropriate confidence intervals, which provide measures of between group comparisons that will be useful in estimating likely effect sizes and sample size requirements in a larger study. Means, medians and confidence intervals can be calculated in the statistical software package, Stata. Where statistical analyses are undertaken these will be paired for within patient comparisons and unpaired for between patient comparisons. Given the small sample sizes we will use non-parametric tests (Mann-Whitney U test for unpaired and Wilcoxon matched pairs for paired data).

3 Trial Medication

3.1 Investigational Medicinal Product and Placebo

The investigational medicinal product in this study is Calcitriol produced by Roche Products Limited, Marketing Authorisation Number PL/0031/0122, 0.25mcg: soft capsules. The soft capsule will be encapsulated into a hard capsule to have the same appearance as the placebo.
Subjects randomised to receive the investigational medicinal product will receive 0.25mcg twice a day orally for 28 days.

The placebo used is matching hard capsules with the same appearance and texture as the Investigational Medicinal Product. Subjects randomised to receive placebo will receive 1 capsule of matching placebo twice a day orally for 28 days.

Subjects will also receive prednisolone 40mg/1.73m2/day (where m2 = Body Surface Area, BSA) for 14 days, to be taken at home. The dose will be calculated as (patient's BSA (m2)/1.73)x 40mg and rounded down to the nearest 5 mg. Prednisolone is produced by Wockhardt UK Ltd, Marketing Authorisation Number PL 29831/0178: flat faced tablets for 14 days.

4 Selection and Withdrawal of Subjects

4.1 Inclusion Criteria

1. Male or Female adults aged between 18 to 75 years.
2. Documented history and typical symptoms of asthma for ≥ 6 months prior to screening.
3. Pre-bronchodilator FEV_1 < 80% predicted and documented variability in airways obstruction of 12% or greater within the previous 5 years or diurnal Peak Flow variability of > 20%.
4. Corticosteroid refractory asthma, as defined by a < 10% improvement in FEV_1 following a 14 day course of prednisolone 40mg/1.73m^2/day[29-31].
5. Written informed consent received.

4.2 Exclusion Criteria

1. Past or present disease, which, as judged by the investigator, may affect the study outcome (other than asthma, rhinitis or eczema).
2. Serum corrected calcium >2.66mmol/L
3. Clinically significant deviation from normal (physical examination or laboratory parameters) as judged by the investigator at the screening visit.
4. Current smoker or an ex-smoker of less than 5 years with a greater than 5 pack year history.
5. Pregnant or lactating females or those at risk of pregnancy (women of childbearing age may be offered a pregnancy test prior to recruitment).
6. History of a respiratory tract infection and/or exacerbation of asthma within 4 weeks of the screening visit requiring oral corticosteroid tablets.
7. Participation in a study involving an investigational medicinal product in the previous 3 months or blood donation within the last year.
8. Current or previous allergen immunotherapy.
9. Concomitant treatment with lithium carbonate or calcium supplements. Thiazide diuretics are a contraindication if the participant is taking calcium supplements at the same time.
10. Inability to understand or comply with the research protocol

4.3 Withdrawal of Subjects

Subjects will be withdrawn from investigational product treatment if they develop hypercalcaemia (corrected serum calcium >2.65 mmol/l confirmed on two samples) during the course of the trial. This will be monitored at each follow-up timepoint.
All outcome data at all subsequent follow-ups will continue to be collected for subjects.
Subjects will be replaced to achieve a recruitment number of at least 40 participants.

4.4 Concomitant Medication

Medication not permitted before and during trial include Benzothiadiazine derivatives and Calcium supplements as per exclusion criteria.

Patients requiring rescue medication for exacerbation of asthma in form of corticosteroids immediately before and during the trial will be excluded or withdrawn from the trial (this includes the four week wash out period).
Throughout the study the participants are allowed to continue their usual asthma medication as prescribed by their doctor (for example short and long acting beta agonists (inhaled and oral form) including slow release, anticholinergica, inhaled corticosteroids, leukotriene receptor antagonists, theophyllines or antihistamines). Changes to background treatment are allowed as prescribed by the participant's doctor.

5 Assessment of Efficacy

Hypothesis
Administration of calcitriol for 4 weeks to moderate/severe asthmatics, who have previously demonstrated clinical refractoriness to systemic steroids:

(a) Significantly increases their clinical response to oral prednisolone therapy administered, during the final 2 weeks of calcitriol treatment, and
(b) The degree of clinical response correlates with prednisolone/calcitriol-induced T cell IL-10 production ex vivo.

5.1 Efficacy Parameters

5.1.1 Primary Outcome Measure/End Point:
The primary outcome measure is the change in FEV_1 at baseline compared to the end of the treatment period.

5.1.2 Secondary Outcome Measure/End Point:
The secondary outcome measures are

1. level of the ex-vivo production of IL-10 and other surrogate biomarkers of outcome or drug effects/process by T-cells.
2. Serological markers of inflammation
3. Fraction of nitric oxide in exhaled air
4. ACQ score

6 Assessment of Safety

6.1 Specification, Timing and Recording of Safety Parameters

Full blood counts and serum biochemistry profiles will be taken on every visit of the study. Results outside the normal ranges of the analysing laboratories will be reported as adverse events only if considered to be clinically significant by the trial investigator. Participants who experience adverse events will be followed up by their usual clinical team or the trial investigators for a clinically appropriate duration, as determined by the trial investigator, although measurements outside the following ranges will be reported as adverse events regardless of their perceived significance:

Sodium <115 or >160 mmol/L
Potassium<2.5 or >6.0 mmol/L
Urea >15 mmol/L
Creatinine >175 umol/L

WBC <2.0 or >20.0 $x10^9$/L
RBC <2.0 or >8.0 $x10^{12}$/L
HB <9 or >19 g/dL
PCV <0.2 or >0.7 L/L
MCV <60 or >120 fL
MCH <20 or >50 pg
RDW <5 or >30
PLT <40 or >600 $x10^9$/L
Neutrophils <0.75 or >20 $x10^9$/L
Lymphocytes <0.5 or >10 $x10^9$/L
Monocytes <0.05 or >5.0 $x10^9$/L
Eosinophils > 5.0 $x10^9$/L
Basophils >5.0 $x10^9$/L

In view of the effects of Calcitriol on serum calcium a further safety parameter is:

1. Specification of the serum corrected calcium:
   Subjects found to have a serum corrected calcium > 2.65mmol/l, which is confirmed by a second blood test, will be withdrawn from the study.
2. The methods and timing for assessing, recording and analysing safety parameters:
   Corrected calcium will be determined at 0,2 and 4 weeks post initiation of calcitriol by staff in the Department of Clinical Biochemistry at Guy's Hospital or at Barts and The London Hospitals using standard analyser. At each follow-up time point the serum corrected calcium result for that day will be awaited before any further dose of calcitriol is administrated. If corrected serum calcium exceeds 2.65mmol/l at any follow-up time point, the test will be repeated. If the second test confirms hypercalcaemia, calcitriol will be withheld for the rest of the study.
3. In addition to the above, patients who develop symptoms of hypercalcaemia (nausea, vomiting, malaise, polydipsia or polyuria) in between follow-up visits will be advised to contact the research study doctor without delay. The study doctor will then arrange for an urgent clinical assessment including determination of corrected serum calcium.
   Participants who experience adverse events will be followed up by their usual clinical team for a clinically appropriate duration, as determined by their consultant. Where an adverse event is attributed to vitamin D, this management may be guided by serial determinations of serum calcium levels. These patients will be followed up again within 28 days after completing the study as part of their usual care. As the elimination half-life of serum calcitriol is 5-8 hours in normal subjects only, it is anticipated that any adverse events attributable to administration of vitamin D will have resolved within a few days.

6.2 Procedures for Recording and Reporting Adverse Events

The Medicines for Human Use (Clinical Trials) Regulations 2004 and Amended Regulations 2006 gives the following definitions:

Adverse Event (AE): Any untoward medical occurrence in a subject to whom a medicinal product has been administered including occurrences which are not necessarily caused by or related to that product.

Adverse Reaction (AR): Any untoward and unintended response in a subject to an investigational medicinal product which is related to any dose administered to that subject.

Unexpected Adverse Reaction (UAR): An adverse reaction the nature and severity of which is not consistent with the information about the medicinal product in question set out in:
The summary of product characteristics (SmPC) for that product (for products with a marketing authorisation)
The Investigator's Brochure (IB) relating to the trial in question (for any other investigational product)
Serious adverse Event (SAE), Serious Adverse Reaction (SAR) or Unexpected Serious Adverse Reaction (USAR): Any adverse event, adverse reaction or unexpected adverse reaction, respectively, that
- Results in death;
- Is life-threatening;
- Required hospitalisation or prolongation of existing hospitalisation;
- Results in persistent or significant disability or incapacity;
- Consists of a congenital anomaly or birth defect.

Reporting Responsibilities
Guy's and St Thomas' NHS Foundation Trust and King's College London have delegated the Sponsor responsibilities of Pharmacovigilance (as defined in Regulation 5 of the Medicines for Human Use (Clinical Trials) Regulations 2004 to the Joint Clinical Trials Office (JCTO).

All SAEs, SARs and SUSARs (excepting those specified in this protocol as not requiring reporting) will be reported immediately by the Chief Investigator to the (JCTO) in accordance with the current Pharmacovigilance Policy.

Death as a result of disease progression and other events that are primary or secondary outcome measures are not considered to be SAEs and should be reported in the normal way, on the appropriate CRF.

The JCTO will report SUSARs (Suspected Unexpected Serious Adverse Reactions) and other SARs to the regulatory authorities (MHRA, competent authorities of other EEA (European Economic Area) states in which the trial is taking place.

The Chief Investigator will report to the relevant ethics committees. Reporting timelines are as follows:

- SUSARs which are fatal or life-threatening must be reported not later than 7 days after the sponsor is first aware of the reaction. Any additional relevant information must be reported within a further 8 days.
- SUSARs that are not fatal or life-threatening must be reported within 15 days of the sponsor first becoming aware of the reaction.

The Chief Investigator will provide an annual report of all SARs (expected and unexpected), and SAEs which will be distributed to the Sponsor (JCTO), MHRA and the REC.

6.3 Treatment Stopping Rules

At each follow- up timepoint the serum corrected calcium result for that day will be awaited before any further dose of trial medication is administered. If corrected serum calcium exceeds 2.65 mmol/l at any follow-up timepoint, the test will be repeated. If the second test confirms hypercalcaemia, study medication will be withheld for the rest of the study.

7 Direct Access to Source Data and Documents

The sponsor will notify the MHRA of any fatal or life-threatening SUSAR arising during the study within 7 days of learning of the event; non-fatal and non-life-threatening SUSAR will be reported within 15 days of learning of the event. The sponsor may also report other safety issues to MHRA where they might alter the current risk-benefit assessment of vitamin D as an adjunct to standard asthma therapy, or alter the overall conduct of the trial. They will ensure that quality standards are met and that personnel are trained for the purpose of data submission, validation, entry and review.

8 Ethics & Regulatory Approvals

The trial will be conducted in compliance with the principles of the Declaration of Helsinki and the principles of GCP.
The study has been submitted to Guy's Research Ethics Committee and has received a favourable opinion.
Any subsequent protocol amendments will be submitted to the REC and Regulatory Authorities for approval. We will comply with regulations, particularly specifying, Pharmacovigilance reporting and providing the REC & MHRA with progress reports, and a copy of the Final Study Report.

8.1 Ethical Considerations

Rationale for research
Ethical research must be informed by existing research, and investigate an important question. We have addressed this issue by thorough review of the existing literature, and by conducting extensive background studies *in vitro* and *in vivo* ourselves.

Design of research
Ethical research must employ the most appropriate design in order to answer the research question. When investigating efficacy of a clinical intervention, a double blind, randomised, placebo-controlled trial is the gold standard methodology.

Minimization of inconvenience, discomfort and risk for participants
Ethical research must seek to minimize potential inconvenience, discomfort and risk that participants may experience during the course of a study. The principle inconveniences of the study arise from the time spent returning for follow-up visits; we have sought to minimize these by providing financial compensation for travel costs and loss of earnings.

The principle discomfort involved arises from giving blood samples; we have sought to minimize this by assuring that venepuncture is performed by either the study doctor or a research nurse trained in phlebotomy. Patients with anaemia or at risk of blood loss or those who gave blood (1 unit) within the past year are excluded from the study.
The principle risk arises from vitamin D-induced hypercalcaemia; we have sought to minimize this by excluding patients known to be at potentially increased risk of developing hypercalcaemia after vitamin D supplementation; by giving a dose of calcitriol, which is in accordance to the British National Formulary; and by frequent monitoring of serum calcium levels.

Confidentiality
Ethical research projects should ensure that participants' personal data remain

confidential. Our procedures for handling, processing, storage and destruction of data are compliant with the Data Protection Act 1998.

8.2 Ownership, Storage and Fate of Donated Samples

Donated samples will be treated as a gift from the participant to King's College London University of London.
King's College London will have control over what happens to them and will retain commercial rights to any profitable invention arising from the research.

Donated samples will be analysed in the laboratory at King's College University of London. Whole blood for DNA extraction will be stored in a freezer until recruitment is complete. On completion of recruitment, DNA will be extracted from whole blood and analysed cytokine gene expression in total blood and purified CD3+CD4+ Tcell preparations using qRT-PCR[27]. On completion of the study, serum and DNA samples will be transported to and stored for up to 15 years in accordance with the Human Tissue Act 2004 and Human Tissue Authority Codes of Practice. Any further investigation, which made use of these samples, will only be conducted with the approval of the ethics committee before the end of this trial. Only laboratory staff will have access to frozen samples. Samples will be labeled with participants' study number, and not with their name. Donated samples, which are not stored will be destroyed according to standard procedures.

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PATIENT INFORMATION SHEET

Study Title: Does active vitamin D3 improve the clinical response to steroids of steroid-resistant asthma patients?

Date 15 Jun 2010

Version 04

You are being invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with your friends, relatives and your GP 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 to take part.

Thank you for reading this.

Why is this study being carried out?

Steroids are the cornerstones of asthma therapy. However, some patients with severe asthma do not improve on steroids at all (steroid resistant), whilst others are unhappy taking high doses of steroids for prolonged periods to control their asthma. We believe that adding Vitamin D, a safe standard vitamin supplement to steroid tablets, will improve the clinical response to steroids in patients who are considered to be steroid resistant. We believe this is because vitamin D increases the production of an anti-inflammatory protein from your white blood cells called interleukin-10 (IL-10). It has been shown that patients with severe asthma, who do not respond to steroids have lower levels of IL-10.

Why have I been chosen?

You have been chosen to participate because you have asthma and we are trying to improve asthma treatment. Altogether, we will recruit 40 patients like you for this study.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you do, we will give you this information sheet to keep and ask you to sign a consent form. You are still free to withdraw from the study at any time without giving a reason. If you decide not to take part, or to withdraw, it will not affect your normal standard of care, either presently or at any time in the future.

Will I get paid to take part?

We will reimburse your travel costs and any inconvenience caused with £40 per visit (£200 in total).

What will happen during the study?

If you agree to take part in this study you will be asked to attend on 5 occasions as described below:

First visit: screening visit 1

This is called the screening visit 1 and will take about 1 hour.
If the breathing test shows you do not have moderate to severe asthma then you cannot be included in this study and will go no further.
We will ask you a few questions relating to your asthma and your smoking history.
We will perform lung function tests, where you will be blowing into a tube. You will be asked to blow again after a dose of salbutamol (Ventolin®) reliever to see how much your asthma improves. You will probably have had this sort of lung function test in the past when you visited the asthma clinic. We will ask you not to use your normal inhalers for 8 hours before the test unless absolutely necessary.
Based on the lung function tests we will see whether you have moderate to severe asthma and we can tell you whether you are suitable for this study.

We will also ask you to record your Peak Flow results during the next coming weeks. We will give you a Peak Flow meter to take home and show you how to use it and record your results. We ask you to do this twice a day (morning and evening).

If your breathing test shows that you have moderate to severe asthma we would like to include you in the study and will perform a blood test (120ml, about half a small teacup full) from your arm. We will use this sample to measure your vitamin D levels, calcium levels, cortisol levels, eosinophil levels (= white blood cells often elevated in asthmatics), and levels of IL-10, the anti-inflammatory marker known to have important anti-asthma effects. You will then have a further breathing test called Nitric Oxide in exhaled air. Nitric oxide is a marker we can measure when you breathe out and is a test to diagnose asthma. Patients with asthma have increased levels of nitric oxide.

We will then give you a steroid tablet called Prednisolone at a dose of 30-60mg (depending on your body weight) once in the morning for 2 weeks.
You might have been prescribed this tablet in the past to treat your asthma. You will continue using your normal inhalers.

Second visit: screening visit 2
This is called the screening visit 2. After 2 weeks of taking Prednisolone we will repeat your breathing test to see whether any blockage or constriction in your airways has improved. If the blockage improves by less than 10% you will qualify for this study. This is what we call steroid resistant (no improvement following steroid treatment). You will then have a further breathing test called Nitric Oxide in exhaled air.
You will also be asked for another blood sample (120ml).
This will take about 1 hour.

Third visit: treatment visit 1
You will attend the clinic again after 4 weeks (during this time you will use your own inhalers as usual) and you will be given either vitamin D (calcitriol) at a standard dose of 25mcg twice a day or a tablet that looks and tastes the same but contains no vitamin D (this is called a placebo). Neither you nor we will know which tablet you have taken (although we can find out if necessary). You will have an equal chance of getting vitamin D or the placebo tablet. You will take the tablet for 4 weeks.
You will have a further blood test (100ml) that day. This will take about 30 minutes.

Fourth visit: treatment visit 2
During this visit we will ask you to answer questions regarding your asthma and how it affects your daily life and what symptoms you get (= asthma quality of life questionnaire).
After 2 weeks you will receive a course of Prednisolone tablets at the same dosage as at the beginning of the study, which you take together with the vitamin/placebo tablet.
We will repeat a blood test (120ml) and measure your lung function.
You will have a further breathing test called Nitric Oxide in exhaled air. This visit will take about 1 hour.

Fifth visit: final visit
We will go through the same questionnaire as during your last visit, inquiring about your asthma-symptoms.
At the end of the study, having taken vitamin D or placebo and Prednisolone we will see you once more for a final blood test (120ml). We will repeat the same breathing tests as during your fourth visit: simple lung function tests (spirometry) and measurement of nitric oxide when you breathe out.
This will take about 1 hour.

Follow up
We will contact you via telephone call or on your next routine visit to the asthma clinic (if this happens to be around 2 months after your first treatment visit) to check whether you remain well and have not suffered any side effects.

What is vitamin D?

This is the vitamin that is made in your skin when you sunbathe; you can also get it from taking cod liver oil. It is important for healthy bones and a strong immune system. The vitamin D that we use has been manufactured and safely prescribed by doctors before.
You should not participate in the study if you are pregnant, have kidney stones, an overactive parathyroid gland, a condition called sarcoidosis, or if you have been diagnosed with liver problems, kidney problems or cancer.

Why do I only have a 1 in 2 chance of getting vitamin D?

The best way to find out if a treatment works is compare a group of patients who get the treatment with a group who do not. In order to make sure the groups are the same to start with, we will use a computer program to allocate you to one group or the other at random (as if tossing a coin).

What are the possible disadvantages and risks of taking part?

High levels of vitamin D have been known to harm the unborn child; you should not take part in this study if you are pregnant or breastfeeding. You mustn't become pregnant during the study and we will advise you on double barrier contraception.
If you do become pregnant during the study you must tell your study doctor immediately.
Potential side effects of the steroid tablet Prednisolone are almost uniquely secondary to prolonged use (> 3months) and include: fluid retention, weight gain, high blood pressure, potassium loss, headache, muscle weakness, puffiness of and hair growth on the face, thinning and easy bruising of the skin, vision problems, heartburn, worsening of diabetes, irregular menses, convulsions, and psychic disturbances.
Calcitriol may cause constipation, thirst, dry mouth, muscle pain, bone pain, weakness, increased urination, rashes, high levels of calcium in your blood, sleepiness, loss of appetite, headaches, vertigo, nausea.
Venepuncture can cause bruising.

What are the possible benefits of taking part?

If you take part in this research we will test your vitamin D level: if we find that this is low, we will inform your doctor at the end of the study and advise you how to prevent deficiency in the future.

Your participation in this trial may also give us information that could improve treatments for other people who suffer from asthma.

What happens when the study stops?

When the study stops, the research doctor will let you know whether you have been taking vitamin D tablets or the placebo tablet. We will also tell you the result of your vitamin D level taken at the beginning of the study.

If your vitamin D level was low at the beginning of the study, and you were given the placebo tablet during the study, we will inform your doctor. All patients who had low levels of vitamin D at the start of the study will get advice about how to prevent deficiency arising in the future.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the doctor in charge of the study. If you remain unhappy and wish to complain formally this should be addressed in writing to:
Professor Chris Corrigan, King's College London School of Medicine (at Guy's, King's and St Thomas' Hospitals), Department of Asthma, Allergy and Respiratory Science, 5th Floor, Thomas Guy House, Guy's Hospital, London, SE1 9RT .

