

The Pharmacogenetic and Immunomodulatory Response to Vitamin D in Tuberculosis

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Abstract

Background

Tuberculosis is a global problem, with little change in antibiotic therapy over the last fifty years, but with the emergence of both multi-drug resistant disease and extensive drug resistant disease further treatments are needed to ensure successful management of the disease.

Severe vitamin D deficiency is prevalent in patients with tuberculosis and the immunomodulatory mechanisms of elements of the vitamin D axis, including vitamin D binding protein (DBP) and vitamin D receptor (VDR) have been explored in part.

Aims

This thesis will ascertain the role of polymorphisms in vitamin D axis genes in determining response to vitamin D in tuberculosis patients. Additionally it will aim to determine whether the interaction between underlying genotype, baseline vitamin D level and other elements of the vitamin D axis has the potential to influence clinical outcome and further investigate in vitro effects of vitamin D and elements of the vitamin D axis in influencing the frequency and functionality of monocytes and T cells, both of which are key elements in the immune response to tuberculosis infection.

Results

Associations between vitamin D baseline and response to supplementation appear to have a genetic association with DBP, VDR and DHCR7 genotypes and varying DBP haplotypes appear to determine the level of DBP at baseline measurement. The effect of vitamin D has an immunomodulatory role in both monocyte response and T regulatory activity, with a clear effect of vitamin D on cytokine response.

Conclusion

There appears to be a role for vitamin D in the treatment of tuberculosis but further questions are raised regarding the benefits and risks of immune response modulation in an inflammatory/cytopathogenic condition such as tuberculosis.

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CHAPTER 1

INTRODUCTION

Tuberculosis (TB) is a worldwide problem with 9 million new cases globally and 1.5 million deaths due to the disease in 2013. The Millennium Development Goals (MDG) for 2015 are that incidence should be decreasing, with prevalence and mortality halved and although globally there is a 1.5% reduction in new cases, 11 of the high burden countries (HBCs) account for 80% of the global burden of TB cases) and are failing to meet the MDG requirements. This is in part due to financial constraints, conflict and HIV epidemics in the regions. [1]. There has been little change in the drugs used to treat TB and the occurrence of both multidrug resistant TB (MDR-TB) and extensive drug resistant TB (XDR-TB) has prompted consideration of additional therapeutic agents. Vitamin D may be one such agent, of broad relevance to infectious disease because of its immunomodulatory properties. Indeed, in the pre- antibiotic era Vitamin D was used with some success as a sole agent [2] and early treatment of TB included light therapy for the skin manifestation lupus vulgaris [3]. More recently roles for variation in its carrier protein (vitamin D binding protein; DBP) [4] and its receptor (vitamin D receptor; VDR) [5] due to genetic polymorphism have been reported to determine risk of disease and response to treatment respectively.

1.1 Tuberculosis

1.1.1 Transmission and causative organism

Tuberculosis is the result of infection with *Mycobacterium tuberculosis* (M.tb) which is spread through droplet infection. The bacteria, carried in a suspension can remain airborne if the particle size is less than 4 micrometres allowing the bacteria to circulate for some time, increasing the likelihood of transfer from person to person, particularly in crowded conditions.

Mycobacteria have a complex lipid rich cell wall (Figure 1.1) consisting of peptidoglycan, arabinogalactan and mycolic acids, which are covalently linked. The structure of the cell wall is thought to aid pathogen survival in the host cell, both structurally and immunologically. The hydrophobic nature of the wall prevents entry of hydrophilic drugs into the cell, whilst mycolic acids are known to influence the differentiation of macrophages to foamy macrophages [6], harbouring M.tb in the granuloma.