Or: Please contact Patient Advisory Liaison Service (PALS) if you have any concerns regarding the care you have received, or as an initial point of contact if you have a complaint.
If you are based at Guy's and Thomas' NHS Foundation Trust: Please phone 020 7188 8801, or email pals@gsst.nhs.uk or visit PALS by asking at any hospital reception
If you are based at The Barts and The London NHS Trust: Please telephone 020 7377 6335, minicom 020 7943 1350, or email pals@bartsandthelondon.nhs.uk, you can also visit PALS by asking at any hospital reception.

Will my taking part in the study be kept confidential?

Maintaining confidentiality is important to us.
Your personal information (for example, your gender, age, the details of your medical conditions) and other information (the data collected by King's College London School of Medicine as part of the study) will be identified by a number (i.e., coded). Your name will not appear in any publications or reports produced from this study. You have the right to ask the study doctor about the data being collected on you for the study and about the purpose of this data. You have the right to ask the study doctor to allow you to see your personal information and to have any necessary corrections made.

We will inform your GP that you have agreed to participate in this study. Should your Vitamin D levels be low we will inform you and your GP about it unless you do not wish so.
Your blood samples are identified by means of a unique number and will not be traceable directly to you.
Results obtained from the samples are treated with the strictest confidence and will not be used for any other diagnostic purpose. All of the samples will be stored securely within our department.

In the event of surplus samples following completion of this study, it will be stored anonymously within the department of Asthma, Allergy & Respiratory Science. Your informed consent to this study allows such surplus material to be used as a gift for future, related departmental studies. The samples would only be used in studies which receive approval of the ethics committee before the end of this study.
This situation will only arise following consent of the doctor in charge of this study.
We will not be using any of the blood for analysis of your genetic code; neither will pharmaceutical companies use any of your tissue for commercial gain.

If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the Institutions sponsoring and organising the research. They may also be looked at by authorised people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty.

What will happen to the results of the research study?

The data collected from you and other study subjects will be used to prepare a detailed research report. The results may be published in the form of a scientific paper. If this is the case, you will not be identified in any results of this study. If you would like to see any published results then please ask your study doctor. These results may not be available for some time, as the study data have to be analysed.

Who is organising and funding the research?

The study is being funded by research grants held by the Department of Asthma Allergy and Respiratory Science at Guy's Hospital and the MRC Asthma UK Centre in Allergic Mechanisms, Centre for Health Sciences at Barts and The London Hospital NHS Trust.

Who has reviewed the study?

Guy's Research Ethics Committee has reviewed this study and has given a favourable opinion for the research to go ahead. The Committee is recognised by the United Kingdom Ethics Committee Authority under the Medicine for Human Use (Clinical Trials) Regulations 2004, and is authorised to carry out the ethical review of clinical trials of investigational medicinal products.

Thank you for taking the time to read this information sheet. If you have any queries concerning this study please contact us on the above number and address. You may contact an impartial third party with any questions you have about the research and your rights as a research subject.

Dr. A. Nanzer, Clinical Research Fellow

Guy's and St Thomas' NHS
NHS Foundation Trust

KING'S College LONDON
University of London

CONSENT FORM

Study title: Does active vitamin D3 improve the clinical response to steroids of steroid-resistant asthma patients?

Date 15-Jun-10, Version 5

LREC Reference number: 08/H0804/84

1.	I confirm that I have read and understand the information sheet (PIS Version 4, dated 15-Jun-10) for the above study and have had the opportunity to ask questions.	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	☐
3.	I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the Sponsor, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records	☐
4.	I agree that my blood may be stored for use in this study.	☐
5.	I agree that my blood may be stored anonymously. I agree that my blood may be stored for use in future studies which have been approved by the Head of Department and received appropriate ethical approval..	☐
6.	I agree to take part in the above study	☐

Name of Patient	Date	Signature
Name of Person taking consent	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes
Chief investigators: Prof. C. Corrigan

SITE: _____

CASE REPORT FORM

(version 03)

Clinical Trial:

Does active vitamin D3 enhance corticosteroid activity in severe asthma?

__ __ / __ __ / __ __ __ __ (Date of visit)	**Patient Number:** **Visit No:**
DEMOGRAPHIC DATA	

Patient's initials:		
Date of Birth: _____/_____/_____ (DD - MM - YYYY)	**Date subject signed written consent form:** _____/_____/_____ (DD - MM - YYYY)	
Origin:	☐ 1 White / Caucasian ☐ 2 Black or African ☐ 3 Oriental ☐ 4 Other, specify: _____	
Sex:	☐ 1 Male ☐ 2 Female	
Smoker:	☐ 1 Yes ☐ 2 No	
Atopic:	☐ 1 Yes ☐ 2 No	

SCREENING VISIT 1	DATE: _ _/_ _/_ _ _ _

PRIOR MEDICAL HISTORY

Condition/illness/surgical procedure	**Start date** (DD/MM/YY)	**Stop date/ongoing** Mark box if ongoing (DD/MM/YY)
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___
	___/___/___	ongoing ☐ ___/___/___

CONCOMMITANT MEDICATIONS

Medication	Start Date (DD/MM/YYYY)	Stop Date (DD/MM/YYYY)	Ongoing
1.			☐
2.			☐
3.			☐
4.			☐
5.			☐
6.			☐
7.			☐
8.			☐
9.			☐
10.			☐

WEIGHT / HEIGHT

Weight: ___ ___ ___ ___ kg **Height:** ___ ___ ___ cm

SPIROMETRY

	Predicted value	Baseline value	% predicted	Value post B_2-agonist	% change post B_2 agonist
FEV_1					
FVC (l/min)					
PEFR (l/min)					
FEV1 /FVC					

Subjects MUST meet the following criteria to be included in the study:		**Yes**	**No**
1.	MODERATE – SEVERE ASTHMA	☐	☐
2.	REVERSIBILITY	☐	☐

EXHALED NO:

_____

LABORATORY TESTS

	Yes	No
Full Blood Count	☐	☐
Renal	☐	☐
Bone Profile	☐	☐
Vitamin D	☐	☐
Blood for Experiments	☐	☐

DRUG TREATMENT

PREDNISOLONE

Dose: _____mg / day

Start date of ingestion: _ _/_ _/_ _ _ _ Time of 1st Dose: _____

Investigator's Signature X_____	**Date of Signature**	__ __ / __ __ / __ __

SCREENING VISIT 2	DATE: _ _/_ _/_ _ _ _

UPDATE MEDICAL HISTORY AND MEDICATION! ☐

ADVERSE EVENTS:

Event No	Event Name	Start date (DD/MM/YY)	Stop date Mark box if ongoing (DD/MM/YY)	Serious?	Outcome	Relation to Study Drug
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes

SPIROMETRY

	Predicted value	Baseline (pre-bronchodilator value)	% predicted
FEV_1			
FVC (l/min)			
PEFR (l/min)			
FEV1 /FVC			

Subjects MUST meet the following criteria to be included in the study:		**Yes**	**No**
1.	STEROID RESISTANT	☐	☐

EXHALED NO:

_____

LABORATORY TESTS

	Yes	**No**
Full Blood Count	☐	☐
Renal	☐	☐
Bone Profile	☐	☐
Vitamin D	☐	☐
Blood for Experiments	☐	☐

Investigator's Signature X_____	**Date of Signature**	_ _ / _ _ / _ _ _ _

TREATMENT VISIT 1	**DATE: _ _/_ _/_ _ _ _**

UPDATE MEDICAL HISTORY AND MEDICATION! ☐

ADVERSE EVENTS

Event No	Event Name	Start date (DD/MM/YY)	Stop date Mark box if ongoing (DD/MM/YY)	Serious?	Outcome	Relation to Study Drug
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes
		____/____/____	ongoing ☐ ____/____/____	☐ No ☐ Yes		☐ No ☐ Yes

LABORATORY TESTS

	Yes	No
Full Blood Count	☐	☐
Renal	☐	☐
Bone Profile	☐	☐
Vitamin D	☐	☐
Blood for Experiments	☐	☐

FEMALE PATIENT: PREGNANCY TEST ISSUED: ☐

DRUG TREATMENT

CALCITRIOL / PLACEBO

Dose: 0.25 mcg / bd

Start date of ingestion: _ _/_ _/_ _ _ _ Time of 1st Dose: _____

Investigator's Signature x_____	**Date of Signature**	__ __ / __ __ / __ __ __ __

TREATMENT VISIT 2	DATE: _ _/_ _/_ _ _ _

UPDATE MEDICAL HISTORY AND MEDICATION! ☐

ADVERSE EVENTS

Event No	Event Name	Start date (DD/MM/YY)	Stop date Mark box if ongoing (DD/MM/YY)	Serious?	Outcome	Relation to Study Drug
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes

SPIROMETRY

	Predicted value	Baseline (pre-bronchodilator value)	% predicted
FEV_1			
FVC (l/min)			
PEFR (l/min)			
FEV1 /FVC			

EXHALED NO:

_____

LABORATORY TESTS

	Yes	No
Full Blood Count	☐	☐
Renal	☐	☐
Bone Profile	☐	☐
Vitamin D	☐	☐
Blood for Experiments	☐	☐

ACQ QUESTIONNAIRE

SCORE_____

DRUG TREATMENT

PREDNISOLONE

Dose: _____mg / day

Start date of ingestion: _ _/_ _/_ _ _ _ Time of 1st Dose: _____

Investigator's Signature X_____	**Date of Signature**	__ __ / __ __ / __ __ __ __

TREATMENT VISIT 3	DATE: _ _/_ _/_ _ _ _

UPDATE MEDICAL HISTORY AND MEDICATION! ☐

ADVERSE EVENTS

Event No	Event Name	Start date (DD/MM/YY)	Stop date Mark box if ongoing (DD/MM/YY)	Serious?	Outcome	Relation to Study Drug
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes

____

____

____

____

SPIROMETRY

	Predicted value	Baseline (pre-bronchodilator value)	% predicted
FEV_1			
FVC (l/min)			
PEFR (l/min)			
FEV1 /FVC			

EXHALED NO:

_____

ACQ QUESTIONNAIRE

Score _____

LABORATORY TESTS

	Yes	No
Full Blood Count	☐	☐
Renal	☐	☐
Bone Profile	☐	☐
Vitamin D	☐	☐
Blood for Experiments	☐	☐

Investigator's Signature X_____	**Date of Signature**	__ __ /__ __ /__ __ __ __

FOLLOW UP	DATE: _ _/_ _/_ _ _ _

How was the patient contacted?

☐1 Phone

☐2 Routine clinic visit

☐2 other________________________

UPDATE MEDICAL HISTORY AND MEDICATION! ☐

ADVERSE EVENTS

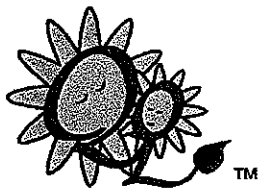
Event No	Event Name	Start date (DD/MM/YY)	Stop date Mark box if ongoing (DD/MM/YY)	Serious	Outcome	Relation to Study Drug
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes
		___/___/___	ongoing ☐ ___/___/___	☐ No ☐ Yes		☐ No ☐ Yes

Investigator's Signature X_______________________________________ _	Date of Signature	__ __ / __ __ / __ __ __ __

Chief Investigator's Signature X________________________________	Date of Signature	__ __ / __ __ / __ __ __ __

ASTHMA CONTROL QUESTIONNAIRE

UK ENGLISH VERSION



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This translation has been made possible through
a grant from YAMANOUCHI
Translated by MAPI RESEARCH INSTITUTE
Senior Translator: Pr Elizabeth Juniper

APRIL 2001

ASTHMA CONTROL QUESTIONNAIRE©
(ENGLISH FOR THE UK)

PATIENT ID:_____

DATE:_____

Please answer questions 1 - 6.

Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?

0 Never
1 Hardly ever
2 A few times
3 Several times
4 Many times
5 A great many times
6 Unable to sleep because of asthma

2. On average, during the past week, how **bad were your asthma symptoms when you woke up** in the morning?

0 No symptoms
1 Very mild symptoms
2 Mild symptoms
3 Moderate symptoms
4 Quite severe symptoms
5 Severe symptoms
6 Very severe symptoms

3. In general, during the past week, how **limited were you in your activities** because of your asthma?

0 Not limited at all
1 Very slightly limited
2 Slightly limited
3 Moderately limited
4 Very limited
5 Extremely limited
6 Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?

0 None
1 A very little
2 A little
3 A moderate amount
4 Quite a lot
5 A great deal
6 A very great deal

5.	In general, during the past week, how much time did you **wheeze?**	0 Never 1 Hardly any of the time 2 A little of the time 3 A moderate amount of the time 4 A lot of the time 5 Most of the time 6 All the time
6.	On average, during the past week, how many **puffs/inhalations of short-acting bronchodilator** (eg. Ventolin/Bricanyl) have you used each day? *(If you are not sure how to answer this question, please ask for help)*	0 None 1 1 - 2 puffs/inhalations most days 2 3 - 4 puffs/inhalations most days 3 5 - 8 puffs/inhalations most days 4 9 - 12 puffs/inhalations most days 5 13 - 16 puffs/inhalations most days 6 More than 16 puffs/inhalations most days

To be completed by a member of the clinic staff

7.	FEV_1pre-bronchodilator: ..... FEV_1predicted:..... FEV_1%predicted:..... (Record actual values on the dotted lines and score the FEV_1 % predicted in the next column)	0 > 95% predicted 1 95 - 90% 2 89 - 80% 3 79 - 70% 4 69 - 60% 5 59 - 50% 6 < 50% predicted

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‡These authors contributed equally to this work as senior authors.

The work was supported by the National Institutes of Health (grant no. 5R01AI065617 and grant no. 1R21AI087627 to T.A.C. and grant no. 1R01AI100315 to R.S.G. and by the Scientific and Technological Research Council of Turkey (grant no. 1059B191300622 to S.K.). This project has been funded in part by federal funds from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, under contract no: HHSN272201000020C.

Disclosure of potential conflict of interest: T. A. Chatila and R. S. Geha's institutions have received grant funding from the National Institutes of Health. G. Dbaibo's institution has received funding from the Dubai-Harvard Foundation for Medical Research. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online April 24, 2014.
http://dx.doi.org/10.1016/j.jaci.2014.03.032

The effects of calcitriol treatment in glucocorticoid-resistant asthma

To the Editor:

Asthma is a chronic inflammatory disease characterized clinically by variable small airways obstruction and hyperresponsiveness and pathologically by airways inflammation and remodeling. The current cornerstone of asthma therapy is anti-inflammatory glucocorticoids. Although glucocorticoids improve clinical features of disease and airways inflammation in most patients, there is a cohort of well-defined asthma patients in whom high-dose glucocorticoid treatment is not only clinically ineffective but also potentially detrimental.[1] Improved understanding and management of glucocorticoid-resistant asthma is vitally important because these patients are very difficult to manage clinically and are at high risk of hospitalization, morbidity, and mortality. A number of mechanisms have been proposed to contribute to glucocorticoid-resistant asthma, including increased expression of nuclear factor kappa B and activating protein 1 (AP-1), increased expression of histone deacetylase, polymorphisms in IL-10, increased expression of the dominant negative isoform of the glucocorticoid receptor beta (GRβ), and vitamin D insufficiency.[2-4]

Our earlier data showed that peripheral blood $CD4^+$ T cells from glucocorticoid-resistant as compared with glucocorticoid-sensitive asthmatic patients failed to synthesize the anti-inflammatory cytokine IL-10 in response to glucocorticoid *in vitro*.[5] The active form of vitamin D (calcitriol; 1,25-dihydroxyvitamin D3 [$1,25(OH)_2D_3$]) when used in combination with glucocorticoid restored this IL-10 response both *in vitro* and *ex vivo* following patient ingestion of calcitriol.[6] These data, together with epidemiologic evidence linking vitamin D insufficiency/deficiency with a poor clinical response to treatment in asthma,[3,4] provided the rationale for this proof-of-concept clinical trial. We characterized a group of patients with severe asthma as glucocorticoid resistant following a standardized, 2-week course of oral prednisolone (Screening) using our previously established and generally accepted criteria.[6] Following a washout period, patients were randomly assigned placebo or 0.25 μg calcitriol twice daily, according to British National Formulary guidelines, for 4 weeks with a repeat course of oral prednisolone during the final 2 weeks. We hypothesized that concomitant calcitriol therapy improves clinical glucocorticoid responsiveness in these patients. It should be noted that calcitriol, a downstream metabolite of 25-hydroxyvitaminD (25[OH]D), would not be expected to restore vitamin D sufficiency, as defined by circulating 25(OH)D: our intention was to address the short-term effects of calcitriol itself. Details of the patient recruitment and study protocol may be seen in the Methods section of this article's Online Repository at www.jacionline.org. There were no differences in patients' demographic characteristics including age, body mass index, atopic status, baseline FEV_1, and inhaled glucocorticoid usage between the 2 randomized groups (see Table E1 in this article's Online Repository at www.jacionline.org). There were no serious adverse events, and all such events were self-limiting (see Table E2 in this article's Online Repository at www.jacionline.org). Differential blood leukocyte counts showed a similar increase in circulating neutrophils and reduction of eosinophils in response to both courses of prednisolone therapy in all patients, confirming compliance with this treatment (data not shown).

On the basis of the primary outcome of change in lung function from the initial screening visit to the end of treatment, we saw no significant difference in % predicted FEV_1 between the 2 groups ($P = .82$; Table I). Nevertheless, a within-group comparison showing the change in lung function during the initial screening in response to 2-week oral prednisolone (Screening) versus the response to an identical course of prednisolone plus either placebo or calcitriol (Treatment) revealed a modest but significant improvement in absolute and predicted FEV_1 within the calcitriol ($P = .03$) but not the placebo arm (Fig 1, *A*). These differences were not apparent in patients randomized to receive calcitriol before the second course of prednisolone, suggesting that calcitriol alone had had no effect on lung function.

TABLE I. Primary and secondary outcomes

Clinical measurement	Screening		Treatment	
	Pre-steroids	Post-steroids	Pre-steroids post-treatment	Post-steroids post-treatment
FEV_1 (L/s)				
Placebo	1.82 (1.44-2.21)	1.82 (1.79-2.44)	1.83 (1.42-2.23)	2.01 (1.61-2.41)
Calcitriol	1.98 (1.67-2.29)	1.88 (1.52-2.23)	1.96 (1.58-2.34)	2.20 (1.64-2.75)
FEV_1 (%)				
Placebo	59.9 (48.8-71.0)	59.9 (48.3-71.3)	60.6 (46.7-74.7)	65.9 (55.0-76.8)
Calcitriol	62.5 (55.2-69.8)	59.5 (49.8-69.2)	61.6 (53.3-69.9)	68.1 (55.4-80.7)
Serum 25(OH)D (nmol/L)				
Placebo	42.6 (26.5-58.6)	44.6 (30.7-58.6)	53.2 (35.4-71.0)	50.8 (31.2-70.5)
Calcitriol	32.5 (21.5-43.5)	34.3 (21.5-43.5)	37.1 (15.9-58.4)	34.4 (19.6-49.2)

Note: Data shown as mean ± 95% CIs.

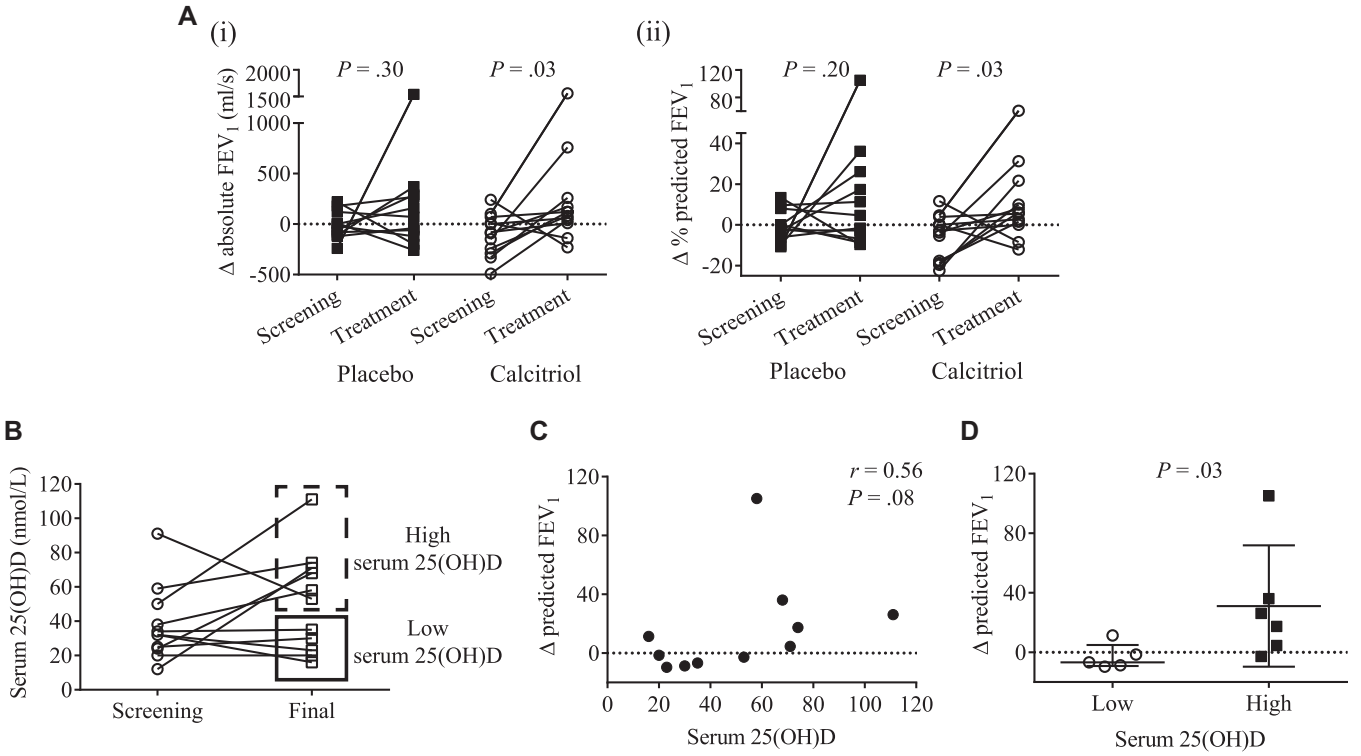

FIG 1. Treatment with calcitriol or vitamin D sufficiency improves clinical response to steroids. **A,** Comparison of change in (i) absolute lung function (FEV_1 [L]) and (ii) predicted lung function after a 2-week course of prednisolone at screening (pretreatment) and treatment (together with placebo OR calcitriol). **B,** Serum 25(OH)D levels of the placebo group at the beginning as compared with the last visit of the clinical trial. **C,** Correlation between serum 25(OH)D and change in lung function (predicted %) after a 2-week course of prednisolone during the treatment phase of the trial (after placebo treatment). **D,** Comparison of change in predicted lung function after a 2-week course of prednisolone at screening (pretreatment) and treatment (together with placebo treatment) in those defined as high serum 25(OH)D (*closed squares*; >50 nmol/L) or low serum 25(OH)D (*open circles*; <50 nmol/L); data shown as median ± interquartile range.

In the placebo cohort, 4 of the 11 patients showed a more than 10% improvement in their lung function post-prednisolone in the treatment phase than in the screening phase. Retrospective analysis of serum concentrations of 25(OH)D in these patients showed that 6 patients exhibited elevation of their baseline serum 25(OH)D concentrations from start to final visit of study (Fig 1, *B*). A trend for a positive correlation between baseline serum 25(OH)D concentrations and change in predicted lung function following prednisolone ($r = 0.56$, $P = .08$; Fig 1, *C*) was observed. Despite the very low numbers, a greater improvement in FEV_1 following prednisolone in those with relatively high (>50 nmol/L; n = 6) as compared with low (<50 nmol/L; n = 5) serum concentrations of 25(OH)D was seen ($P = .03$; Fig 1, *D*).

This study represents the first demonstration to our knowledge that the clinical responsiveness of asthmatic patients to glucocorticoid therapy is subject to manipulation in the short term. Our data show that treatment with a short course of $1,25(OH)_2D_3$

(calcitriol) may modestly improve the clinical glucocorticoid responsiveness in asthma, even in patients classified as clinically glucocorticoid resistant.

This study was not designed to correct or take account of the patients' vitamin D status. Nevertheless, all but 1 of the patients were vitamin D insufficient (<75 nmol/L) and 16 of our patients were deficient (<50 nmol/L)[7] at the commencement of the study and, as shown, unexpectedly 6 of the 11 patients randomized to placebo showed considerable improvement in vitamin D status (insufficient/deficient to sufficient) by the final visit, for reasons one can only speculate on, but that are likely to include exposure to sunlight, dietary intake, and supplementation. Even so, we observed a trend for 25(OH)D concentrations to correlate positively with clinical glucocorticoid responsiveness. A similar observation was made in an independent recent study using asthma exacerbations as a clinical outcome.[8]

Our data indicate that $1,25(OH)_2D_3$ (calcitriol) may improve the clinical response to glucocorticoids in resistant asthma patients. They raise important questions about the design of future studies. On the one hand, the decision to study a well-characterized cohort of glucocorticoid-resistant asthmatic patients produced significant challenges with recruitment and retention, but on the other hand may have facilitated our ability to observe a clinical effect that may be manifest most clearly in this small but important subset of patients. Future studies must address wider cohorts of asthmatic patients and consider other end points. Although our study suggests an impact on glucocorticoid responsiveness, 2 independent studies implicate vitamin D in reducing asthma exacerbation rates.[8,9] Despite the low power of this study, these preliminary clinical data are encouraging and warrant further investigations in larger, ideally clinically well-defined cohorts, including in pediatric asthma patients for whom epidemiologic data are arguably the strongest.

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We acknowledge the support of Asthma UK through project grant 08/040. A.M.N. was funded by Asthma UK. E.S.C. was funded by an MRC British Thoracic Society/Morriston Davies Trust Capacity Building PhD Studentship and is now funded by an MRC Centenary Fellowship. K.R. was in receipt of an MRC Clinical Training Research Fellowship. The research was supported by the National Institute for Health Research (NIHR) Clinical Research Facility at Guy's & St Thomas' National Health Service (NHS) Foundation Trust and NIHR Biomedical Research Centre based at Guy's & St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department for Health. We acknowledge funding from the Guy's and St Thomas' Charity contributed toward the purchase of placebo.

Disclosure of potential conflict of interest: E. S. Chambers has received travel support from the Federation of Clinical Immunology Societies. K. Ryanna has received research support from the Medical Research Council Clinical Research Training Fellowship. C. J. Corrigan has received research support and personal fees from Novartis Pharma and has received personal fees from GlaxoSmithKline and Boehringer-Ingelheim. C. M. Hawrylowicz has received research support from Asthma UK and Guy's and St Thomas' Charitable Trust; is employed by the Academy of Finland and the University of Nottingham, United Kingdom; and has received payment for lectures from the Transatlantic Airways Conference. The rest of the authors declare that they have no relevant conflicts of interest.

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Available online April 29, 2014.
http://dx.doi.org/10.1016/j.jaci.2014.03.015

Immunologic response and safety in birch pollen sublingual versus oral vestibule immunotherapy: A pilot study

To the Editor:

To date the efficacy of sublingual immunotherapy (SLIT) in the form of allergy immunotherapy tablets is well documented with a large number of clinical trials and it is accepted as an effective alternative to subcutaneous immunotherapy in the treatment of type I–mediated respiratory allergies in Europe, but is currently not likewise accepted in the United States.[1] The attention of many scientists and clinicians is directed toward how immunologic mechanisms mediate the effect of SLIT and improvement of its efficacy, while reducing adverse effects. Although the immunologic mechanisms of SLIT as well as subcutaneous immunotherapy remain to be elucidated in detail, the induction of regulatory T cells as well as a shift from an allergy-mediating T_H2 toward an allergy-preventing T_H1 immune response is propagated along with the induction of allergen-specific IgG.[2] Moreover, antibodies with IgE-blocking activities—quantified as the IgE-blocking factor—have been described recently within the allergen-specific IgG fraction, which are hypothesized to correlate with the clinical response to immunotherapy.[3] The common link between these immune reactions are professional

METHODS

Study participants

Study participants were recruited at Guy's and St Thomas' National Health Service Foundation Trust and Barts Health National Health Service Trust, tertiary health care centers in London, United Kingdom. The study was approved by the London Bridge Research Ethics Committee (REC ref 06/Q0605/83), and written informed consent was obtained from all participants before enrolment. The study is registered with the UK Clinical Research Network (International Standard Randomized Controlled Trial Number 2937824).

Patients aged 18 to 75 years with a documented history and typical symptoms of asthma for more than 6 months before screening were assessed for eligibility to participate. All study subjects had a prebronchodilator FEV_1 of less than 80% predicted and variability in airways obstruction (≥12% in response to bronchodilator or >20% diurnal peak expiratory flow rate variability) documented within the previous 5 years. Individuals were excluded if they suffered from past or present disease, which, as judged by the investigator, might affect the study outcome (other than asthma, rhinitis, or eczema); if serum-corrected calcium was more than 2.65 mol/L; if they were a current cigarette smoker or ex-smoker of less than 5 years with a more than 5 pack-year history; if they were pregnant or lactating females or at risk of pregnancy; if they had a history of a respiratory tract infection and/or exacerbation of asthma within 4 weeks of the screening visit requiring oral glucocorticoid therapy; if they had participated in a study involving an investigational medicinal product in the previous 3 months or had made a blood donation within the last year; if they were currently receiving, or had received, allergen immunotherapy or treatment with lithium carbonate or calcium supplements; or if they were unable to understand or comply with the research protocol. Patients were asked, and agreed not to take any vitamin supplements for the duration of the study. Patients remained on their regular maintenance medication for the duration of the study, including the 4-week washout period. On the day of screening, 8 (34.7%) patients showed more than 20% peak flow variability and 15 (65.3%) showed reversibility to a bronchodilator response. However, all 8 patients who were enrolled on the basis of peak flow variability had shown significant bronchodilator response within 5 years prior to enrolling into the study. All patients who went through the screening period (ie, 2 weeks of oral corticosteroids) had confirmed bronchodilator reversibility.

Glucocorticoid-resistant asthma was defined as less than 10% improvement in baseline FEV_1 following a 14-day course of oral prednisolone (Wockhardt UK Ltd, Wrexham, United Kingdom; 40 mg/1.73 m^2/d) in eligible patients. Routine spirometry was measured before and after the course of prednisolone using a PC-based spirometer and software (MIR Medical International Research, Rome, Italy/WinspiroPRO). In addition, differential full blood cell counts were performed before and after the course of prednisolone using a LH750 hematology analyzer (Beckman Coulter, Brea, Calif).

Fifty patients were screened for eligibility between April 2009 and September 2012. Twenty-five patients were excluded before or during the screening period: 7 patients did not meet inclusion criteria, 3 declined to participate, 3 were noncompliant with the protocol, and 12 were steroid sensitive (>10% improvement in FEV_1 compared with baseline). One patient was lost to follow-up after completion of the screening visits. Twenty-four patients were randomized: 11 were allocated to placebo, 13 to intervention. One patient in the treatment group withdrew because of an adverse event, thought to possibly be related to the study drug (Fig E1; clinical flow diagram), making it 12 patients to complete the treatment group in that arm. There were no serious adverse events, and all events were self-limiting (Table E1).

Study protocol

The study outline is summarized in Fig E1. Participants who met the eligibility criteria for glucocorticoid-resistant asthma returned following a 4-week washout period and were randomly allocated to commence treatment with either calcitriol 0.25 μg soft capsules (Rocaltrol; Roche Pharmaceuticals, Welwyn Garden City, United Kingdom) or organoleptically identical lactose placebo generated in-house (Pharmacy Production Unit, St Thomas' Hospital NHS Trust, London, United Kingdom) twice daily. Patients were randomized in a 1:1 ratio using a computerized random plan generated by a physician not involved in the trial. Patients and trial investigators were blinded to treatment allocation. Following 2 weeks of calcitriol or placebo treatment, patients were given a second course of oral prednisolone identical to the first while calcitriol or placebo was continued. Spirometry was performed at the beginning and end of this second course of oral prednisolone as before. Serum concentrations of calcium, corrected calcium, albumin, total protein, phosphate, sodium, potassium, urea, creatinine, and 25(OH)D were analyzed. Concentrations of 25(OH)D2 and 25(OH)D3 were determined by isotope-dilution liquid chromatography–tandem mass spectrometry and summed to give values for total 25(OH)D. Sensitivity for this assay was 10 nmol/L. Full blood cell counts were performed as described above. Albumin, phosphate, and total serum calcium concentrations were determined using an Architect ci8200 analyzer (Abbott Diagnostics, Chicago, Ill). Calcium concentration was corrected for serum albumin concentration using the formula: corrected calcium (mmol/L) = total calcium (mmol/L) + 0.02 × (40 – albumin [g/L]).

Power calculations predicted that the study of 40 glucocorticoid-resistant participants would enable detection of an improvement in change in FEV_1 from 0.4% to 10% with 80% power, tested at the 2-sided 5% significance level. Because of difficulties with patient recruitment, however, the decision was made to terminate the study after 24 patients completed.

The primary outcome measure was change in FEV_1 from screening to the final visit. Secondary outcomes measured included asthma control questionnaire and serum 25(OH)D measurements. There was no difference observed in the asthma control questionnaire scores between the calcitriol and placebo treatment groups. Additional analysis of glucocorticoid response was performed within (screening vs treatment phases) and between groups (calcitriol vs placebo), expressed as absolute values and percentage change. Study subjects were asked to report any adverse events from the day of commencement of the first course of oral prednisolone until 4 weeks after the second course by telephone or e-mail or at study visits.

Statistical methods

The differences in % predicted FEV_1 at 28 days postrandomization between treatment groups was assessed using a linear regression model adjusted for screening values of % predicted FEV_1 value and vitamin D status. The analysis was performed on the complete case population, and statistical significance was assessed at the 5% level.

For subsequent subanalyses, data were assessed for Gaussian distribution and equality of variance. Then, for normally distributed data, assessment was done by either an unpaired or a paired (where appropriate) Student *t* test and for non-normally distributed data, Wilcoxon matched-pairs signed test (paired data) and Mann-Whitney *U* test (unpaired). Correlations were nonnormally distributed and were assessed by Spearman rank correlation test. Normally distributed data were presented as the mean ± 95% CIs, and nonnormally distributed data were shown as median ± interquartile range.

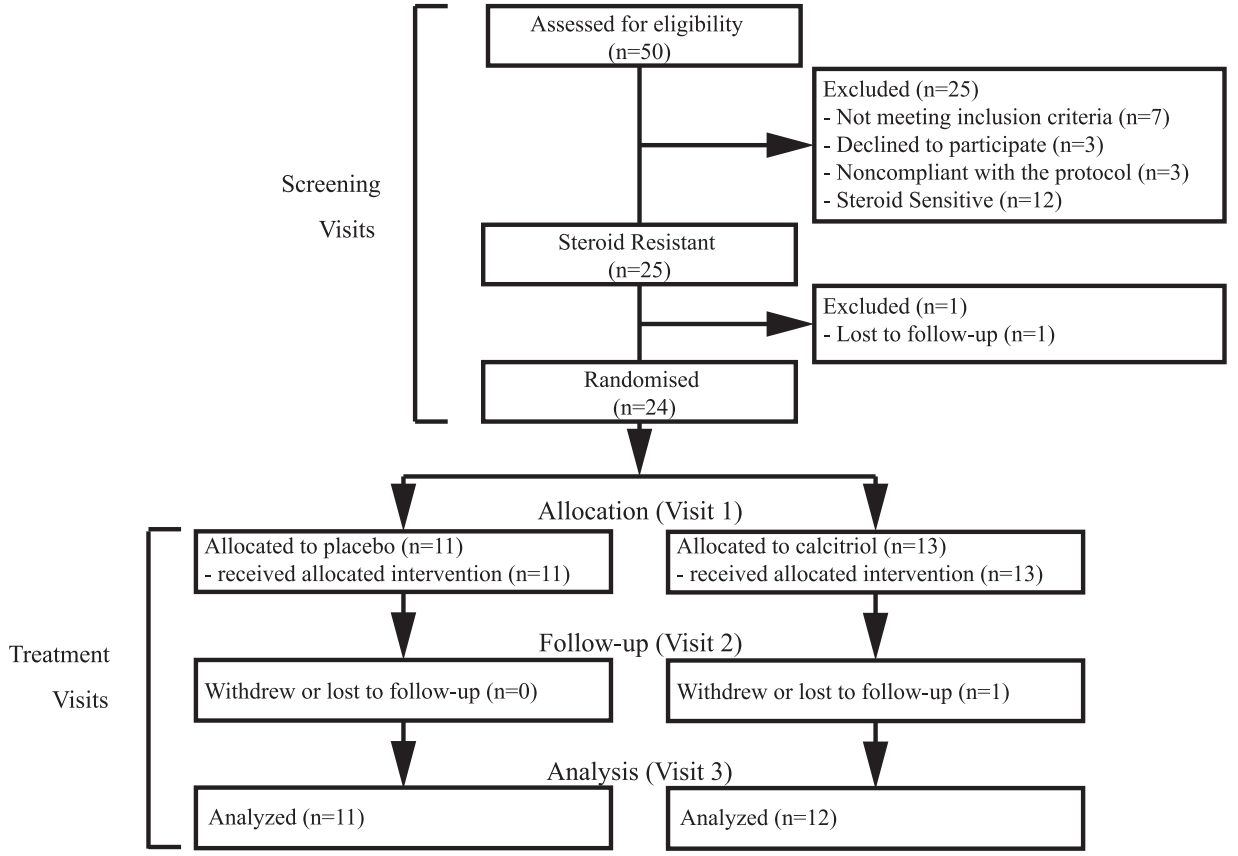

FIG E1. Schematic of clinical trial design.

TABLE E1. Patients' characteristics

Characteristic	Placebo	Calcitriol
Age (y)	53.5 (47.8-59.1)	50.3 (39.1-61.6)
Ethnic origin		
Caucasian	9	8
African	2	3
Asian	0	1
Sex		
Male	7	7
Female	4	6
Atopic	10	9
BMI	29.1 (25.0-33.2)	27.4 (25.5-29.2)
Inhaled corticosteroid dose	1236 (830-1643)	1267 (849-1684)
FENO	19.9 (15.7-24.1)	38.4 (20.7-56.0)
	n = 11	n = 12

Note: Data are presented as the mean and 95% CI. Atopy was defined by skin prick testing. The dose of inhaled corticosteroids was calculated according to the British Thoracic Society - Scottish Intercollegiate Guidelines Network (BTS-SIGN) Guideline on the management of asthma (Table 8b: Equivalent doses of inhaled steroids relative to BDP and current licensed age indications). Patients were on beclomethasone 1600 μg/d, budenoside 1600 μg/d, or fluticasone 800 μg/d.
BDP, Beclomethasone dipropionate; *BMI*, body mass index; *FENO*, fractional exhaled nitric oxide; *25(OH)D*, 25-hydroxyvitamin D.

TABLE E2. Side effects documented throughout the trial

Adverse event	Study phase	Study drug	Related to study drug
Mild indigestion	Screening	Prednisolone	Yes
Nausea	Screening	Prednisolone	Possible
Increased hunger	Screening	Prednisolone	Yes
Coryzal symptoms	Treatment	Placebo	No
Diarrhea	Treatment	Placebo	No
Coryzal symptoms	Treatment	Calcitriol	No
Coryzal symptoms	Treatment	Calcitriol	No
Coryzal symptoms	Treatment	Calcitriol	No
Constipation, back pain	Treatment	Calcitriol	Possible
Bang to head	Treatment	Calcitriol	No

Note: All effects were nonsevere and resolved spontaneously.

Enhanced production of IL-17A in patients with severe asthma is inhibited by 1α,25-dihydroxyvitamin D3 in a glucocorticoid-independent fashion

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Background: T_H17 cells are proposed to play a role in the pathology of asthma, including steroid-resistant (SR) disease. We previously identified a steroid-enhancing function of vitamin D in patients with SR asthma in restoring the impaired response to steroids for production of the anti-inflammatory cytokine IL-10.
Objective: We sought to investigate the production of the T_H17-associated cytokines IL-17A and IL-22 in culture in patients with moderate-to-severe asthma defined on the basis of their clinical response to steroids and the susceptibility of this response to inhibition by steroids and the active form of vitamin D, 1α,25-dihydroxyvitamin D3 ($1,25[OH]_2D3$).
Methods: PBMCs were stimulated in culture with or without dexamethasone and $1,25(OH)_2D3$. A cytometric bead array, ELISA, and intracellular cytokine staining were used to assess cytokine production. The role of CD39 in inhibition of the T_H17 response was studied by using quantitative real-time PCR, flow cytometry, and addition of the antagonist POM-1 to culture.
Results: Asthmatic patients synthesized much higher levels of IL-17A and IL-22 than nonasthmatic control subjects, with patients with SR asthma expressing the highest levels of IL-17A. Glucocorticoids did not inhibit IL-17A cytokine expression in patients and enhanced production in cultures from control subjects. Treatment with $1,25(OH)_2D3$ with or without dexamethasone significantly reduced both IL-17A and IL-22 levels. An antagonist of the ectonucleotidase CD39 reversed $1,25(OH)_2D3$-mediated inhibition of the IL-17A response.
Conclusion: Patients with severe asthma exhibit increased levels of T_H17 cytokines, which are not inhibited by steroids. $1,25(OH)_2D3$ inhibits T_H17 cytokine production in all patients studied, irrespective of their clinical responsiveness to steroids, identifying novel steroid-enhancing properties of vitamin D in asthmatic patients. (J Allergy Clin Immunol 2013;132:297-304.)