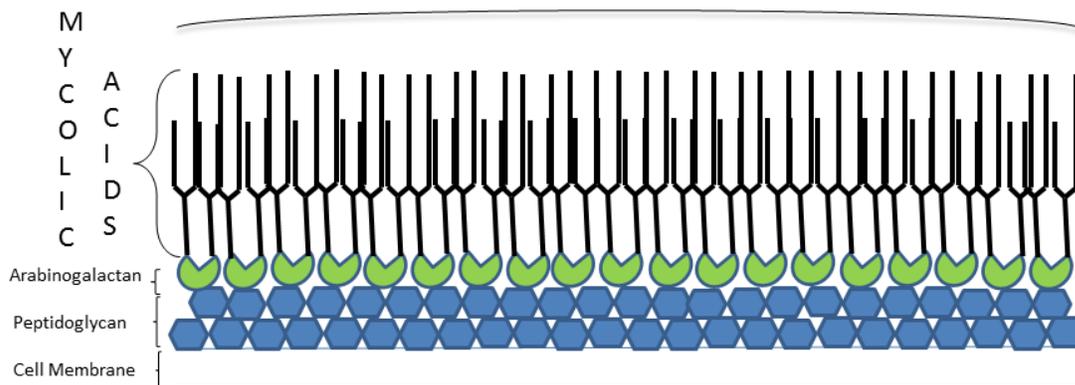


Figure 1.1 Schematic diagram of *Mycobacterium tuberculosis* cell wall.

Components of the mycobacterial cell wall include a peptidoglycan layer, the arabinogalactan layer and mycolic acid covalently bonded together forming the mycolic acid-arabinogalactan-peptidoglycan complex (MAP-c). The peptidoglycan layer contains *N*-acetyl glucosamine–*N*-acetyl muramic acid (NAG–NAM) which is heavily cross linked and surrounds the cell membrane. The arabinogalactan layer, surrounding the peptidoglycans, consists of repeating disaccharide units (galactan) modified with arabinan polymers. This can undergo further modifications to increase pathogenicity of the mycobacteria. The arabinan is bound to long carbon chain mycolic acids, which form the waxy layer around the mycobacteria and contribute to its hydrophobic nature and impermeability of the thick wall.

1.1.2 Infection with mycobacterium tuberculosis

Infection with M.tb results in an increase in alveolar macrophages, dendritic cells, monocytes, small macrophages and granulocytes within the lung [7]. The risk of infection with M.tb is influenced by several factors including the immune status of the individual, ethnicity, excessive alcohol consumption, drug use, residing in an urban area and homelessness [8].

The normal immune response to infection involves both the innate and cell mediated immune system. The innate immune response is non-specific and involves the general recruitment of immune cells to sites of infection. The cell-mediated (adaptive) immune response involves antigen presentation and subsequent T cell activation secondary to this specific stimulus. On entering the lung, the M.tb bacilli are initially taken up by local alveolar macrophages and dendritic cells and thus move to an intracellular environment. Phagocytosis of a pathogen by the macrophage may occur when the pathogen is recognised by a particular pathogen associated molecular pattern (PAMP) or secondary to opsonisation of the pathogen by complement. Internalisation of the pathogen then occurs via receptors including mannose receptors and complement receptors [9]. A phagosome is formed, which in turn matures and fuses with a lysosome to form a phagolysosome.

In addition to the innate immune response, cellular immunity is essential in the formation of a granuloma (consisting of naïve T cells encasing the infected macrophages), which is thought to control dissemination of the bacillus. It is believed that although the cell mediated response is important in the formation of the granuloma, this can also occur in the presence of innate immune activity alone [10]. The action of cytokines such as tumour necrosis factor alpha (TNF α), produced by both macrophages and T cells, is essential in maintaining granuloma structure [11] and studies in

mice have shown that TNF α knockout animals succumb to overwhelming infection due to uncontrolled replication and dissemination of mycobacteria. The inability to form granulomas results in the formation of necrotic lesions [12].

M.tb uses a number of strategies to evade the host response and these contribute to its potential survival and persistence in the host. For instance, M.tb is capable of causing an arrest in phagosome maturation [13] and resisting acidification in the phagolysosome [14].

1.1.3 Clinical features

Tuberculosis has a variety of clinical presentations. In individuals who have not been previously exposed to M.tb, *primary tuberculosis* occurs which may be subclinical in an immune competent individual. The initial infection results in multiplication of the bacillus in the lung, known as the Ghon focus. Local involvement of peri-bronchial lymph nodes results in the primary complex. The infected person may eradicate the disease or through persistence of the bacillus in the macrophage, develop latent tuberculosis with the risk of reactivation at a later time. Primary infection may result in clinically evident (active) infection in the form of pulmonary / pleural manifestations, hypersensitivity reactions or disseminated extra-pulmonary manifestations such as tuberculous adenopathy, meningitis, pericarditis or miliary TB [15].

The risk of reactivation of latent infection is around 0.084 per 100,000 person years [16] and results in *post-primary tuberculosis*. Post primary infection typically results in pulmonary disease with cavitation and lung tissue destruction. The patient is likely to have positive smear microscopy for acid fast bacilli in the sputum [17]. Extra-pulmonary manifestations may occur in reactivation such

as cervical lymphadenopathy, pleural, pericardial, meningeal, bone or abdominal / gastrointestinal disease. Other tissues and organs may more rarely be infected.

In addition to reactivation, a person may develop post–primary tuberculosis through reinfection as a result of a second exposure to tubercle bacillus.

1.1.4 HIV associated tuberculosis

13% of new cases of tuberculosis worldwide in 2013 were co-infected with HIV with 25% of all TB deaths related to HIV co-infection [1]. It is recommended by the Stop TB Partnership (a collaboration of nearly 1000 groups working together to improve diagnosis and treatment of TB), that all patients with tuberculosis should be tested for HIV [18]. TB in HIV infected persons can be difficult to diagnose due to lower rates of sputum smear positivity and the lack of classical pulmonary features typically associated with post-primary infection. In addition, higher treatment failure rates are seen due to an increased risk of adverse drug reaction and lack of health care support in developing regions [19].

1.1.5 Diagnosis of Tuberculosis

Latent tuberculosis occurs when an individual is infected with M.tb but the immune response is adequate to control replication and the disease remains ‘dormant’. When the immune response is no

longer able to provide effective control the disease may become active and systemic / localising features of disease are usually present. These methods concern the diagnosis of active disease.

History

A suggestive history may include symptoms of cough, haemoptysis, weight loss, sweats, lethargy or manifestations such as palpable lymphadenopathy.

Microscopic detection

Detection of tubercle bacilli can be done using auramine-rhodamine or Ziehl-Neelson stain, resulting in the common term for TB appearances on microscopy of ‘acid fast bacilli’. Microscopy is an inexpensive method of identification and can be done easily with relatively simple equipment and a microscope. Microscopy also diagnoses the most infectious patients (i.e. those with bacilli in the sputum). Although microscopy is highly specific, it has had variable reported sensitivities [20] and cannot differentiate viable from non-viable organisms or give information on drug susceptibility, which requires culture of the organism.

At least three sputum samples for microscopy are recommended. A systematic review evaluating the yield of serial microscopy found that regardless of the method of data stratification, the mean incremental yield in smear positivity and mean sensitivity of the third specimen were between 2% and 5%, suggesting that when considered in light of the additional manpower required to process the third specimen, in low-resource areas with heavy workloads, two specimens may be a more feasible option [21].

Culture

Culture of *M.tb* is preferred for diagnosis as it is the most sensitive of the diagnostic techniques and gives information on antibiotic sensitivity. It has traditionally been done on solid media (Lowenstein–Jensen) but is associated with prolonged culture times (6-8 weeks) due to the slow growth of *M.tb*. Liquid culture (manual, automated or semi-automated), more recently introduced, has the advantage of higher turnaround time in the laboratory and increased recovery rate of mycobacteria [22, 23].

Tuberculin skin testing (TST)

Intradermal injection of Purified Protein Derivative (PPD) results in delayed hypersensitivity reaction in those who have been exposed to mycobacterial infection previously. The BCG (Bacille Calmette Guerin) vaccination, administered to selected individuals is derived from *Mycobacteria bovis* (*M.bovis*) and due to cross reactivity previous vaccination may result in a positive response to PPD. An induration of the skin is positive at 5mm or larger, regardless of BCG vaccination history. It is easy to perform and inexpensive but lacks the specificity of interferon gamma release assays (IGRA) described below.

Interferon gamma release assay (IGRA)

IGRA tests are blood assays based on the release of interferon gamma from T-cells when activated by specific M.tb antigens ESAT-6 and CFP-10, complexes of which add structural stability to the mycobacterial cell wall. There are two commonly used IGRA tests in the UK – the T-spot®.TB (Oxford Immunotec, UK) and the Quanti-FERON®-TB Gold (Cellestis). A high level of agreement has been found between the T-SPOT.TB and the QuantiFERON-TB Gold assays [24]. The benefits of IGRA testing are that it has better sensitivity than TST [25], appears to maintain its sensitivity even in immune compromised individuals [26] and has high specificity in the BCG vaccinated [27]. These tests are not currently recommended as the sole test for diagnosis except in certain sub-groups where latent, rather than active TB is suspected [22].