Key words: *Asthma, vitamin D, IL-17A, steroid resistant, steroid sensitive, T_H17, glucocorticoids*

Asthma is broadly defined as bronchial hyperresponsiveness with reversible expiratory airflow limitation. Historically, asthma has been described as having an underlying T_H2-mediated inflammatory profile; however, there is increasing evidence for heterogeneity in asthma phenotypes, with a significant proportion of asthmatic patients demonstrating a low or non–T_H2-mediated phenotype that appears less sensitive to control by glucocorticoids.[1,2] Although glucocorticoids are currently the most effective treatment for asthma, steroid-resistant (SR) asthma is a significant clinical problem. These patients generally require prolonged systemic treatment, their asthma is less stable and more difficult to control, and they are subject to higher morbidity and mortality.[3-6] The precise cause or causes of SR asthma are unknown, but several mechanisms have been proposed to account for it,[7-10] including genetic susceptibility, defects in glucocorticoid receptor (GR) binding, increased expression of the functionally inactive GR-β, activation of transcription factors (eg, activator protein 1), or decreased synthesis of immunoregulatory cytokines, such as IL-10. Although it is difficult to investigate SR asthma in patients with milder disease because its definition is based on improvement in originally abnormal lung function, it is reasonable to suppose that SR asthma is likely to be associated with severe and difficult-to-control disease.

T_H17 cells are critical for defense against bacterial and fungal infections.[11] Mouse models demonstrate that animals deficient in IL-17A or IL-17RA are highly susceptible to infection with mucosal pathogens, such as *Klebsiella pneumoniae* and *Candida albicans*.[12,13] IL-17A–mediated activation of the innate immune system and neutrophil influx can be protective against infection but can also lead to damage of the surrounding tissues associated with immune pathology.[14,15]

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Supported by Asthma UK through project grants 08/040 and 11/040. A.M.N. is funded by Asthma UK. E.S.C. is funded by an MRC British Thoracic Society/Morriston Davies Trust Capacity Building PhD Studentship. K.R. was in receipt of an MRC Clinical Training Research Fellowship. The research was funded/supported by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

Disclosure of potential conflict of interest: E. S. Chambers has received an MRC Centenary Award and has received a BSI International Travel Grant. K. Ryanna has received an MRC Clinical Research Training Fellowship. C. J. Corrigan has received honoraria for consultancy from Meda and from Chiesi, has received one or more grants from or has one or more grants pending with and has received one or more payments for travel/accommodations/meeting expenses from Novartis, and has received honoraria for lectures from Allergy Therapeutics. C. M. Hawrylowicz has been supported by one or more grants from Asthma UK and has received an Ancillary grant from the National Institutes of Health, a Wellcome Trust Clinical Research Training Fellowship, and MRC PhD studentships. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 14, 2012; revised March 18, 2013; accepted for publication March 26, 2013.

Available online May 15, 2013.

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0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2013.03.037

Abbreviations used
Foxp3: Forkhead box protein 3
GR: Glucocorticoid receptor
$1,25(OH)_2D3$: 1α,25-Dihydroxyvitamin D3
SR: Steroid resistant
SS: Steroid sensitive
Treg: Regulatory T

A body of circumstantial evidence consistent with the hypothesis that IL-17A exacerbates asthma, reduces patients' responsiveness to therapy, or both already exists. Thus IL-17A expression in sputum or bronchial mucosal biopsy specimens has been correlated with bronchial hyperresponsiveness,[16] granulocyte infiltration,[17] and production of fibrogenic mediators by bronchial fibroblasts.[18] Furthermore, increased serum IL-17A appears to be both a marker[19] and an independent risk factor for severe asthma.[16-18,20,21] Involvement of T_H17 cells in patients with severe SR asthma is proposed from murine and human studies.[22,23] In addition to IL-17A, IL-22 is commonly produced by T_H17 cells. In various animal models of disease, IL-22 can elicit damaging or protective responses, possibly dependent on the location, cellular source, cytokine milieu, and timing of its expression.[24-26]

There is great interest in the therapeutic potential of vitamin D to regulate the severity of asthma and to reduce the amount of anti-inflammatory medication required for disease control. Several studies have pointed out striking links in populations between vitamin D deficiency and asthma severity or requirements for anti-inflammatory medication.[27-30] In previous studies investigating the immunologic basis of this relationship, we have shown that 1α,25-dihydroxyvitamin D3 ($1,25[OH]_2D3$) exerts immunomodulatory effects on T cells from patients with SR asthma, enhancing the impaired steroid-induced IL-10 response in these subjects,[31] whereas others have highlighted the capacity of this molecule to inhibit T_H17 responses.[32-35]

Building on these studies, we hypothesized that $1,25(OH)_2D3$ inhibits increased IL-17A production by blood T_H17 cells from patients with severe asthma independently of glucocorticoids *in vitro* and independently of their clinical responsiveness to glucocorticoid therapy *in vivo*. To address this hypothesis, we studied the effects of $1,25(OH)_2D3$ and the glucocorticoid dexamethasone on the production of IL-17A and its common coproduct, IL-22, by peripheral blood $CD4^+$ T cells from a cohort of patients with severe asthma who were carefully characterized for their clinical glucocorticoid responsiveness and nonasthmatic control subjects. Finally, in an attempt to elucidate possible mechanisms of the effect of $1,25(OH)_2D3$ in altering IL-17A production, we measured its effects on the expression of the ectonucleotidase CD39, increased expression of which has previously been reported to be associated with inhibition of IL-17A production in patients with autoimmune disease.[36] We hypothesized that dexamethasone, but not $1,25(OH)_2D3$, reduces CD39 expression on blood T cells and that inhibition of its ectonucleotidase activity abolishes any effects of $1,25(OH)_2D3$ on IL-17A expression.

METHODS

Subjects

Healthy adults (study approved by the local research ethics committee, 09/H0804/77) and asthmatic patients (08/H0804/84) were recruited. All healthy control subjects and patients provided written informed consent. Asthmatic patients had moderate-to-severe asthma for at least 6 months on therapy step 3 or 4 of the British Thoracic Society guidelines on the management of asthma diagnosed by a specialist physician. The average inhaled corticosteroid dose in beclomethasone diproprionate equivalents was 1177 μg for patients with SR asthma and 1180 μg for patients with steroid-sensitive (SS) asthma. All patients had a prebronchodilator FEV_1 of less than 80% of predicted value, had documented airway variability of greater than 12% after 400 μg of short-acting bronchodilator, and had undergone detailed assessment to exclude a diagnosis other than asthma and comorbidities affecting asthma control. Patients were not taking oral corticosteroids for 4 weeks before the study. Steroid sensitivity was defined by an increase in FEV_1 of greater than 10% from baseline after a 2-week course of prednisolone at 40 mg/1.73 m^2 body surface area. Steroid resistance was defined by a less than 10% increase in FEV_1 from baseline after a 2-week course of prednisolone at 40 mg/1.73 m^2 body surface area. Compliance with prednisolone during the study was assessed with cortisol serum levels before and after steroids. Patients undergoing immunotherapy, smokers, or patients who had a respiratory tract infection or asthma exacerbation during or 4 weeks before enrollment into the study were excluded.

Eighteen patients with SR asthma and 10 patients with SS asthma were assessed with a mean age of 54 (SR) and 50 (SS) years. Mean prebronchodilator FEV_1 before and after a course of oral corticosteroids was 1.99 L (64.56%) and 1.96 L (64.05%) in the patients with SR asthma and 1.72 L (57.1%) and 2.19 L (73.2%) in the patients with SS asthma (poststeroid FEV_1, patients with SR vs patients with SS asthma: $P < .001$, analysis of covariance). Ten healthy control subjects were assessed for comparison (mean age, 41 years).

Cell isolation and culture

Human PBMCs were isolated, as previously described.[31] Briefly, CD8-depleted PBMCs were obtained by means of negative selection with $CD8^+$ Dynalbeads (Invitrogen, Paisley, United Kingdom). $CD4^+$ T cells were purified by means of positive selection with Dynalbeads. Cells (1×10^6 cells/mL) were cultured in RPMI (containing 10% FCS, 2 mmol/L L-glutamine, and 50 μg/mL gentamicin) and stimulated with plate-bound anti-CD3 (1 μg/mL, OKT-3) plus 50 U/mL recombinant human IL-2 (Eurocetus, Harefield, United Kingdom) in the presence or absence of dexamethasone (Sigma-Aldrich, Gillingham, United Kingdom) and $1,25(OH)_2D3$ (BIOMOL Research Labs, Exeter, United Kingdom) at the indicated concentrations in a 24-well plate for 7 days. There was no significant difference between viability between the different conditions (data not shown). Where indicated, cells were recultured at the same density of 1×10^6/mL after 7 days with plate-bound anti-CD3 and IL-2 alone in a 48-well plate, and supernatants were harvested at 48 hours for cytokine analysis.

Flow cytometry

The following antibodies were used for cell-surface phenotyping: CD3 (SK7; BD Biosciences, Oxford, United Kingdom) and CD39 (ebioA1; eBioscience, Hatfield, United Kingdom). For *ex vivo* staining, red blood cells were lysed with FACS Lysing Solution (working dilution, 1:10; BD Biosciences) and then further stained for intranuclear forkhead box protein 3 (Foxp3; PCH101, eBioscience) by using the Foxp3 staining kit per the manufacturer's instructions (eBioscience).

For intracellular cytokine staining on day 7, cells were restimulated for 4 hours with 5 ng/mL phorbol 12-myristate 13-acetate and 500 ng/mL ionomycin, with 2 μmol/L monensin (Sigma-Aldrich) added for the final 2 hours. Cells were washed, fixed, and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) and stained with the fluorescently labeled mAbs to IL-22 and IL-17A (22URTI and eBio64CAP17, eBioscience). Dead cells (7-aminoactinomycin D positive, Sigma-Aldrich) were gated out. Analysis was performed with a FACSCalibur flow cytometer (BD Biosciences).

Quantitative real-time PCR

RNA was extracted from cell pellets with the RNeasy Mini kit (Qiagen, Crawley, United Kingdom), according to the manufacturer's instructions. The RNA was quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Del), and 250 ng of RNA was reverse transcribed into

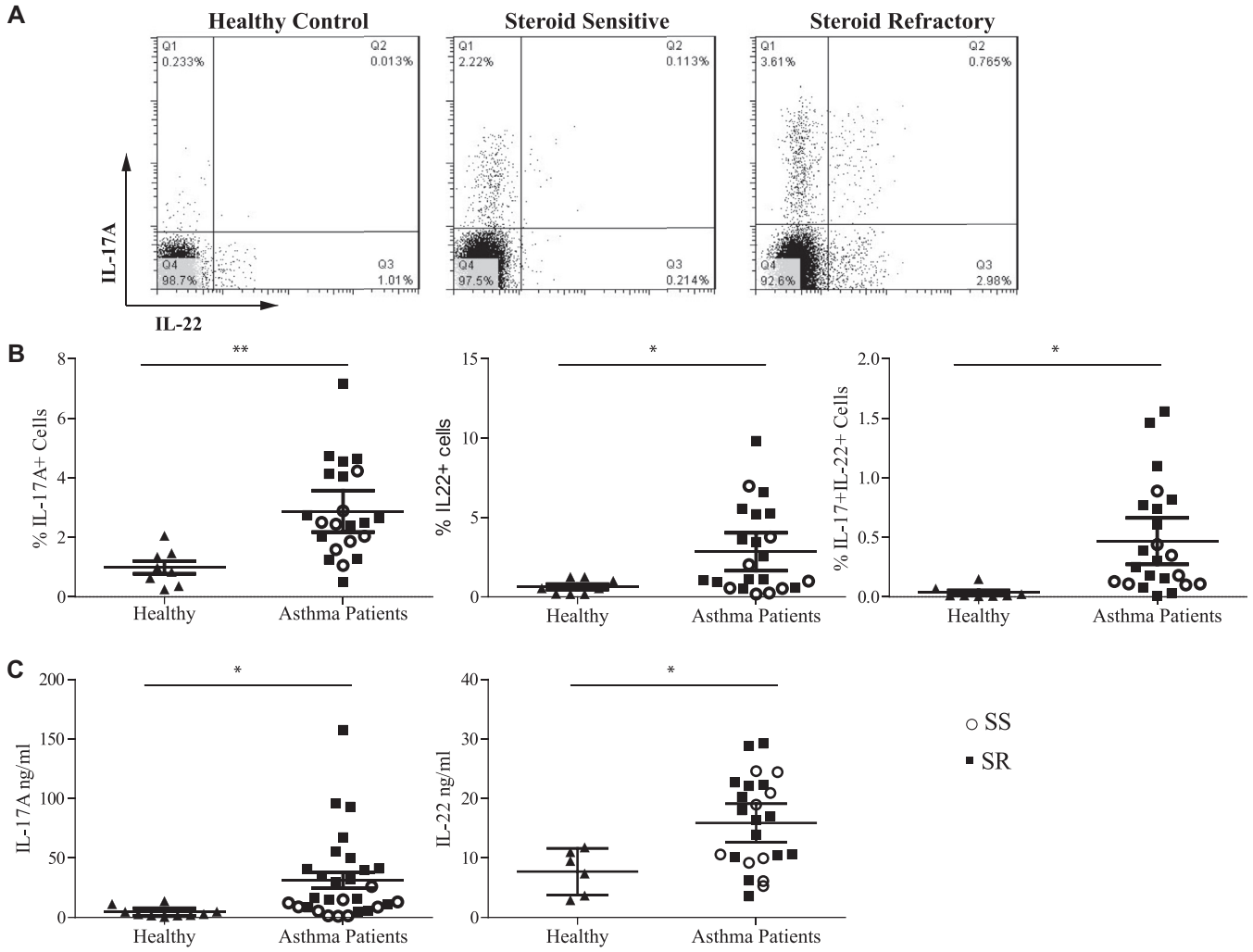

FIG 1. Patients with severe asthma express higher IL-17A and IL-22 levels. Control and asthmatic cultures were assessed for IL-17A and IL-22 production by using intracellular flow cytometry and an antibody capture assay. **A,** Representative dot plots. **B** and **C,** Cumulative data of cytokine-positive cells (healthy subjects, n = 8; patients with SS asthma, n = 8; and patients with SR asthma, n = 14; Fig 1, *B*) and secreted cytokines (healthy subjects, n = 10; patients with SS asthma, n = 10; and patients with SR asthma, n = 14; Fig 1, *C*). **P* < .05 and ***P* < .001, Student *t* test. *Open circles*, Patients with SS asthma; *solid squares*, patients with SR asthma; and *solid triangles*, healthy control subjects.

cDNA. Quantitative real-time PCR was performed in triplicate by using an Applied Biosystems 7900 HT system and FAM-labeled Assay-on-Demand reagent sets for *CD39* (Hs00969559_m1). Quantitative real-time PCR reactions were multiplexed with VIC-labeled 18S primers and probes (Hs99999901_s1) as an endogenous control and analyzed with SDS software, version 2.1 (Applied Biosystems, Foster City, Calif), according to the $2^{-\Delta\Delta Ct}$ method.

Cytokine analysis

IL-22 levels were measured by using ELISA and matched antibody pairs (BD Biosciences). The lower limit of detection for IL-22 was 100 pg/mL. IL-17A, IL-10, IFN-γ, and IL-5 levels were measured with the Cytometric Bead Array (BD Biosciences), according to the manufacturer's protocol; the lower limit of detection was 1.5 pg/mL.

Statistics

Data are presented as means ± SEMs unless indicated. Data were assessed for equivalence to a Gaussian distribution and equality variance, after which the appropriate parametric or nonparametric test was performed (see individual figure legends). Differences were considered significant at the 95% confidence level.

RESULTS

Comparison of IL-17A and IL-22 production by PBMCs in culture from patients with SS and patients with SR asthma

Details of the 2 cohorts of patients with moderate-to-severe asthma, defined as SS or SR asthma on the basis of changes in lung function after a 2-week course of oral prednisolone, and of healthy nonasthmatic control subjects are provided in the Methods section. Notably, these 2 patient groups exhibited comparable disease severity based on impairment of lung function and were taking comparable doses of inhaled corticosteroids (total dosages based on beclomethasone dipropionate equivalence).

PBMCs from the asthmatic patients were evaluated for their capacity to synthesize the T_H17-associated cytokines IL-17A and

IL-22 in culture, and comparisons were made with control subjects. PBMCs depleted of $CD8^+$ T cells were stimulated *in vitro* with anti-CD3 and IL-2 for 7 days and then stained for intracellular cytokine expression. Significantly greater percentages of IL-$17A^+$ and IL-22^+ expressing cells were observed in cultures from asthmatic patients compared with healthy control subjects (Fig 1, *A* and *B*). When patients were subdivided based on clinical responsiveness to glucocorticoids, only patients with SR asthma showed a significant difference in IL-17A immunoreactivity (SR asthma, $P = .003$; SS asthma, $P = .142$) and IL-22 immunoreactivity (SR asthma, $P = .037$; SS asthma, $P = .701$) compared with control subjects (Fig 1, *B*). Cells coexpressing IL-17A and IL-22 are proposed to represent the most pathogenic population, and their numbers were significantly increased in all asthmatic patients and patients with SR asthma compared with healthy control subjects (all asthma, $P = .029$; SR asthma, $P = .009$). At day 7, cells were also harvested and recultured at equal cell densities for 48 hours with anti-CD3 and IL-2 alone. Culture supernatants from asthmatic patients contained significantly higher quantities of secreted IL-17A and IL-22 compared with those from healthy control subjects (Fig 1, *C*). Production of IL-17A and IL-22 was significantly increased in patients with SR asthma compared with that seen in control subjects (IL-17A, $P = .009$; IL-22, $P = .036$). Supernatants from patients with SR asthma had significantly more IL-17A compared with those from patients with SS asthma ($P = .016$), whereas culture supernatants from patients with SS and patients with SR asthma contained comparable levels of IL-22 (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org).

Glucocorticoids do not inhibit IL-17A and IL-22 synthesis in culture

The capacity of glucocorticoids to alter production of IL-17A and IL-22 was next examined. Addition of 10^{-7} mol/L dexamethasone to cultures from healthy control subjects significantly increased the mean percentage of cells expressing IL-17A but did not alter the low percentage of cells expressing IL-22 (Fig 2, *A*). IL-17A and IL-22 secretion was increased in a concentration-dependent manner by dexamethasone in cultures from healthy donors (Fig 2, *B* and *C*). IL-2 is present in the stimulation cultures, and addition of IL-2 plus IL-4 has previously been described to promote steroid insensitivity in culture.[37] Therefore the effects of dexamethasone were assessed in the presence or absence of anti-CD3, IL-2, or both on IL-17A and a range of additional cytokines (see Fig E2 in this article's Online Repository at www.jacionline.org). Notably, IL-17A (and IL-10) enhancement by dexamethasone only occurred when the combination of anti-CD3 and IL-2 was present. Dexamethasone inhibited the production of IFN-γ, IL-13, and IL-5, although the inhibition of IFN-γ in particular was less marked when anti-CD3 plus IL-2 compared with anti-CD3 or IL-2 alone was present in these cultures.

The effects of dexamethasone in asthmatic patient cultures were less marked, where notably there was a greater than 7-fold higher basal level of IL-17A synthesis compared with that seen in nonasthmatic cultures (Fig 2, *B*). A trend toward increased IL-17A secretion ($P = .102$; Fig 2, *B*) and a significant increase in the frequency of IL-$17A^+$ cells in patient cultures containing 10^{-7} mol/L dexamethasone was observed (Fig 3, *A*). An effect observed in both the SS asthma and SR asthma subgroups was

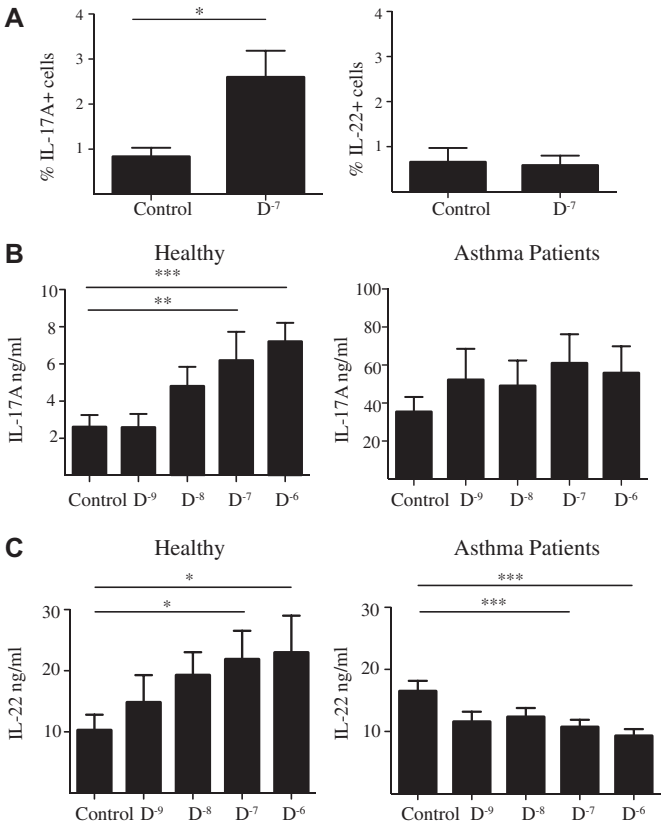

FIG 2. Dexamethasone does not inhibit IL-17A production. **A,** Cumulative data of the effects of dexamethasone on the frequency of IL-$17A^+$ and IL-22^+ cells in nonasthmatic cultures (healthy subjects, n = 8). **B** and **C,** IL-17A and IL-22 secretion in control *(left)* and asthmatic *(right)* cultures (healthy subjects, n = 10; asthmatic patients, n = 28). $*P < .05$, $**P < .01$, and $***P < .001$; Fig 2, *A*, paired *t* test, and Fig 2, *B* and *C*, 1-way ANOVA with the Tukey post test. D^{-x}, Dexamethasone at 10^{-x} mol/L.

partial inhibition by dexamethasone of IL-22 secretion (Fig 2, *C*, and see Fig E1).