Nucleic acid amplification test (NAAT) (Xpert-MTB/RIF)

This is a fully automated diagnostic molecular test which was strongly endorsed by the WHO (World Health Organisation) in 2010 for use in patients with suspected MDR-TB or HIV/TB. It is a rapid test carried out on untreated sputum which provides information on the presence of M.tb and rifampicin resistance in less than two hours [28].

Radiology

Chest radiographs are often suggestive of active disease. They classically show upper zone consolidation but may also provide information on mediastinal and para-tracheal lymphadenopathy. Computed tomography (CT) is used for further evaluation of possible mediastinal lymphadenopathy for example, in order to determine preferable sites for biopsy. It also provides further information on the presence of disease within other organs. Magnetic resonance imaging (MRI) is commonly used for imaging the spine if spinal TB is suspected and for evaluating skeletal disease. It can also be used for imaging the brain in greater detail than CT.

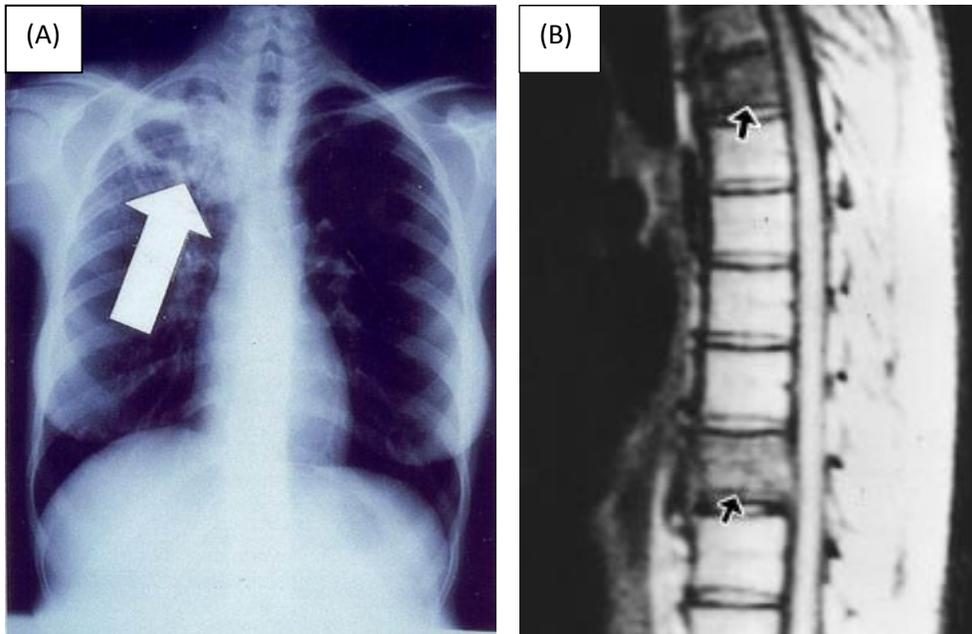


Figure 1.2 (A) Upper zone cavity on chest radiograph (B) change in MRI signal / oedema in vertebrae secondary to tuberculosis.

Radiological changes such as upper zone cavitation or consolidation may be suggestive of tuberculosis but microbiological sampling is essential. Although supportive, radiology cannot give a conclusive diagnosis and changes may mimic other conditions such as malignancy.

1.1.6 Management of Tuberculosis

Active fully sensitive tuberculosis is treated with standard quadruple therapy (rifampicin, isoniazid, pyrazinamide and ethambutol). Standard treatment is 2 months initiation phase with four drugs followed by 4 months continuation with rifampicin and isoniazid alone [29]. This schedule is extended to 12 months treatment in TB meningitis. Treatment schedules will also vary depending on the presence of drug resistance, which inevitably prolongs the regime and requires the use of second line drugs.

Latent infection can be treated with isoniazid as a sole agent for six months or with a combination of rifampicin and isoniazid for three months. A variety of preparations are available which combine the antibiotics and these can be administered as a daily regime or more recently as a weekly regime [30].

Multidrug resistant tuberculosis (MDR-TB), resistant to both of the first line drugs rifampicin and isoniazid, requires the use of an injectable agent for around 6 months along with the use of the remaining first line along with second line drugs, resulting in an extended treatment regime of at least 18 months. This presents difficulties in adherence and treatment completion rates [31]. Newer drugs are being marketed for MDR-TB and XDR-TB such as bedaquiline which inhibits mycobacterial ATP synthase [32] and delamanid which inhibits mycolic acid synthesis [33].

1.2 Vitamin D

The majority of Vitamin D is obtained from sunlight with just 10% obtained from the diet, predominantly from fish, meat and fortified foods. During exposure to sunlight, UVB is absorbed by 7-dehydrocholesterol which forms pre-vitamin D₃. This is then converted to vitamin D₃ and moves from the skin to the extracellular space [34]. This, bound to DBP, moves into the vascular system and is converted by hydroxylation to 25-hydroxyvitamin D₃ (25(OH)D₃) in the liver and further hydroxylated in the kidneys to form active 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). This has the effect of increasing calcium absorption and osteoclastic activity. Vitamin D receptors (VDRs) are present in tissues of the central nervous system, endocrine system, epithelium, muscle and adipose [35] as well as all cells of the immune system including activated T and B cells [36, 37] where VDR levels increase in the presence of 1,25(OH)₂D₃[38]. Targeted gene expression, through binding to vitamin D response elements (VDREs), triggered by the presence of vitamin D generates key antimicrobial products in the immune response including cathelicidin, and defensin B4 (DEFB4) [39]. This process is illustrated in Figure 1.3.