1α,25-dihydroxyvitamin D3 inhibits IL-17A and IL-22 expression

The capacity of $1,25(OH)_2D3$ to inhibit IL-17A production alone and in combination with dexamethasone was next investigated in cultures from asthmatic patients. $1,25(OH)_2D3$ significantly decreased the mean percentages of cells that were immunoreactive for IL-17A or IL-22 in culture (Fig 3). Although dexamethasone did not reduce IL-22 or IL-17A immunoreactivity compared with control cultures, the combination of dexamethasone and $1,25(OH)_2D3$ led to a marked reduction in the frequency of cytokine-positive cells that was not significantly different from cultures with $1,25(OH)_2D3$ alone (Fig 3). Protein expression in culture supernatants after $1,25(OH)_2D3$ treatment alone or in combination with dexamethasone demonstrated a comparable trend, with no significant difference in the mean concentration of IL-17A or IL-22 between cultures containing $1,25(OH)_2D3$ or $1,25(OH)_2D3$ plus dexamethasone (Fig 3). The effects of $1,25(OH)_2D3$ on IL-17A and IL-22 synthesis were comparable in the patients with SS asthma and those with SR asthma (see Fig E1, *B*). The reduction in IL-17A production by $1,25(OH)_2D3$ is unlikely to be explained by inhibition of proliferation (see Fig E3 in this article's Online Repository at www.jacionline.org) or

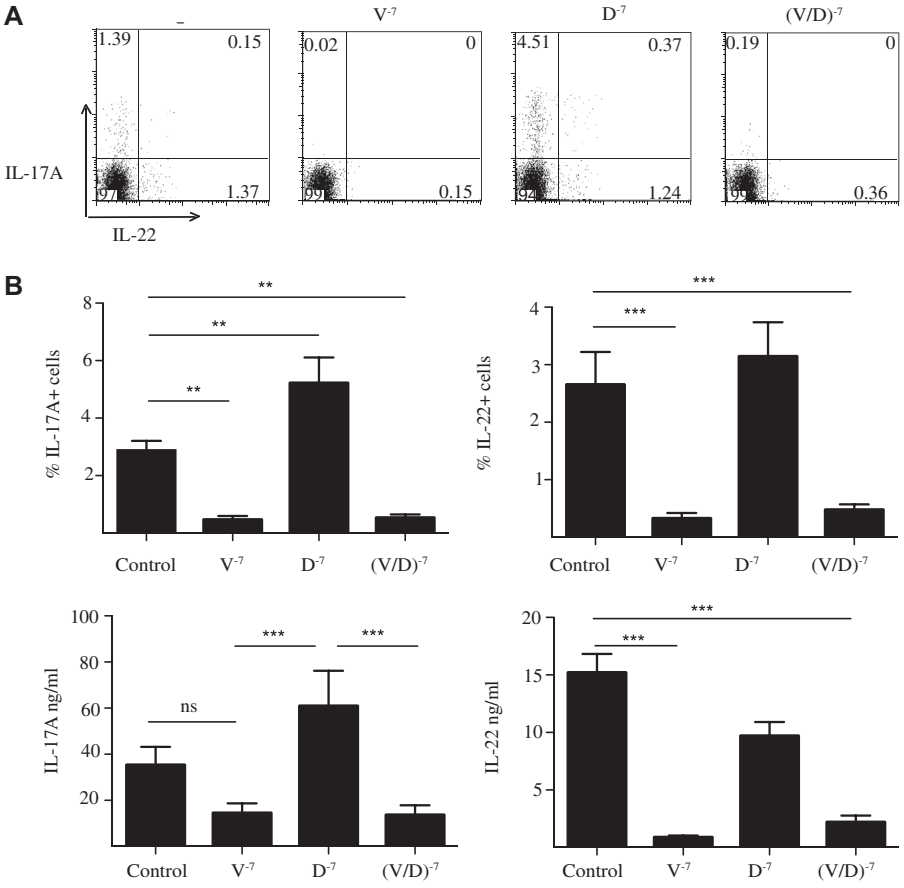

FIG 3. 1,25(OH)$_2$D3 inhibits IL-17A and IL-22 in cultures from asthmatic patients. **A,** Representative dot plots. **B,** Cumulative percentage of cytokine-positive cells (n = 22) or cytokine secretion (n = 28). 1,25(OH)$_2$D3 reduced IL-17A and IL-22 levels in asthmatic cultures with or without dexamethasone. $^{**}P < .01$ and $^{***}P < .001$, 1-way ANOVA with the Tukey post test. *ns*, Not significant; D^{-x} and V^{-x}, dexamethasone and 1,25(OH)$_2$D3 respectively, at 10^{-x} mol/L.

decreased viability (data not shown) because 1,25(OH)$_2$D3 at the concentration used did not inhibit either, as recently reported.[38] These data highlight the capacity of 1,25(OH)$_2$D3 to counteract any detrimental effect or lack of effect of dexamethasone on T_H17-associated cytokine synthesis in cell cultures from asthmatic patients. 1,25(OH)$_2$D3 effectively inhibited IL-17A production in cultures from healthy control subjects (data not shown), as previously reported by others.[33-35]

1,25(OH)$_2$D3 enhances expression of CD39$^+$ T cells, a potential mechanism for IL-17A inhibition

Expression of the ectonucleotidase CD39 by Foxp3$^+$ regulatory T (Treg) cells has previously been reported to be associated with inhibition of IL-17A production.[36,39,40] Analysis of e*x vivo* expression of CD39 demonstrated a significantly higher frequency of CD3$^+$CD39$^+$ T cells in the peripheral blood of healthy donors compared with that of patients with severe asthma ($P = .003$). CD39 can be expressed on both CD4$^+$ memory and Foxp3$^+$ T cells,[36,39] and therefore we further analyzed expression on Foxp3$^+$ and Foxp3$^-$ populations. A significantly higher frequency of CD39-expressing cells was detected in both CD3$^+$Foxp3$^-$ and CD3$^+$Foxp3$^+$ T cells in the nonasthmatic donors compared with the patients with severe asthma (Fig 4, *A*) in contrast to the higher IL-17A synthesis observed in the patients.

Next, the effect of 1,25(OH)$_2$D3 and dexamethasone on CD39 expression was investigated. CD4$^+$ T cells cultured in the presence of 1,25(OH)$_2$D3 demonstrated a significantly increased gene expression of CD39 (Fig 4, *B*). When surface expression of CD39 was assessed by using flow cytometry, there was a modest but significant increase in the expression level of CD39, as determined based on mean fluorescence intensity, but there were no differences in the overall frequency of CD39$^+$ T cells. In comparison, addition of dexamethasone reduced the percentage of CD39$^+$ T cells and mean fluorescence intensity (Fig 4, *C* and *D*). To investigate the role of CD39 in 1,25(OH)$_2$D3 inhibition of IL-17A production, we used POM-1, a known inhibitor of CD39 ectonucleotidase activity. POM-1 partially abrogated the inhibition of IL-17A synthesis effected by 1,25(OH)$_2$D3 in cultured T cells. This effect was specific for IL-17A because the production of IL-10 and other T_H1 and T_H2 cytokines was not affected (Fig 4, *E*).

DISCUSSION

Our data demonstrate that the production of IL-17A and IL-22 by human peripheral blood CD4$^+$ T cells is increased in patients with severe asthma. Notably, IL-17A levels, but not IL-22 levels, were approximately 5-fold greater in patients with SR asthma compared with those seen in patients with SS asthma. Strikingly,

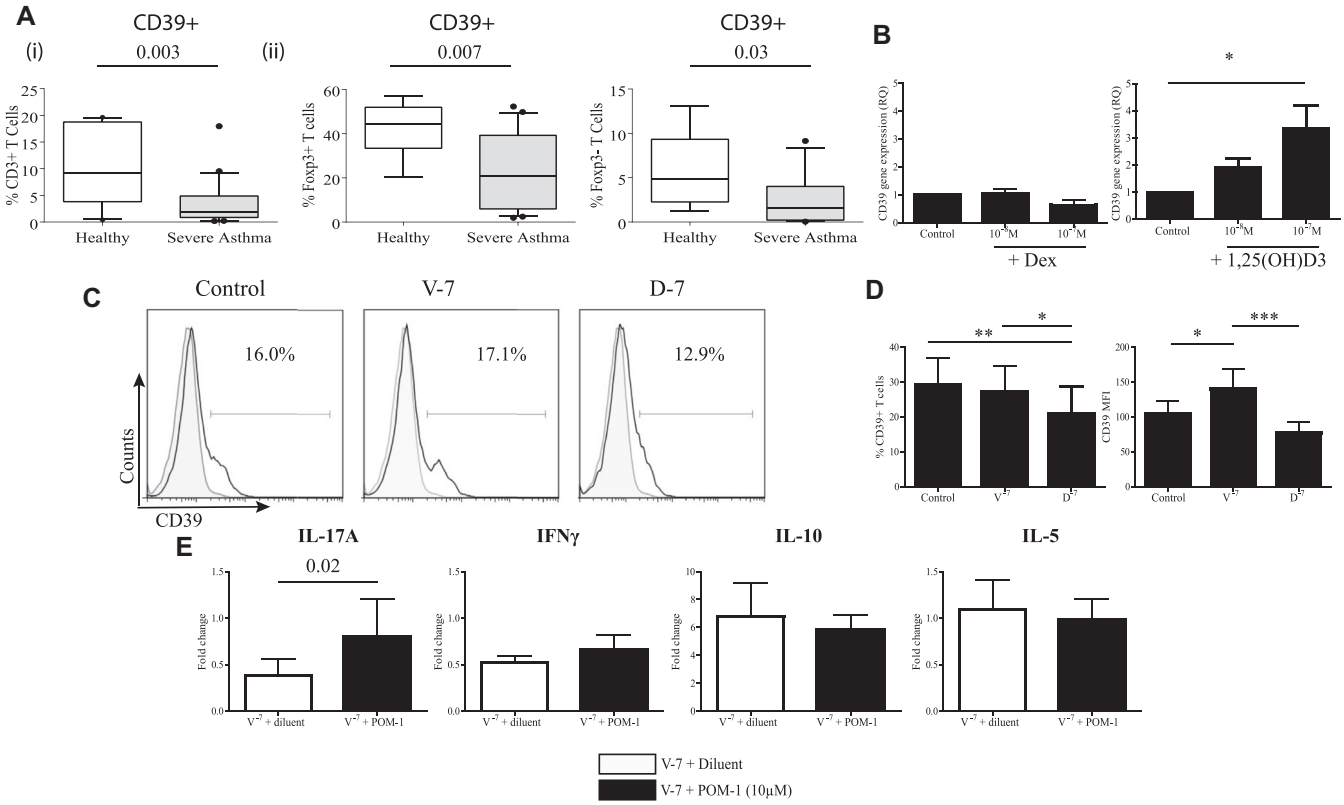

FIG 4. 1,25(OH)$_2$D3 increases CD39 expression by CD4$^+$ T cells. **A,** The frequency of CD39$^+$ and CD3$^+$ T cells *(i)* and CD39$^+$ and Foxp3$^+$ and Foxp3$^-$ T cells *(ii)* in the peripheral blood of healthy donors (n = 8) and patients with severe asthma (n = 19). **B-D,** Effects of treatment with dexamethasone or 1,25(OH)$_2$D3 in culture on healthy nonasthmatic donors. Fig 4, *B*, Relative CD39 mRNA (n = 5). Fig 4, *C*, Representative histograms. Fig 4, *D*, Cumulative data on CD39 expression (n = 6). **E,** Cumulative data (n = 8) on effects of POM-1 (10 μmol/L, CD39 antagonist) on 1,25(OH)$_2$D3-modulation of IL-17A, IFN-γ, IL-10, and IL-5 expressed as fold change from the no-drug condition. *$P < .05$, **$P < .01$, and ***$P < .001$; Fig 4, *B* and *D*, 1-way ANOVA with the Tukey post test, and Fig 4, *E*, Wilcox matched pairs signed-rank test.

the glucocorticoid dexamethasone significantly enhanced the frequency of IL-17A$^+$ cells in culture in both nonasthmatic control subjects and asthmatic patients, although IL-17A secretion was only significantly increased by dexamethasone in control cultures. In contrast, the active form of vitamin D reduced levels of both cytokines directly and also when dexamethasone was present in culture. Importantly, this *in vitro* effect was equivalent in clinically glucocorticoid-resistant and glucocorticoid-sensitive asthmatic patients, with greater than 80% inhibition of the T_H17 response in both patient cohorts. The data support a potential role for the ectonucleotidase CD39 in 1,25(OH)$_2$D3-mediated inhibition of the IL-17A response. Together, our data are consistent with the hypothesis that 1,25(OH)$_2$D3, through inhibition of IL-17A, improves disease control in asthmatic patients independently of their glucocorticoid responsiveness.

The present study extends earlier observations showing an association between increased IL-17A production and severe asthma by investigating 2 asthmatic patient cohorts with clearly defined clinical responsiveness or nonresponsiveness to steroids at the time of study but comparable disease severity. Although blood T cells from all asthmatic patients demonstrated increased IL-17A and IL-22 synthesis, the most striking differences from the control group were observed in the patients with SR asthma. Analysis of cytokine production from patients with SS asthma alone demonstrated a nonsignificant trend for enhanced IL-17A and IL-22 production in comparison with that seen in healthy control subjects. In comparisons between patients with SS asthma versus patients with SR asthma, the most notable differences were in the frequency of the levels of secreted IL-17A and IL-17A$^+$IL-22$^+$ double-positive cells. Double-positive conventional T cells are proposed to represent the more pathogenic population, with studies showing that IL-22 enhances the proinflammatory properties of IL-17A.[32,41]

Independent studies have suggested that T_H17 cytokine production plays a mechanistic role in increasing asthma severity and reducing corticosteroid sensitivity.[22,42] Nevertheless, we cannot completely exclude from the present data the possibility that increased IL-17A production is associated with inhaled corticosteroid administration. Although dexamethasone increased the frequency of IL-17A$^+$ cells in both nonasthmatic and asthmatic cultures, the levels secreted were only significant in nonasthmatic cultures, although a trend for an increase was observed ($P = .102$). A major difference between these 2 subject groups was the baseline level of IL-17A synthesis, which was more than 7-fold lower in the nonasthmatic cultures compared with the asthmatic cultures and might contribute to the capacity to visualize the enhancement by dexamethasone. Furthermore, in a retrospective analysis of our data, we observed an association between inhaled corticosteroid dosages (beclomethasone equivalent before the trial of oral

prednisolone therapy) and the amounts of blood PBMC IL-17A released *in vitro* (Pearson correlation $r = 0.459$, $P = .014$), supporting the possibility that at least some of the IL-17A produced by PBMCs in asthmatic patients results from corticosteroid exposure.

Our data are also broadly complementary of studies in an animal model, demonstrating that IL-17A production by PBMCs was not inhibited by dexamethasone in culture and that airways inflammation induced in mice by adoptive transfer of T_H17 cells was not inhibited by dexamethasone, which is in contrast to that mediated by the adoptive transfer of T_H2 cells.[22] IL-17A might contribute to asthma pathogenesis through a number of different mechanisms, such as induction of neutrophilia and stimulation of lung innate and structural cells, to secrete proinflammatory cytokines.[15,43,44] Also worthy of note is the association of IL-17A with subepithelial fibrosis, a feature of airway remodeling shown in some studies to correlate with asthma severity.[45]

IL-17A has been proposed to affect global corticosteroid responsiveness through effects on the GR. GR-α is ubiquitously expressed and responsible for the induction and repression of target genes, whereas GR-β is expressed at much lower levels and is a dominant negative inhibitor of GR-α. Increased expression of GR-β has been linked to severe asthma in some studies,[42,46] and a recent study suggested that IL-17A and IL-17F increase the expression of GR-β, an effect that was more prominent in asthmatic patients than in healthy control subjects.[23] These data imply a complex and potentially detrimental relationship between IL-17A, steroid treatment, and responsiveness in patients with severe asthma, on which vitamin D might have a positive effect.

We investigated a possible mechanism for the inhibition of T-cell IL-17A synthesis by $1,25(OH)_2D3$. The ectonucleotidase CD39, which is expressed by memory T cells, as well as $Foxp3^+$ Treg cells, has previously been reported to inhibit IL-17A synthesis.[36,39,40] In the present study treatment with $1,25(OH)_2D3$ increased the mean intensity of CD39 expression by human $CD4^+$ T cells, whereas the CD39 antagonist POM-1 partially abrogated $1,25(OH)_2D3$-mediated inhibition of the IL-17A production, supporting a mechanistic role for this pathway but not excluding others. In contrast, dexamethasone tended to reduce CD39 expression.

Manipulation of vitamin D status for therapeutic benefit in asthmatic patients and patients with other respiratory conditions is currently highly topical. This arises in part from studies demonstrating that patients with poorly controlled asthma, poor responsiveness to corticosteroids, or both have significantly reduced vitamin D levels, at least as assessed by the circulating precursor 25-hydroxyvitamin D3.[27-30,47] We and others have previously demonstrated an association with $25(OH)_2D3$ status and airways smooth muscle mass.[27,28,48] Bronchial smooth muscle hypertrophy and hyperplasia are prominent features of airway remodeling, as is subepithelial fibrosis, and IL-17A is a key mediator implicated in lung structural changes with profibrotic activity within the bronchial epithelium.[45] Understanding the various mechanisms through which vitamin D controls respiratory health and steroid responsiveness is central in targeting this pathway therapeutically, and our results provide support for an additional beneficial effect. We believe that evidence of $1,25(OH)_2D3$ downregulating proinflammatory cytokines, such as IL-17A and IL-22, and its capacity to enhance antimicrobial pathways, Treg cells, and other homeostatic mechanisms, such as CD200,[49] are all likely to contribute to promoting homeostasis in the airway and lung health.