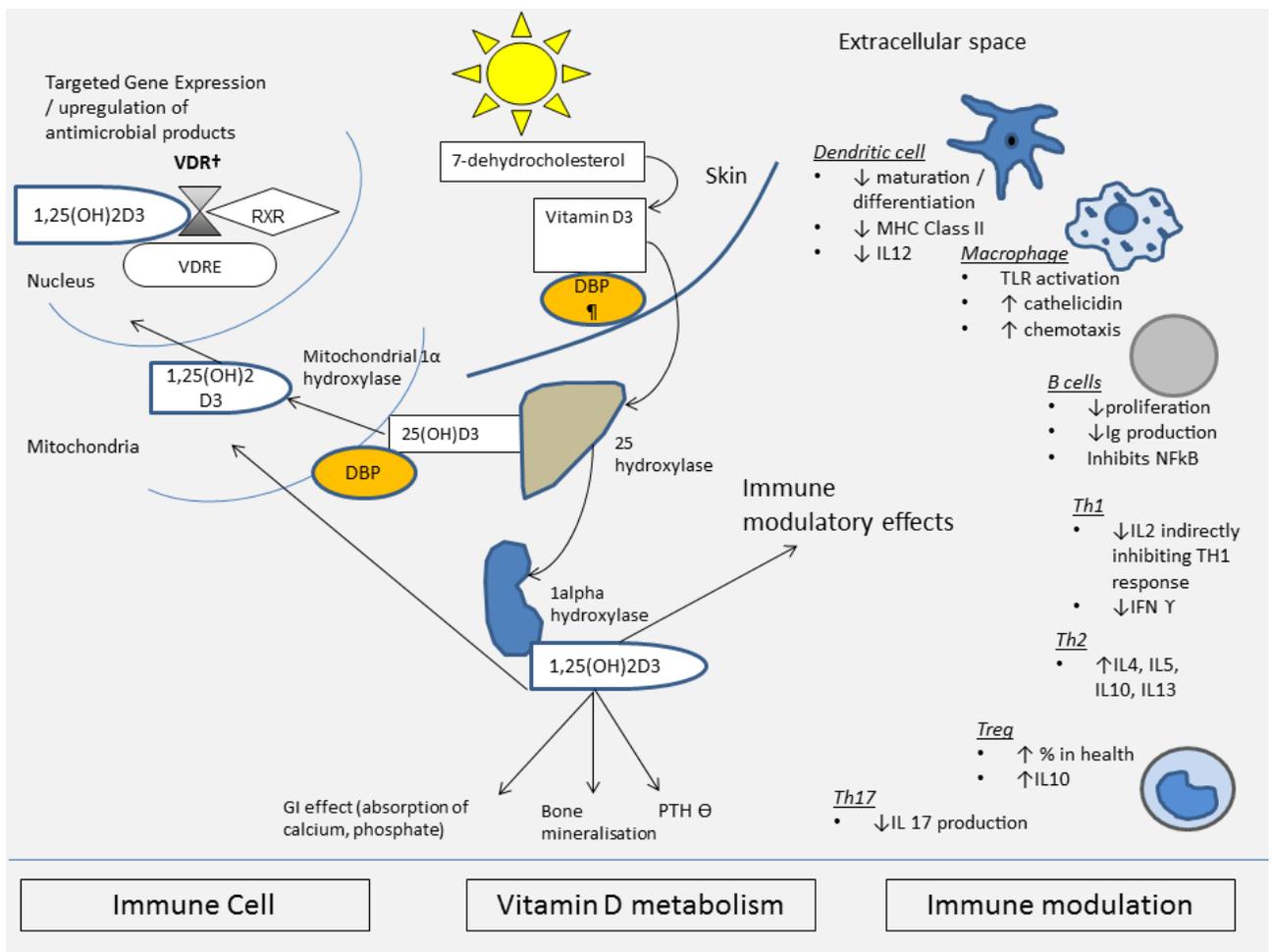


Figure 1.3 The vitamin D axis and the effect of DBP and VDR on immune mechanisms.

Sunlight converts 7- dehydrocholesterol in the skin to a pre-vitamin D₃, which forms Vitamin D₃. This is then hydroxylated in the liver to form 25(OH)D₃ and further hydroxylated by CYP27b1, in the kidney to form 1,25(OH)₂D₃ (other cells e.g. macrophages also have this capability). 25(OH)D₃ can enter the cell by binding with DBP and transporter proteins cubulin or megalin or it can enter the cell by diffusion. It can also be converted within the mitochondria to 1,25(OH)D₃. On entering the nucleus 1,25(OH)₂D₃ binds to VDR, causes a heterodimer to form with retinoid X receptor and binding to the VDREs of gene promoters. The result of this is the alteration of gene expression (up to 9000 genes), upregulation of antimicrobial products such as cathelicidin, alterations in TH1-TH2 balance, activity of T regulatory cells and reduced cellular proliferation.

VDR: Vitamin D receptor; DBP: Vitamin D Binding Protein; RXR: Retinoid X Receptor; VDRE: Vitamin D Response Element

¶ DBP actions include sterol binding, G actin binding, chemotaxis and as macrophage activating factor (MAF) precursor.

† VDR functions include control of transcription of target genes in innate immunity (cathelicidin production) and epidermal / hair differentiation.

1.2.1 Definition of Vitamin D Deficiency

Serum 25(OH)D is the indicator used for assessing vitamin D levels because it best represents whole body vitamin D levels [40]. In contrast 1,25(OH)₂D₃ is tightly regulated by the parathyroid hormone system, and as such varies little. 25(OH)D is generally measured by one of two techniques: competitive immunoassay or chromatographic separation followed by non-immunological direct detection (tandem mass spectrometry). It is best measured by tandem mass spectrometry because it can be automated and is highly accurate when validated [41].

Deficiency can result in the development of diseases such as osteomalacia and rickets, and in the past the critical blood level to cause these diseases has defined deficiency. Levels of <10ng/ml (<25nmol/l) are known to result in rickets but are often lower in practice [42]. However a single cut off value for deficiency is difficult to produce, given the multifunctional effects of vitamin D; indeed the levels required to affect risk of extra-articular manifestations may be higher than those required to prevent bone loss [43]. A recent expert panel review recommended 25(OH)D level >30ng/ml (>75nmol/l) in those with or at risk of musculoskeletal, autoimmune, cardiovascular disease and cancer [44]. For this purpose during this thesis adequate circulating levels will be taken as ≥ 20ng/ml (≥ 50nmol/l) as recommended recently by the Institute of Medicine to meet the needs of 97.5% of the population [45] (see table 1.1). More recently in 2014 NICE produced a set of guidelines, highlighting the importance of identifying and supplementing vitamin D in at risk groups [46], reiterating the statement by the Scientific Committee on Nutrition in 2007 [47].