Key messages

- **PBMC cultures from steroid refractory asthma patients synthesize significantly higher levels of IL-17A than steroid sensitive patients and nonasthmatic control cultures.**
- **Glucocorticoids do not inhibit IL-17A synthesis in patient cultures and enhance synthesis in nonasthmatic cultures.**
- **$1,25(OH)_2D3$ inhibits the IL-17A response in a glucocorticoid-independent manner, suggesting additional benefits of vitamin D to control severe asthma.**

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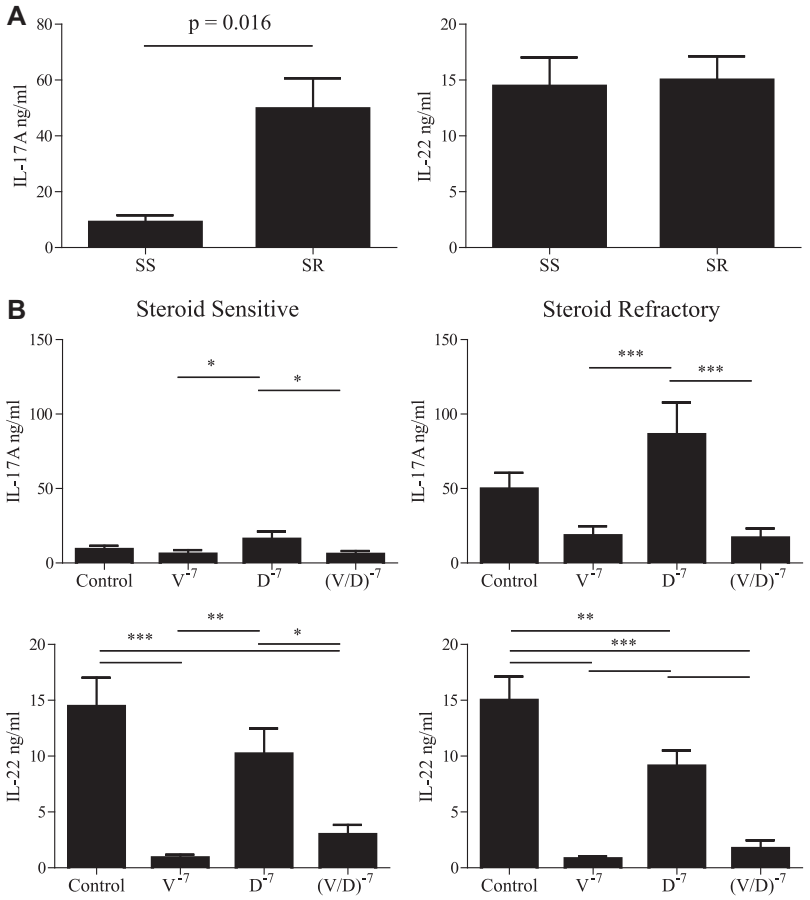

FIG E1. 1,25(OH)$_2$D3 inhibits T_H17-associated cytokines in patients with SS severe asthma and patients with SR severe asthma. **A,** Cumulative data showing higher IL-17A expression in patients with SR asthma and comparable IL-22 levels in cultures from patients with SS asthma and patients with SR asthma (SS asthma, n = 10; asthma, n = 18). **B,** Secreted IL-17A and IL-22 from cultures of patients with SS asthma *(left)* and patients with SR asthma *(right)* containing dexamethasone and 1,25(OH)$_2$D3 (SS asthma, n = 10; SR asthma, n = 18). **P* < .05, ***P* < .01, and ****P* < .0001; Fig E1, *A*, unpaired *t* test, and Fig E1, *B*, 1-way ANOVA with the Tukey post test. D^{-x} and V^{-x}, Dexamethasone and 1,25(OH)$_2$D3, respectively, at 10^{-7} mol/L.

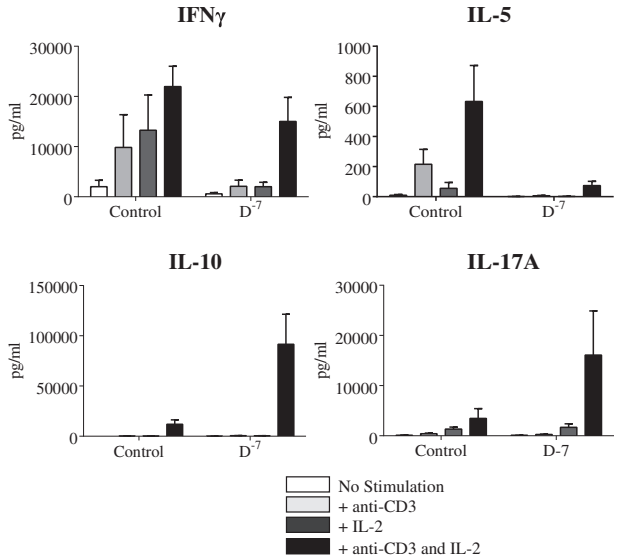

FIG E2. Effect of IL-2 in culture on dexamethasone modulation of IL-10, IFN-γ, and IL-5. Levels of secreted IFN-γ, IL-10, IL-13, IL-5, and IL-17A under different stimulation conditions in healthy control subjects are shown (n = 6). D^x, Dexamethasone at 10^{-7} mol/L.

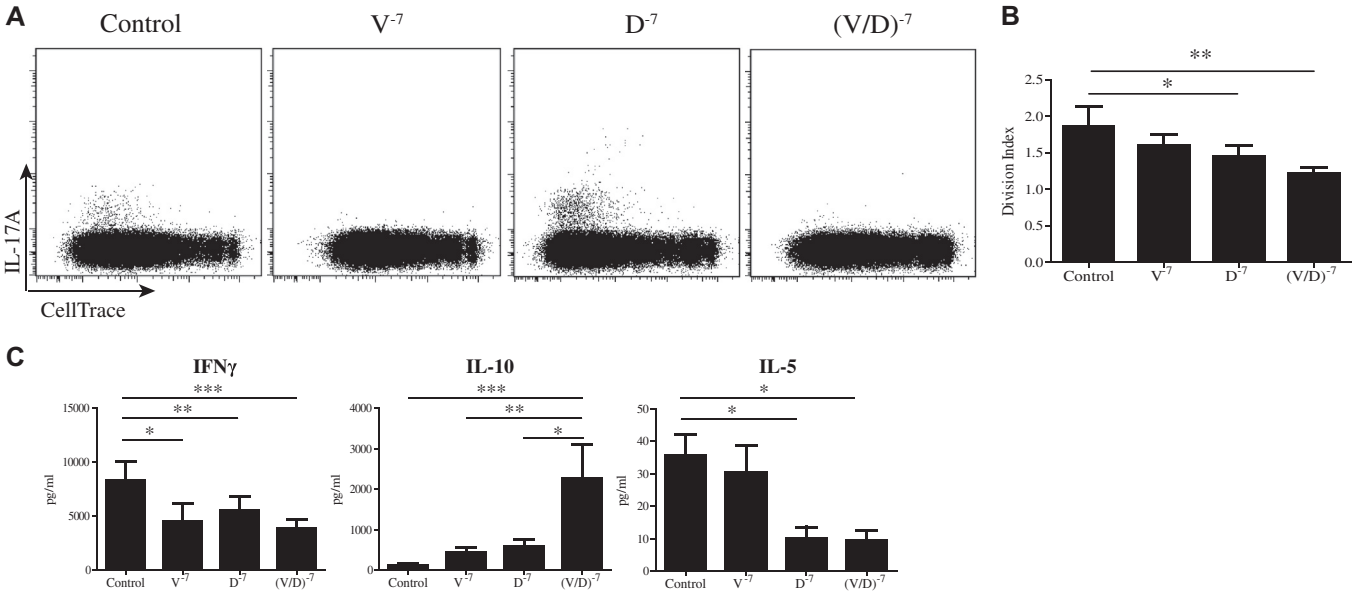

FIG E3. Dexamethasone, but not 1,25(OH)$_2$D3, inhibits PBMC proliferation. **A,** Representative dot plots. **B,** Cumulative data showing proliferation of PBMCs in the presence or absence of dexamethasone and 1,25(OH)$_2$D3 in healthy control subjects (n = 4). **C,** Cumulative data cytokine secretion data of IFN-γ, IL-10, and IL-5 secretion in healthy donors (n = 8). **P* < .05, ***P* < .01, and ****P* < .001; Fig E3, *B* and *C*, 1-way ANOVA with the Tukey post test. D^x and V^x, Dexamethasone and 1,25(OH)$_2$D3, respectively, at 10^{-x} mol/L.

Distinct endotypes of steroid-resistant asthma characterized by IL-17A^{high} and IFN-γ^{high} immunophenotypes: Potential benefits of calcitriol

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Background: A small population of patients with severe asthma does not respond to glucocorticoids (steroid resistant [SR]). They have high morbidity, highlighting an urgent need for strategies to enhance glucocorticoid responsiveness.
Objective: We investigated the immunologic differences between steroid-sensitive (SS) and SR asthmatic patients and the effect on immunophenotype of oral calcitriol treatment because it has been previously shown to beneficially modulate the clinical response to glucocorticoids in patients with SR asthma.
Methods: CD8-depleted PBMCs were isolated from 12 patients with SS and 23 patients with SR asthma and cultured for 7 days with anti-CD3 and IL-2 with or without dexamethasone. Cytokine production was assessed in supernatants by using the Cytometric Bead Array. Patients with SR asthma were subsequently randomized to oral calcitriol or placebo therapy, and identical studies were repeated.
Results: Patients with SR asthma produced significantly increased IL-17A and IFN-γ levels compared with those in patients with SS asthma, although it was evident that cells from individual patients might overproduce one or the other of these cytokines. Production of IL-17A was inversely and production of IL-13 was positively associated with the clinical response to prednisolone. Oral calcitriol, compared with placebo, therapy of the patients with SR asthma significantly improved dexamethasone-induced IL-10 production *in vitro* while suppressing dexamethasone-induced IL-17A production. This effect mirrored the previously demonstrated improvement in clinical response to oral glucocorticoids in calcitriol-treated patients with SR asthma.
Conclusions: IL-17A^{high} and IFN-γ^{high} immunophenotypes exist in patients with SR asthma. These data identify immunologic pathways that likely underpin the beneficial clinical effects of calcitriol in patients with SR asthma by directing the SR cytokine profile toward a more SS immune phenotype, suggesting strategies for identifying vitamin D responder immunophenotypes. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *Asthma, steroid resistant, steroid sensitive, glucocorticoids, IL-17A, vitamin D*

Asthma is an inflammatory lung disease characterized by airways hyperresponsiveness and remodeling and is one of the most common long-term medical conditions. The majority of asthmatic patients achieve control of their disease with β_2-adrenergic agonists and glucocorticoids (steroids) and therefore have steroid-sensitive (SS) asthma.[1] However, there is a proportion of patients who do not show improvement in symptoms and lung function after compliant treatment with inhaled steroids, oral steroids, or both; these patients with steroid-resistant (SR) asthma experience considerable ill health and impart a considerable financial burden on the health care system.

Asthma is a heterogeneous disease, and phenotype-specific therapies have been suggested to enhance the likelihood of therapeutic success in patients relatively refractory to conventional therapy.[2] Recently, significant effort has been directed at defining severe asthma and, in particular, its endotypes or phenotypes.[3] A number of newer biological agents are currently being tested in clinical trials, and although therapeutic benefit has been observed with some of them, they appear to target small subsets of patients.[4-9] Furthermore, they are expensive to manufacture and administer. On the other hand, steroid-sparing or enhancing strategies for refractory patients remain relatively unexplored.

Asthma historically was characterized as being a CD4$^+$ T_H2-mediated disease with increased production of IL-4, IL-13,

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E.S.C. was funded by an MRC British Thoracic Society/Morriston Davies Trust Capacity Building PhD Studentship and subsequently funded by an MRC Centenary Fellowship. A.M.N. was funded by Asthma UK (08/019 and 11/040) awarded to C.M.H. P.E.P. was the recipient of a Wellcome Trust Clinical Research Training Fellowship. The research was funded/supported by the National Institute for Health Research (NIHR) Clinical Research Facility at Guy's & St Thomas' National Health Service (NHS) Foundation Trust and NIHR Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health.

Disclosure of potential conflict of interest: E. S. Chambers was funded by an MRC British Thoracic Society/Morriston Davies Trust Capacity Building PhD Studentship and subsequently funded by an MRC Centenary Fellowship; she has also received funding from the Biomedical Research Centre. A. M. Nanzer was funded by Asthma UK. P. E. Pfeffer holds a Wellcome Trust Clinical Research Training Fellowship. A. R. Martineau's institution has received funding from Asthma UK. C. J. Corrigan has received consultancy fees from Novartis and Chiesi, as well as payment for delivering lectures from Allergy Therapeutics. C. M. Hawrylowicz's institution has received funding from Asthma UK (Grant ID 08/019, 11/040); she has received payment for delivering lectures from Boehringer Ingelheim, as well as payment for manuscript preparation. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication September 28, 2014; revised January 28, 2015; accepted for publication January 29, 2015.

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0091-6749

http://dx.doi.org/10.1016/j.jaci.2015.01.026

Abbreviations used
BAL: Bronchoalveolar lavage
GR-β: Glucocorticoid receptor β
25[OH]D: 25-Hydroxyvitamin D
1,25$(OH)_2$D3: 1,α25-Dihydroxyvitamin D
ROC: Receiver operating characteristic
SR: Steroid resistant
SS: Steroid sensitive

and IL-5. More recently, these studies have been refined to identify clinical and immunologic subtypes (phenotypes and endotypes, including immunophenotypes). For example, there is established evidence of an IL-17A^{high} phenotype in patients with severe asthma. Subgroups of patients with severe asthma have been demonstrated to have increased concentrations of the proinflammatory cytokine IL-17A in sputum and bronchoalveolar lavage (BAL) fluid.[10-13] Additionally, IL-17A production has been correlated with airways hyperresponsiveness.[14] Although T_H17 cells have been shown to be important for defense against fungal and bacterial lung infections, a critical balance between this and an association with various immune pathologies, including autoimmune disease, appears to exist. Studies in mice suggest an association between steroid resistance and T_H17-mediated disease: adoptive transfer of T_H17 cells resulted in increased concentrations of chemokines and granulocyte colony-stimulating factor in the BAL fluid of mice with severe combined immunodeficiency, and treatment with dexamethasone resulted in increased neutrophil infiltration but no improvement in airways hyperresponsiveness.[15] More recently, we showed that blood T-cell IL-17A expression was increased 7-fold in patients with SR asthma compared with that seen in patients with SS asthma.[16] Additionally, we found that although the synthetic glucocorticoid dexamethasone did not inhibit IL-17A production, calcitriol (1,α25-dihydroxyvitamin D [1,25$(OH)_2$D3]), the active form of vitamin D, significantly inhibited IL-17A production in a glucocorticoid-independent manner.[16] Additionally SR asthma has been associated with increased expression of the transcriptionally inactive glucocorticoid receptor β (GR-β),[17] whereas increased GR-β expression has been reported in response to exogenous IL-17A and IL-23 *in vitro*, an effect that was more prominent in asthmatic patients than in healthy control subjects.[18,19] Together, these studies constitute a firm platform for implicating IL-17A in the pathogenesis of SR asthma.

There is a large body of epidemiologic data suggesting that vitamin D insufficiency, as defined by the serum 25-hydroxyvitamin D (25[OH]D) concentration, is strongly associated with impaired respiratory health,[20] risk of asthma,[21,22] asthma severity, and refractoriness to current therapy.[23-27] We have previously shown that patients with SR asthma have a defective T-cell IL-10 response to dexamethasone *in vitro*[28] and that this could be restored by 1,25$(OH)_2$D3.[29] These data, along with the epidemiologic evidence, prompted us to design a proof-of-concept, placebo-controlled clinical trial assessing whether treatment with calcitriol could restore clinical responsiveness to a standard 2-week trial of oral prednisolone in patients with SR asthma. Indeed, in this trial we showed that treatment with calcitriol resulted in significant improvement in steroid responsiveness in these patients.[30]

The aim of the present study was to use PBMC samples from the patients in this trial to further assess the immunophenotypes of SS and SR asthma at baseline and after calcitriol therapy in the patients with SR asthma to identify potential immunophenotypes of steroid refractoriness and also the likely effect of treatment with calcitriol.

METHODS

Subjects

Asthmatic patients receiving step 3 or 4 therapy according to the British Thoracic Society guidelines and receiving optimized care by tertiary care respiratory physicians were recruited and provided written informed consent (ethics 08/H0804/84). All patients had a prebronchodilator FEV_1 of less than 80% of predicted value and documented airway variability of greater than 12% after 400 μg of short-acting bronchodilator and had undergone detailed assessment to exclude a diagnosis other than asthma and comorbidities affecting asthma control. Patients had not received oral glucocorticoids (steroids) for at least 4 weeks before the study. Patients receiving immunotherapy, smokers, or patients with a respiratory tract infection or asthma exacerbation during the 4 weeks before enrollment were excluded.

Steroid resistance was defined as less than 10% improvement in baseline prebronchodilator FEV_1 after a 14-day course of oral prednisolone (40 mg/1.73 m^2/d; Wockhardt UK, Wrexham, United Kingdom) in eligible patients. Routine spirometry was measured before and after the course of prednisolone by using a PC-based spirometer and software (WinspiroPRO Medical International Research, Rome, Italy).

Participants who were identified as having SR asthma were randomized to receive either 0.25-μg calcitriol soft capsules (Rocaltrol; Roche, Mannheim, Germany) or organoleptically identical lactose placebo generated in house (Pharmacy Production Unit, St Thomas' Hospital NHS Trust, London, United Kingdom) twice daily for 4 weeks after a 4-week washout period (full details of the clinical trial study outline and patient details can be found in the article by Nanzer et al[30]; see Fig E1 in this article's Online Repository at www.jacionline.org for a study schematic). After 2 weeks of calcitriol or placebo treatment, patients were given a second course of oral prednisolone identical to the first while calcitriol or placebo was continued. Spirometry was performed at the beginning and end of this second course of oral prednisolone, as before.

Flow cytometry

The following antibodies were used for *ex vivo* phenotyping of peripheral blood obtained from asthmatic donors: CD3, CD4, CD8, and CD19 (SK7, RPA-T4, RPA-T8, and HIB19, respectively; BD Biosciences, Oxford, United Kingdom). Red blood cells were lysed after staining with BD FACS lysing solution; the samples were subsequently assessed on a FACSCalibur (BD Biosciences). Absolute and differential blood leukocyte counts were performed routinely with an LH750 hematology analyzer (Beckman Coulter, Brea, Calif) and analyzed in conjunction with flow cytometric data to calculate cell numbers.

Cell isolation and culture

Human PBMCs were isolated, as previously described.[29] Briefly, CD8-depleted PBMCs were obtained by means of negative selection with $CD8^+$ Dynabeads (Invitrogen, Paisley, United Kingdom). Cells (1×10^6 cells/mL) were cultured in RPMI (containing 10% FCS, 2 mmol/L L-glutamine and 50 μg/mL gentamicin) and stimulated with plate-bound anti-CD3 (1 μg/mL, OKT-3) plus 50 U/mL recombinant hIL-2 (Eurocetus, Harefield, United Kingdom) in the presence or absence of dexamethasone at indicated concentrations (Sigma-Aldrich, Gillingham, United Kingdom) and/or 10 ng/mL hIL-4 in a 24-well plate for 7 days. There was no significant difference in cellular viability under all culture conditions (data not shown). Where indicated, after this initial 7-day period, cells were harvested and readjusted to the same density of 1×10^6/mL viable cells and then cultured

for a further 48 hours with plate-bound anti-CD3 and IL-2 alone in 48-well plates, after which supernatants were harvested for cytokine analysis.

Cytokine analysis

IL-17A, IL-10, IFN-γ, and IL-13 concentrations in culture supernatants were measured by using the Cytometric Bead Array (BD Biosciences), according to the manufacturer's protocol. The lower limit of detection for each analyte was 1.5 pg/mL.

Statistics

Data were assessed for equivalence to a Gaussian distribution and equality of variance, after which the appropriate parametric or nonparametric test was performed (see individual figure legends) with GraphPad Prism 6 software (GraphPad Software, La Jolla, Calif). Differences were considered significant at the 95% confidence level. Data are presented as means, with error bars representing 95% CIs.

RESULTS

To investigate the immunologic phenotypes of SS and SR asthma, we recruited patients with moderate-to-severe asthma who were defined as having either SS or SR asthma based on their changes in lung function after 2 weeks of therapy with oral prednisolone at pharmacodynamically uniform dosages (for a clinical trial schematic, see Fig E1).[30] The patients with SS and SR asthma were similar in terms of demographics, mean body mass index, mean equivalent inhaled glucocorticoid dosages, and mean FEV_1 at baseline. The only clinical difference between patients in the 2 groups was their changes in lung function after oral prednisolone (mean ΔFEV_1 percent predicted); the patients with SS asthma showed a significant improvement (from 56.0% [95% CI, 47.4% to 64.6%] to 70.8% [95% CI, 62.6% to 79.0%], $P < .0001$), whereas the patients with SR asthma did not (from 61.3% [95% CI, 55.3% to 67.3%] to 59.7% [95% CI, 52.8% to 66.5%], $P = .18$; Table I).[30,31] There was no significant difference in mean peripheral blood eosinophil counts in the patients with SS asthma compared with those in the patients with SR asthma. Oral prednisolone therapy was associated in both groups with a significant increase in mean blood neutrophil counts and a reduction in mean blood eosinophil counts (see Fig E2 in this article's Online Repository at www.jacionline.org).

TABLE I. Patients' characteristics

	Patients with SS asthma (n = 12)	Patients with SR asthma (n = 23)	*P* value
Age (y)	49.0 (40.7-57.3)	51.8 (45.9-57.8)	
Ethnic origin			
White	9 (75.0)	17 (73.9)	
African	3 (25.0)	5 (21.7)	
Asian	0	1 (4.4)	
Sex			
Male/female	4 (33.3)/8 (66.6)	14 (60.8)/9 (39.2)	
Atopic*	10 (83.3)	19 (82.6)	
BMI (kg/m^2)	31.8 (28.0-35.6)	28.2 (26.2-30.2)	.06
Inhaled corticosteroid dose (BDP)	1113 (798-1469)	1225 (985-1520)	.60
FEV_1 (L)			
Before steroids	1.7 (1.4-1.9)	1.9 (1.7-2.1)	.13
After steroids	2.1 (1.8-2.4)	1.8 (1.6-2.1)	
FEV_1 (%)			
Before steroids	56.0 (47.4-64.6)	61.3 (55.3-67.3)	.97
After steroids	70.8 (62.6-79.0)	59.7 (52.8-66.5)	
Serum 25(OH)D (nmol/L)	38.8 (27.6-50.1)	36.9 (27.6-46.2)	.51

Where applicable, data are means with 95% CIs or frequencies (percentages). *P* values are shown for noncategorical clinical parameters assessed by using an unpaired *t* test. *BDP*, Daily beclomethasone dipropionate equivalent dosage according to the British Thoracic Society/SIGN guidelines on the management of asthma[31]; *BMI*, body mass index.
*Atopy was defined based on skin prick test responses.