<u>25(OH)D levels</u>	<u>Vitamin D Status</u>
<10ng/ml (<25nmol/l)	Deficient
<20ng/ml (<50nmol/l)	Insufficient
≥20ng/ml (≥50nmol/l)	Sufficient

Table 1.1 Vitamin D status according to 25(OH)D levels

For the purpose of this study, sufficiency is determined as ≥50nmol/l as suggested by the Institute of Medicine.

1.2.2 Vitamin D Levels and Tuberculosis

Numerous studies have assessed vitamin D status in TB in different populations and ethnicities. In most studies the level of 25(OH)D₃ was measured to assess the vitamin D status of the patient. 1,25(OH)₂D₃ and 24,25(OH)D₃ were also measured in other studies [48]. The definitions of severe, moderate and mild vitamin D deficiency varied but there are a number which noted significantly lower vitamin D levels in patients with tuberculosis.

1.2.3 Vitamin D levels prior to and during anti-tuberculous therapy

A systematic review and meta-analysis of this topic in 2008 concluded that vitamin D levels were highly likely to be lower in tuberculosis compared to health, although few studies met their inclusion criteria [49]. A further small systematic review in 2014 resulted in similar conclusions [50]. Some of the key original studies contributing to these, together with subsequent work are summarised in table 1.2.

Study	Population	Sample size	25(OH)D levels ng/ml	Result
Davies <i>et al.</i> [51]	Predominant white, UK.	(P) and (C) 40	Median (P) 6.4 (C) 10.9	Difference between patients and control 25(OH)D levels highly significant : P<0.005
Davies <i>et al.</i> [52]	Kenyan	(P) and (C) 15	Median (P) 15.9 (C) 26.2	Significant difference in 25(OH)D levels between patients and controls P<0.05
Davies <i>et al.</i> [53]	Thai	(P) and (C) 51	Mean (P) 27.8 (C)38.2	Significant difference in 25(OH)D levels between patients and controls p<0.001
Chan <i>et al.</i> [54]	Chinese	(P) 23 (C) 22	Mean (P) 18.6 (C) 20.9	No significant difference in 25(OH)D levels between groups.
Sasidaran <i>et al.</i> [55]	Indian	(P) 35 (C) 16	Mean (P) 10.7 (C) 19.5	Statistically significant difference in mean 25(OH)D levels p<0.005
Wilkinson <i>et al.</i> [56]	Gujurati, London	(P)103 (C) 42	Median (P) 17 (C) 12	OR for 25(OH)D level < 10 nmol/l (25ng/ml) in patients to controls = 2.9 ([95% CI 1.3–6.5], p=0.008)
Grange <i>et al.</i> [57]	Indonesian	(P) 40 (C) 38	Median (P) 26.3 (C) 27.8	No significant difference between groups
Ho-Pham <i>et al.</i> [58]	Vietnamese	(P) 166 (C) 219	Mean Log[25(OH)D] (P);M=3.49,F=3.39 (C);M=3.6, F=3.40	Statistically significant difference in 25(OH)D levels only in the male group. P=0.001
Sita-Lumsden <i>et al.</i> [59]	UK, predominantly Black or Asian	(P) 178 (C) 130	(P) 8.1 (C) 12.3	25(OH)D levels were significantly lower in patients than control group. P<0.001

Study	Population	Sample size	25(OH)D levels ng/ml	Result
Talat <i>et al.</i> [60]	Pakistani	(P) 20 (C) 120	Median (P) 7.9 (C) 9.6	Significant when further 8 patients included in patient group who were on treatment for TB or had a past history of TB treatment.
Hong <i>et al.</i> [61]	Korean	(P) 94 (C) 282	Median (P) 9.86 (C) 16.03	Median baseline 25(OH)D in the TB group (9.86 ng/ml, IQR 7.19–14.15) was lower than in controls (16.03 ng/ml, IQR 12.38–20.30, P < 0.001)
Mastala <i>et al.</i> [62]	African (Malawi)	(P) 161 (C) 157	Mean (P) 23.9 (C) 33.7	Statistically significant difference in mean levels comparing medical patients with a 2008 cohort of TB patients.
Kim <i>et al.</i> [63]	Korean	(P) 165 (C) 197	Mean (P) 13.2 (C) 18.7	Statistically significant difference in baseline 25(OH)D levels between cases and controls.

Table 1.2 Summary of studies of vitamin D levels in tuberculosis patients prior to anti-tuberculous therapy.