Increased expression of IL-17A and IFN-γ in patients with SR asthma compared with patients with SS asthma

Because asthma is believed to be a $CD4^+$ T cell–mediated disease, we used CD8-depleted PBMC cultures with T-cell stimulation, as performed in our previous studies,[16,29] to compare T-cell immunophenotypes in both patients with SS and those with SR asthma. Blood samples for these studies were taken at screening visit 1 (see Fig E1). CD8-depleted PBMCs from both groups were cultured for 7 days with anti-CD3 and IL-2 in the presence or absence of IL-4. After 7 days, cells were washed, recounted, and replated at 1×10^6/mL to allow for any differential changes in the numbers of cells between the groups and stimulated for a further 48 hours. Cytokine protein expression in culture supernatants was then assessed by using the Cytometric Bead Array for IL-17A, a T_H17 cytokine; IL-13, a T_H2 cytokine; and IFN-γ, a T_H1 cytokine, as well as the anti-inflammatory cytokine IL-10. There were no significant differences in *ex vivo* mean numbers of blood T cells (including $CD4^+$ and $CD8^+$ cells) and B cells in the asthmatic patients classified as having SS or SR asthma (see Fig E2, *B*).

Under these conditions, CD8-depleted PBMCs from the patients with SR asthma produced significantly increased mean concentrations of both IL-17A and IFN-γ compared with the those seen in the patients with SS asthma, although the mean production of IL-10 and IL-13 did not significantly differ. This difference in IFN-γ production between the 2 groups was no longer observed when exogenous IL-4 was included in the culture conditions (Table II). Attempts were made to detect IL-17A in the cultures containing exogenous IL-4, but concentrations were low or undetectable (data not shown). This is in line with earlier studies showing that IL-4 inhibited IL-17A production, whereas anti–IL-4 enhanced IL-17A production from $CD4^+$ T cells.[32] Conversely, IL-4 signaling through the IL-4 receptor is known to enhance IL-10 responses.[33]

Differential response to the glucocorticoid dexamethasone in patients with SR asthma compared with that in patients with SS asthma

We next examined whether there were differences in responsiveness to the synthetic steroid dexamethasone *in vitro* between patients with SS asthma and patients with SR asthma that might reflect the differences seen clinically. Cells from the patients with SR asthma compared with those from the patients with SS asthma continued to show increased mean production of IL-17A and IFN-γ across a range of dexamethasone concentrations. Indeed, high concentrations of dexamethasone further significantly increased IL-17A production by cells from the patients with SR asthma, a phenomenon not observed in cells

TABLE II. Cytokine production in cultures from both patients with SS and those with SR asthma at baseline

	CD3/IL-2			CD3/IL-2 + IL-4		
Cytokine	**Patients with SS asthma**	**Patients with SR asthma**	***P* value**	**Patients with SS asthma**	**Patients with SR asthma**	***P* value**
IFN-γ	11.0 (8.3-13.7)	19.7 (14.2-25.2)	.02	8.5 (4.9-12.1)	11.6 (9.0-14.2)	.15
IL-10	1.3 (0.2-2.4)	0.8 (0.5-1.2)	.51	1.2 (0.5-2.0)	1.6 (0.6-2.6)	.61
IL-13	3.6 (0.7-6.6)	2.6 (0.8-4.4)	.89	5.1 (1.7-8.6)	4.3 (2.7-5.8)	.59
IL-17A	11.3 (1.5-21.0)	47.0 (27.8-66.2)	.007	ND	ND	

Cytokine production (in nanograms per milliliter) by cells either stimulated with CD3 plus IL-2 or additionally in the presence of IL-4 in culture is shown. Data are means and 95% CIs.
ND, Not detected/measured.

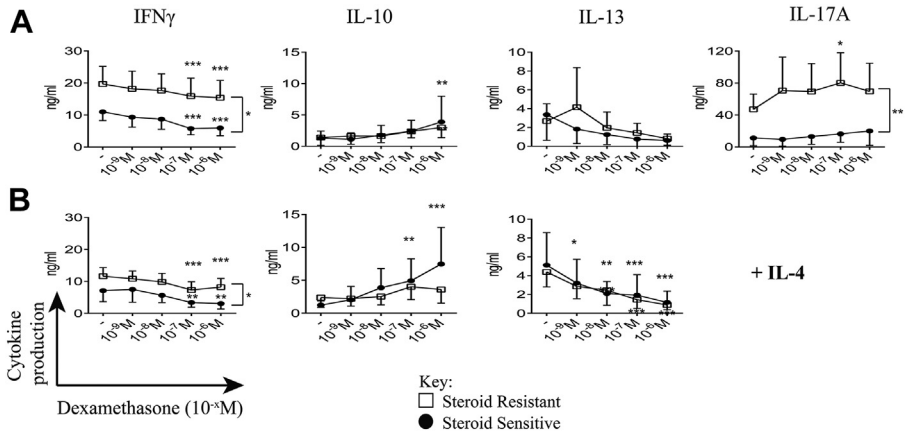

FIG 1. Differential cytokine production by both patients with SS and those with SR asthma in response to dexamethasone *in vitro*. Cytokine production by CD8-depleted PBMCs *(y-axis)* in the presence of dexamethasone (10^{-x} mol/L) *in vitro (x-axis)* stimulated with CD3 plus IL-2 **(A)** and additional IL-4 **(B)** is shown. *Open squares*, Patients with SR asthma; *solid circles*, patients with SS asthma. Data are means and 95% CIs and assessed by using 2-way ANOVA with Dunnett multiple comparisons tests of cytokine production in the presence of different concentrations of dexamethasone compared with no dexamethasone. $*P \leq .05$, $**P < .01$, and $***P < .001$.

from the patients with SS asthma. Although increased production of IFN-γ by cells from the patients with SR asthma was retained in the presence of dexamethasone, high concentrations of dexamethasone nevertheless inhibited IFN-γ production in both the patients with SS and those with SR asthma. Dexamethasone significantly inhibited IL-13 production by cells cultured in the presence of IL-4 from patients in both groups. Conversely, dexamethasone increased IL-10 production only in cells from the patients with SS asthma and strikingly so in the presence of exogenous IL-4 ($P = .008$), which is in line with our earlier observations (Fig 1, *B*).[28]

IL-13 and IL-17A production associate positively and negatively with clinical responsiveness to glucocorticoid therapy

We next investigated whether these observed differences correlated with clinical measurements. Cytokine production was analyzed according to absolute lung function in both the patients with SS and those with SR asthma, and there was no significant association with production of any cytokine (see Fig E3 in this article's Online Repository at www.jacionline.org). However, when the patients with severe asthma were segregated into quartiles according to their change in lung function after 2 weeks of oral prednisolone therapy (ΔFEV_1 [in liters]), it was apparent that cells from those asthmatic patients in the lowest quartile whose lung function actually decreased with steroid therapy produced a significantly increased mean quantity of IL-17A at baseline compared with those who showed an improvement. There was a similar trend with IFN-γ production, although this was not statistically significant. Conversely, cells from those asthmatic patients who were in the upper quartile of lung function improvement produced a significantly increased mean quantity of IL-13 at baseline. No such association was observed with IL-10 production (Fig 2, *A*), although those patients whose cells produced the highest mean ratios of IL-10/IL-17A, as well as IL-13/IL-17A, were more likely to fall in the upper quartile of the degree in lung function improvement after taking oral prednisolone (Fig 2, *B*).

Production of IL-17A and IFN-γ predict clinical glucocorticoid responsiveness

Because increased production of both IL-17A and IFN-γ by CD8-depleted PBMCs was associated with clinical unresponsiveness to glucocorticoid therapy, we compared production of these cytokines in the entire population of asthmatic patients (both SS and SR asthma). Contrary to our expectations, there was no correlation between overproduction of IL-17A and IFN-γ (Fig 3, *A*), suggesting that both immunophenotypes contribute to corticosteroid responsiveness. Indeed, combined production of both cytokines correlated inversely with change in absolute FEV_1

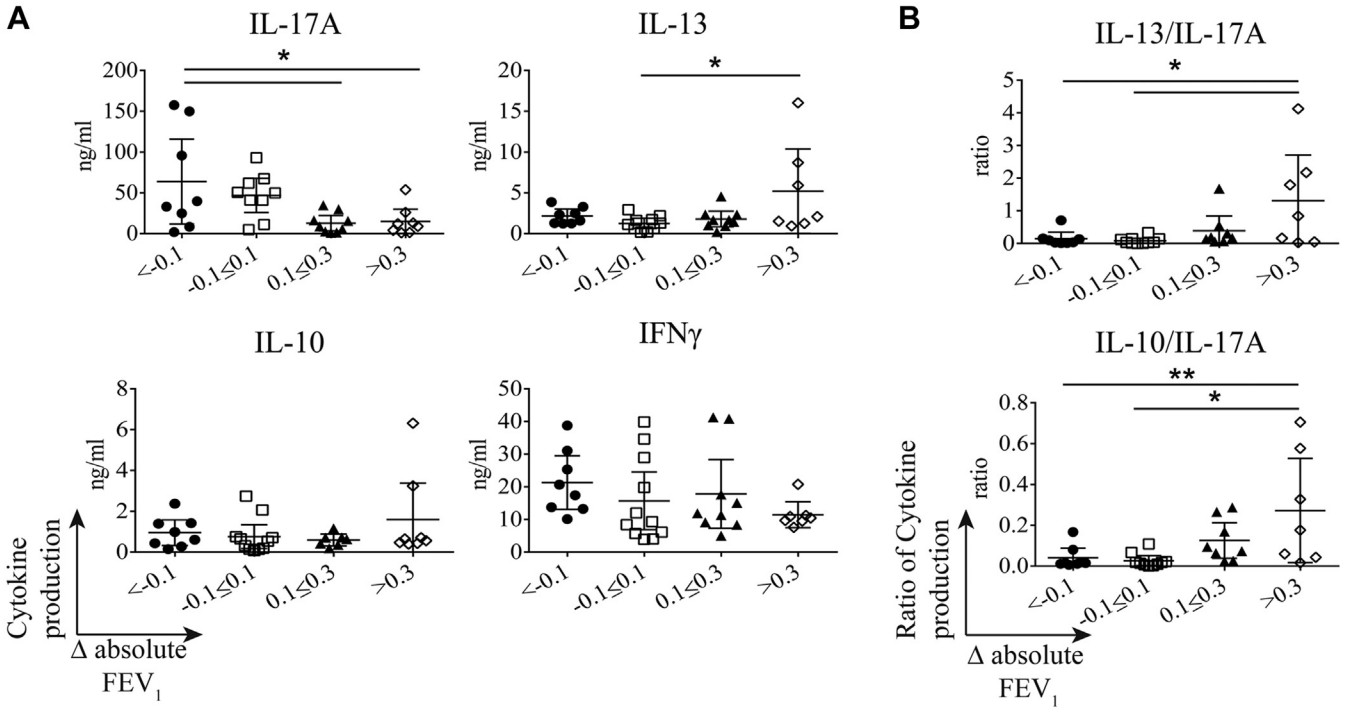

FIG 2. IL-17A is negatively and IL-13 is positively associated with clinical response to 2 weeks of prednisolone. Cytokine production **(A)** and ratio of IL-13/IL-17A and IL-10/IL-17A cytokine production **(B)** separated based on change in absolute lung function in response to 2 weeks of prednisolone (ΔFEV_1) are shown. Data are means and 95% CIs and assessed by using Kruskal-Wallis 1-way ANOVA with the Dunn multiple comparison *post hoc* statistical test. *$P \leq .05$ and **$P < .01$.

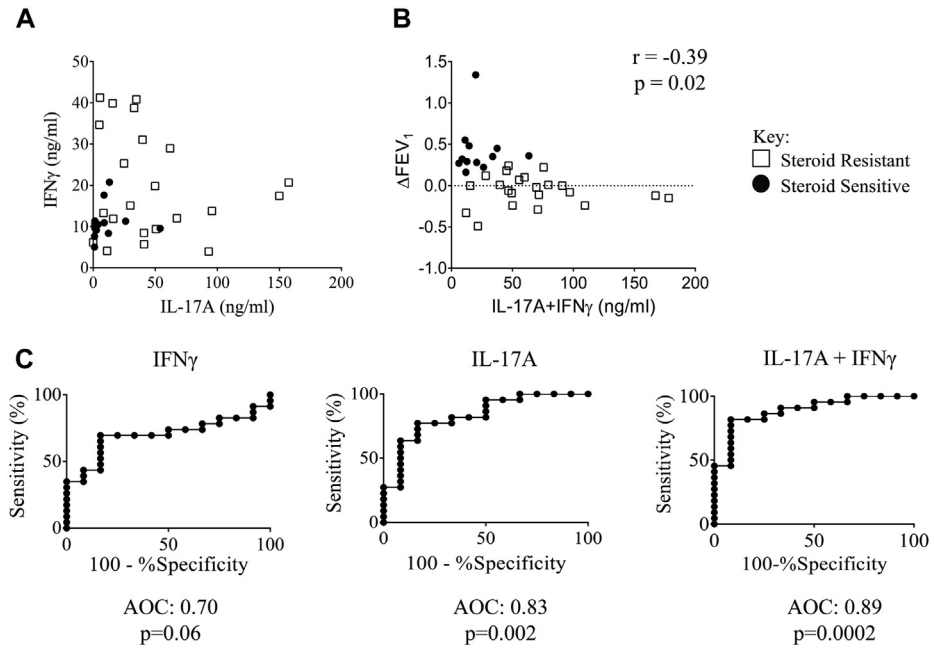

FIG 3. IL-17A and IFN-γ production predict glucocorticoid responsiveness. **A,** Comparison of IL-17A and IFN-γ production in patients with SS asthma *(solid circles)* and patients with SR asthma *(open squares)*. **B,** Combined production of IL-17A and IFN-γ correlated with change in absolute lung function (ΔFEV_1) in response to 2 weeks of prednisolone, as assessed by using the Spearman rank correlation statistical test. **C,** ROC curves for prediction of steroid resistance based on production of IFN-γ, IL-17A, and their combination.

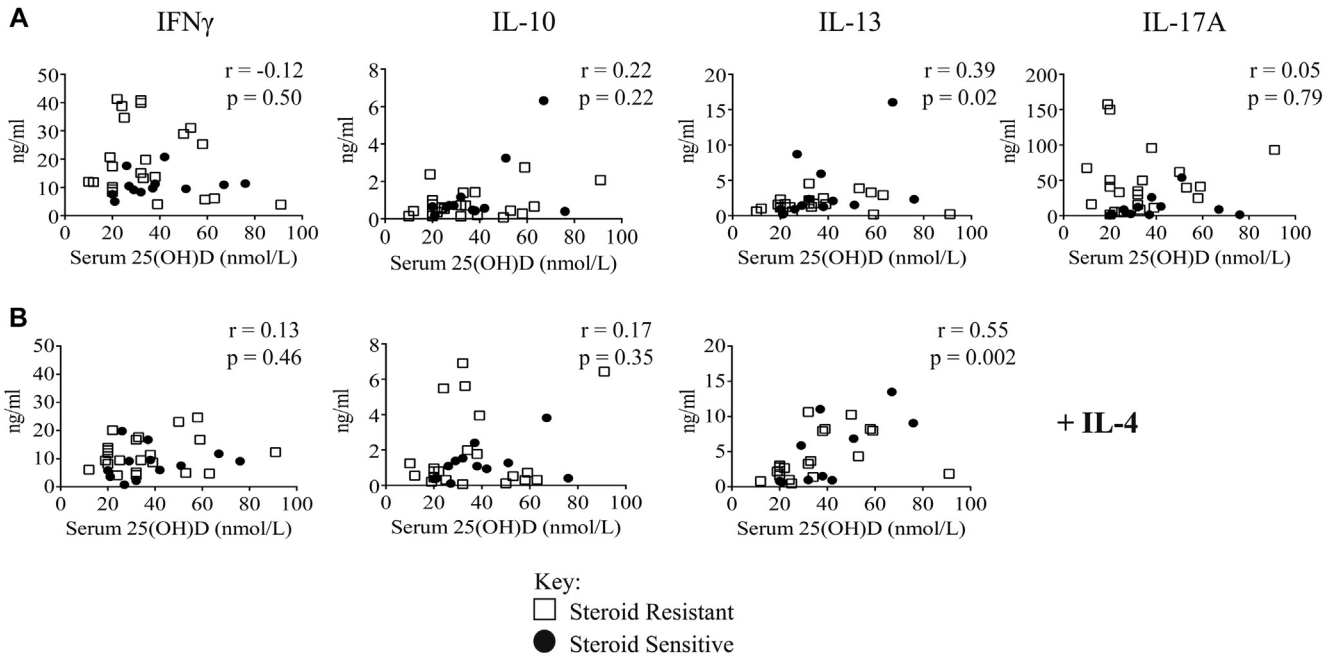

FIG 4. Serum 25(OH)D concentration correlates with IL-13 expression *in vitro*. Cytokine production in cell-culture supernatants was assessed by using the Cytometric Bead Array and correlated with baseline vitamin D status (defined as the serum 25[OH]D concentration). **A,** Cells stimulated with CD3 plus IL-2. **B,** Cells stimulated with addition of IL-4 in culture. *Open circles*, Patients with SR asthma; *solid squares*, patients with SS asthma. Data were assessed by using Spearman rank correlation.

after glucocorticoid therapy (Fig 3, *B*). Additionally, we performed analysis of the data in Fig 3, *B*, in the absence of the potential outlier (with $\Delta FEV_1 = 1.34$), and interestingly, in the absence of this point, the correlation was statistically stronger, with a new *r* value of −0.41 and a *P* value of .018.

Consequently, we analyzed the sensitivity and specificity of IL-17A and IFN-γ production, both separately and in combination, to predict the clinical effect of oral prednisolone therapy in changing FEV_1. In the case of IL-17A, receiver operating characteristic (ROC) analysis was highly significant ($P = .002$): an IL-17A production threshold of greater than 28.1 ng/mL produced a sensitivity of 63.6% and a specificity of 91.7% for detecting glucocorticoid resistance. In the case of IFN-γ, ROC analysis provided less robust outcomes ($P = .06$): an IFN-γ production threshold of greater than 20.7 ng/mL produced a sensitivity of 34.8% and a specificity of 91.7%. The combination of IL-17A and IFN-γ production proved to be a better test: ROC analysis was highly significant ($P = .0002$), showing that a production threshold of greater than 38.5 ng/mL for the sum of both cytokines predicted clinical glucocorticoid resistance with a sensitivity of 81.8% and specificity of 91.7% (Fig 3, *C*). This degree of sensitivity and specificity is at least comparable with that of noninvasive markers, which have been claimed to predict glucocorticoid-resistant asthma.[34]

IL-13 production positively correlates with serum 25(OH)D concentrations

Vitamin D has been reported to inhibit lymphocyte production of IL-17A and IFN-γ *in vitro*[16,29,35] while enhancing T_H2 cytokine production.[36] To examine such possible effects *in vivo*, we next compared cytokine production in all of the study participants with their vitamin D status based on serum 25(OH)D concentrations before calcitriol or placebo therapy. Serum 25(OH)D concentrations did not correlate with production of IL-10, IL-17A, or IFN-γ, but we did observe a significant positive correlation with IL-13 production that was further strengthened in the presence of exogenous IL-4 (Fig 4). After removal of a potential outlier (67 nmol/L; 16 ng/mL), IL-13 still significantly correlated (r = 0.35; $P = .047$) with serum 25(OH)D levels in the cultures without exogenous IL-4.

Calcitriol therapy of patients with SR asthma reverses induction of IL-17A and augments IL-10 production in response to dexamethasone

In our recent clinical report of the same patients used in the present study, we showed that treatment of our patients with SR asthma with calcitriol compared with placebo improved their clinical responsiveness to glucocorticoid therapy.[30] We used samples collected from these patients, and notably, no difference was observed between patients with SR asthma subsequently allocated to either placebo or calcitriol treatment at baseline before any treatments with regard to their lymphocyte populations at screening visit 1 (see Fig E2, *C*). We studied these patients to establish the possible effect of oral calcitriol therapy on the immunologic phenotype of peripheral blood cells. We found that after 4 weeks of therapy with calcitriol/placebo and an additional identical 2-week course of oral prednisolone for the final 2 of these 4 weeks (treatment visit 3, see Fig E1), there was significant induction by dexamethasone of IL-17A production in the placebo-treated group, as seen at baseline (screening visit 1). Strikingly, this effect was no longer evident in the calcitriol-treated group (Fig 5, *A*). Furthermore, as

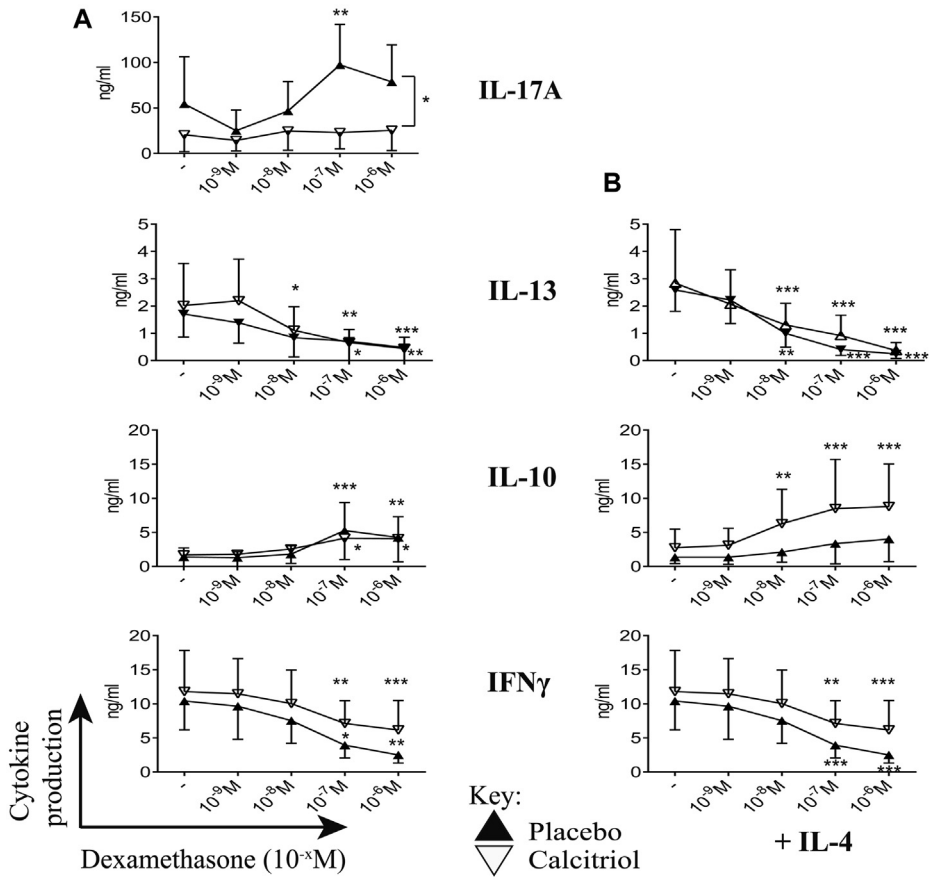

FIG 5. Calcitriol treatment decreases expression of IL-17A in cultures from patients with SR asthma. Cytokine production by CD8-depleted PBMCs from patients with SR asthma in the presence of dexamethasone (10^{-x} mol/L) *in vitro* after 4 weeks of calcitriol or placebo therapy and 2 weeks of oral prednisolone therapy and cells stimulated with CD3 plus IL-2 **(A)** and additional IL-4 **(B)** is shown. *Open triangles*, Calcitriol; *solid triangles*, placebo. Data are means and 95% CIs. Data were assessed by using 2-way ANOVA with Dunnett multiple comparisons tests. $*P \leq .05$, $**P < .01$, and $***P < .001$.

predicted from our earlier work,[28,29] cells from the calcitriol-treated patients with SR asthma now showed a significant increase in IL-10 production in response to dexamethasone, an effect that was most marked in the presence of IL-4 in culture (Fig 5, *B*). Although IL-13 production correlated with serum 25(OH)D concentrations at baseline, as shown above, there was no apparent effect of 4 weeks of calcitriol treatment on production of IL-13 or its inhibition by dexamethasone. Similarly, dexamethasone modestly but significantly inhibited IFN-γ production as before and equivalently in the calcitriol- and placebo-treated patients. Although the numbers are small, further analysis of the calcitriol-treated patients with SR asthma showed that the patients whose improvement in lung function was in the upper quartile in response to the second course of oral prednisolone were those whose cells produced the highest mean quantities of IL-13 and the lowest mean quantities of IL-17A (see Fig E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

The data presented in this article demonstrate that there is a significantly higher production of IL-17A and IFN-γ in PBMC cultures from patients with SR asthma compared with those from patients with SS asthma. Notably, these appear to reflect 2 distinct immunophenotypes of SR asthma, an IL-17A^{high} and an IFN-γ^{high} profile, which are largely nonoverlapping. We have previously reported,[30] and develop here, evidence that glucocorticoids can aggravate the excessive production of proinflammatory T_H17 by blood lymphocytes in patients with SR asthma, which characterizes a subgroup of these patients. We demonstrate that the levels of IL-17A synthesis in the PBMC cultures negatively and IL-13 synthesis positively correlate with the absolute change in lung function (FEV_1) after 2 weeks of oral prednisolone treatment in our adult asthma cohort. Our data further support earlier findings that T_H2^{high} asthma, as characterized by increased IL-13 production, is more likely to be associated with glucocorticoid responsiveness.[37]

The second major finding of this study relates to the capacity of calcitriol to reverse the SR immune phenotype to one more closely aligned to that of patients with SS asthma. Thus the IL-17A^{high} profile is abrogated by calcitriol therapy in a glucocorticoid-independent manner. Conversely, calcitriol therapy also restores the impaired, corticosteroid-induced anti-inflammatory IL-10 response that characterizes patients with SR asthma, extending our earlier findings in this area.[29] In addition to effects on IL-10 synthesis, calcitriol might reduce the induction of IL-17A through enhancement of a number of

regulatory mechanisms, including the inhibitory CD39/adenosine pathway and regulatory T cells.[16,35,38-40]

Several mechanisms have been proposed to contribute to clinical glucocorticoid refractoriness in asthmatic patients at the cellular level, particularly in T cells, including overexpression of proinflammatory transcriptional regulators, such as nuclear factor κB and activator protein 1; increased expression of histone deacetylases, polymorphisms of the *IL10* gene; increased expression of the dominant negative isoform of the GR-β; overexpression of T_H2 cytokines; and vitamin D insufficiency.[17,24,25,41-44] More recent evidence also suggests that IL-17A overexpression can be both a marker and a risk factor for severe and SR asthma.[11-14,16] In this study we confirm the latter association and show an independent association with overexpression of the T_H1 cytokine IFN-γ. Previous mechanistic studies in autoimmune models of disease have suggested that pathologic IL-17A production is by IL-17A$^+$IFN-γ$^+$ coexpressing cells,[45-47] but our data were not consistent with this, rather suggesting separate "immunophenotypes" of SR asthma. It is notable from our study that the IL-17A^{high} phenotype might be enhanced by glucocorticoids in some subjects and that patients with this phenotype were more likely to show deterioration in lung function after oral prednisolone, raising the possibility that oral glucocorticoid therapy is not only unhelpful but also detrimental in some of these patients. Although lymphocyte cultures might not be practical as routine clinical biomarkers, further research might be anticipated to reveal more practical novel biomarkers of IFN-γ^{high} and IL-17A^{high} SR asthma similarly to the development of periostin as a biomarker of T_H2^{high} asthma.[48]

The mechanisms behind glucocorticoid enhancement of IL-17A production by CD4 cells are not fully understood and represent an area of active research. A recent article described a detailed transcriptional time course during T_H17 differentiation, suggesting a number of potential candidates for further study.[49] Previous data from our laboratory suggest that glucocorticoids do not inhibit proliferation of IL-17A–expressing cells; the mechanisms behind these differential effects on proliferation are unknown and currently under investigation. The differences observed in this study are not due to differences in the *ex vivo* baseline populations of the major lymphocyte groups because no differences were observed; however, we cannot rule out there being an enrichment of certain subsets of memory cells. The only difference we have observed previously was in the frequency of FoxP3–positive regulatory T cells,[39] where we showed that there was a higher frequency of FoxP3–positive T cells in the peripheral blood of patients with SS asthma compared with that seen in patients with SR asthma. However, it seems unlikely that this small population of cells can account for the large differences observed in this study.

It is important to note that a somewhat contradictory scientific literature exists on the capacity of vitamin D to inhibit and/or enhance T_H2 responses experimentally and in animal models (reviewed by Lange et al[50]). Our data indicate that serum concentrations of 25(OH)D positively correlate with IL-13 synthesis in culture. Importantly, however, administration of oral calcitriol did not lead to enhancement of IL-13 production in culture. Although IL-13 is implicated in asthma pathogenesis, its production is strongly inhibited by glucocorticoids[16,51]; indeed, the T_H2^{high} asthma phenotype is now widely regarded as a marker of glucocorticoid responsiveness.[37] Therefore our data support the concept that vitamin D improves responsiveness to glucocorticoids at least partly by altering the immunophenotype away from a glucocorticoid-resistant T_H17 phenotype toward a more responsive T_H2 immunophenotype. Thus although calcitriol does not improve lung function in asthma directly, it does improve responsiveness to glucocorticoid therapy.[30]

A limitation to this study was that the data observed were collected from peripheral blood, although we were able to show significant correlations between cytokine production in culture and changes in lung function. Future studies need to be directed at the target organ to gain full insight into changes in cytokine expression in these patients. It would be informative to have studied BAL samples, as well as peripheral blood, providing more information about the tissue of interest. However, bronchoscopy with BAL carries risks and side effects, especially in patients with severe asthma, and therefore was not part of the current study design. One approach we took to counteract this limitation was to perform our peripheral blood cultures in the presence or absence of IL-4 in an attempt to simulate the T_H2-like environment of the asthmatic bronchial mucosa.[52] In our previous work we showed that the vitamin D–induced effects on IL-10 were most profound when IL-4 was present in culture.[28,40,53]

Data from several clinical trials investigating the clinical efficacy of vitamin D in asthma are emerging.[30,54-56] The biggest published trial to date (408 patients randomized) was the VIDA trial. In this placebo-controlled trial patients were supplemented with an initial bolus of 100,000 IU oral vitamin D3 and then subsequently supplemented with 4,000 IU daily. The primary outcome was time to first asthma treatment failure, and no significant improvement in this outcome after vitamin D supplementation was observed. However, the trial also assessed changes in the clinical response to inhaled glucocorticoids, and vitamin D3 supplementation modestly decreased the overall dose of steroid used.[54] Additionally, a striking reduction in the cumulative number of exacerbations was observed in patients within the treatment arm. Two recent smaller studies showed a more positive role for vitamin D supplementation in asthmatic patients in which vitamin D therapy improved lung function and reduced asthma exacerbations in the treatment arms compared with the placebo arm.[56,57] Another interesting earlier trial investigated the effect of vitamin D3 supplementation (1,200 IU/d) on seasonal influenza A infection and, despite low numbers, in a secondary outcome measure a significant reduction in the number of asthma attacks in the group that was receiving vitamin D3 was reported.[55]

The studies reported to date have used different forms of vitamin D at different concentrations and dosing regimens over varying periods of time. In several studies many patients were not or were borderline vitamin D deficient, which will affect the efficacy of vitamin D treatment. Clearly, further investigation is required to fully understand the effect of vitamin D treatment on asthma control and prevention. What is unique about our study was the use of the active form of vitamin D, calcitriol, which we have previously shown to improve the SR asthmatic response to 2-week prednisolone therapy. Calcitriol is an attractive short-term adjunct to oral glucocorticoid therapy for severe exacerbations of disease in patients with SR asthma, particularly those already taking maintenance oral glucocorticoids. Although there are concerns about long-term prescription of this form of vitamin D, short-term therapy might be a viable strategy, particularly because expression of Cyp27B1, the catalytic enzyme that converts circulating 25(OH)D into active calcitriol

(1,25[OH]$_2$D3), is inhibited by glucocorticoids[58]; treatment of patients with SR asthma with oral calcitriol would bypass this effect.

In summary, comparison of immunophenotypes in both patients with SS and those with SR asthma highlights both IL-17A^{high} and IFN-γ^{high} immunophenotypes as independent indicators of glucocorticoid refractoriness and suggests that calcitriol ameliorates the glucocorticoid responsiveness of IL-17A^{high} patients. We believe the data from this study, although from a small cohort, justify further investigation of calcitriol therapy, and comparison with 25(OH)D supplementation in patients with SR asthma identified with biomarkers indicative of nonresponsiveness to glucocorticoids.

Key messages

- **IL-17A^{high} and IFN-γ^{high} immunophenotypes are indicative of SR asthma.**
- **Calcitriol potentially improves the clinical response to glucocorticoids through reduction in IL-17A production and enhancement of steroid-induced IL-10.**

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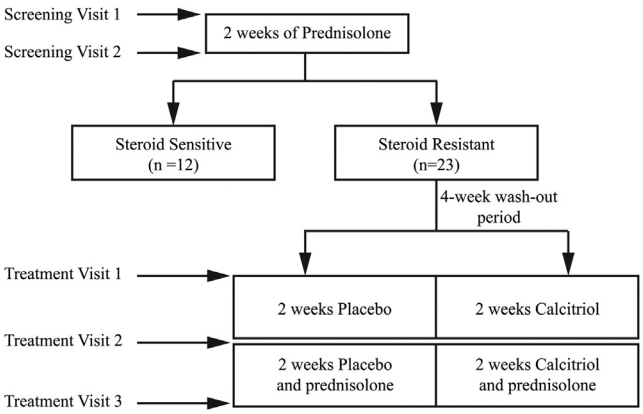
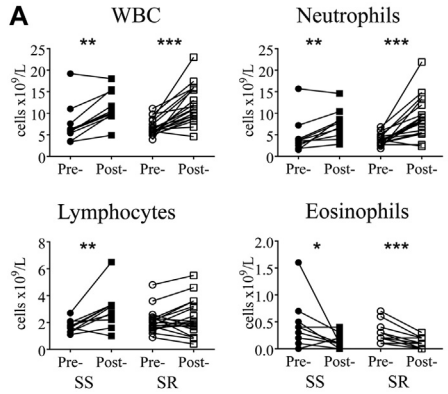


FIG E1. Clinical trial schematic. Patients with severe asthma were recruited and given prednisolone at screening visit 1 and returned 2 weeks later at screening visit 2 when patients with SS asthma were excluded from the trial and patients with SR asthma were retained. After a 4-week washout period, the patients with SR asthma were randomly assigned to receive calcitriol or placebo for 1 month (treatment visit 1). After 2 weeks (treatment visit 2), the patients were started on additional oral prednisolone and then returned 2 weeks later for the final visit (treatment visit 3). Lung function was assessed at all clinical trial visits except treatment visit 1. CD8-depleted PBMCs were assessed from peripheral blood collected at screening visit 1 and treatment visit 3.



B

Lymphocyte population (x10^8 cells/L)	Steroid Sensitive	Steroid Resistant	p value
B cells	1.5 (1.0-1.9)	1.6 (1.0-2.3)	0.70
T cells	10.6 (8.4-12.8)	11.4 (8.7-14.1)	0.69
CD4+ T cells	7.1 (5.2-9.0)	7.0 (5.1-8.9)	0.92
CD8+ T cells	2.8 (2.0-3.5)	3.0 (1.5-4.5)	0.31

C

Lymphocyte population (x10^8 cells/L)	Placebo	Calcitriol	p value
B cells	2.0 (0.8-3.2)	1.6 (0.7-2.4)	0.55
T cells	13.4 (9.0-17.7)	11.3 (7.1-15.5)	0.19
CD4+ T cells	9.6 (5.9-13.3)	7.0 (4.4-9.6)	0.10
CD8+ T cells	3.0 (2.3-3.8)	3.7 (1.8-5.5)	0.96

FIG E2. Peripheral leukocyte counts. **A,** Peripheral blood total and differential leukocyte counts in the patients with SS asthma *(solid symbols)* and patients with SR asthma *(open symbols)* before and after 2 weeks of prednisolone. **B** and **C,** Total B cells (CD19$^+$), T cells (CD3$^+$), and CD4$^+$ and CD8$^+$ T cells were assessed by using *ex vivo* flow cytometric analysis of peripheral blood from screening visit 1 of both patients with SS and those with SR asthma (Fig E2, *B*), as well as in the same patients with SR asthma separated based on their assignment to calcitriol or placebo treatment (Fig E2, *C*). Data were assessed by using the Wilcoxon matched-pairs statistical test (Fig E2, *A*) or the Mann-Whitney *U* statistical test (Fig E2, *B* and *C*). *$P \leq .05$, **$P < .01$, and ***$P < .001$.

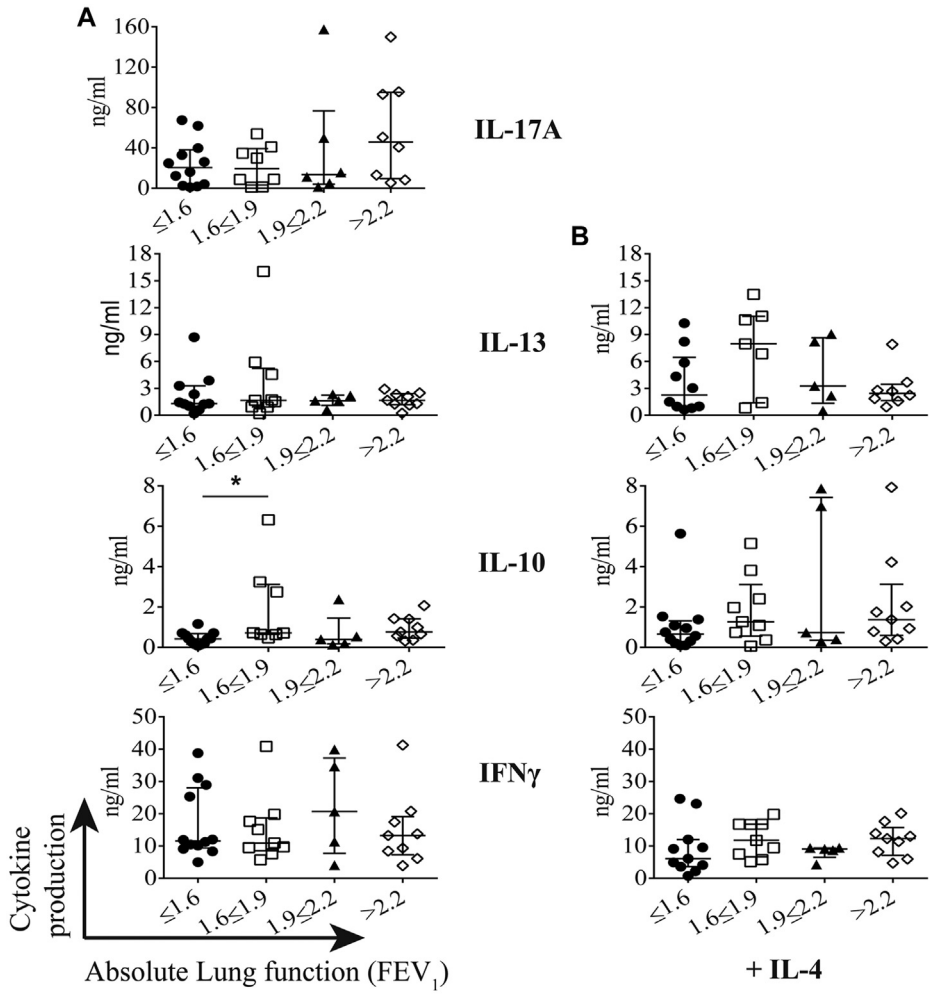


FIG E3. Cytokine production does not associate with lung function. Cytokine production from all patients with severe asthma separated according to their absolute lung function at baseline (FEV_1) is shown. Data were assessed by using Kruskal-Wallis 1-way ANOVA with the Dunn multiple comparison *post hoc* statistical test.

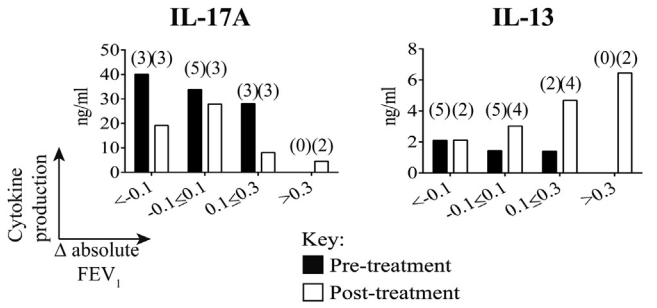


FIG E4. Effects of calcitriol on lung function and IL-13 and IL-17A levels. Cytokine production was separated based on individual changes in absolute lung function (ΔFEV_1) in response to 2 weeks of prednisolone before (baseline; *black bars*) and after (after 4 weeks of calcitriol and 2 weeks of prednisolone; *white bars*) treatment. Numbers of patients with SR asthma are indicated in parentheses above.