The table summarises the findings of key studies comparing vitamin D levels in patient (smear or culture positive tuberculosis) and control groups. (P: patient; C: control; M: male; F: female). The first seven studies were included in 2008 meta-analysis, two of which show no statistical significance between patient and control groups. The remaining studies were carried out subsequently. Overall the findings suggest lower vitamin D levels in tuberculosis patients.

Whether the serum 25(OH)D₃ levels should be measured prior to or during treatment is a contentious issue. Yamshchikov *et al* retrospectively measured 25(OH)D₃ levels from stored serum before and during treatment with standard anti-tuberculous therapy in a non-controlled trial. It was noted that the mean baseline serum 25(OH)D₃ levels were insufficient (20.4ng/ml; range 6.9-53.1ng/ml) and that 86% of patients had serum levels <30ng/ml. However no significant change in these levels was noted during treatment [64], suggesting that assessment at either time point may be valid for comparisons to control groups. This finding was also noted in a study by Banda *et al* where no significant difference was found between duration of treatment and vitamin D levels [65] and by Kim *et al* who noted no significant change in 25(OH)D levels one year after TB treatment, despite an improvement in nutritional status [63] .

Despite this, other studies of 25(OH)D levels during TB treatment have been inconsistent in their results, unlike those performed prior to treatment. There are numerous factors which may explain these inconsistencies, such as socioeconomic status, sun exposure, diet, gender and ethnicity – illustrated by the following studies: Wejse's study of a West African population of TB patients in Guinea Bissau noted hypovitaminosis (25(OH)D₃<50nmol/l) in 46% of TB patients and 39% of controls. However there were no significant group differences when adjusted for socioeconomic and demographic factors [66]. The sampling mean lag time of seven days after commencing therapy may have affected serum 25(OH)D levels. Another factor to consider is that high levels of UVB exposure in both patients and controls may influence serum 25(OH)D levels; differences may be more difficult to detect in this population compared to those in sun-deprived regions [66] and the research is lacking regarding ultraviolet radiation exposure and anti-mycobacterial effect. Dietary intake of vitamin D in certain populations (e.g. Inuits who consume relatively large quantities of oily fish, which is high in vitamin D) may influence pre-treatment serum 25(OH)D levels and this may

be the reason that in a large case control study in Greenland there were no patients with severe deficiency and only one with levels <20ng/ml (<50nmol/L) despite the potential lack of sun exposure [67].

Gender may also influence results, as noted by Ho-Pham *et al*, where a statistically significant difference in serum 25(OH)D levels between the case and control group was only noted in the male group studied [58] and in the study by Talat *et al*, which noted significantly higher baseline 25(OH)D values in males compared to females [60, 68]. This may perhaps be attributed to higher sun exposure in males at work etc. and traditional dress which may be worn and reduce sun exposure in females.

Vitamin D levels were noted to be low in a case-note analysis by Ustianowski [69], where 56% of unselected patient with tuberculosis had undetectable levels of 25(OH)D₃ (<13nmol/L) and 76% had levels below normal (<22nmol/L). Somalian, East African Asian and Indian patients demonstrated significantly lower levels when compared to White Europeans, Chinese or SE Asians, suggesting a plausible role of ethnicity / skin colour on vitamin D levels.

1.2.4 Vitamin D Supplementation in Tuberculosis

Three major trials of vitamin D supplementation have been carried out in TB, as well as a number of smaller trials, observational work, and some studies looking at its role at a cellular level. The latter studies suggested that increasing vitamin D levels may be beneficial in TB treatment, and preceded the trials by some years. In vitro, vitamin D enhances the immunity to mycobacteria [70] and inhibit

the growth of mycobacteria [71]. Data such as this was the starting point to determine whether supplementation could influence levels in TB patients, although the 2011 (and 2015) Cochrane review on the use of nutritional supplements suggest that although blood levels of some vitamins may be low in patients starting treatment for active tuberculosis, there was a lack of evidence to promote supplementation above the recommended daily amount [72].

The first study was small, involving just eleven patients tested within seven days of starting anti-tuberculous treatment, in whom there was a significant increase in 25 hydroxyvitamin D levels post supplementation with a single dose of 2.5mg (100000 units) of Vitamin D₂ / ergocalciferol at one week, when compared with placebo [73]. This effect declined by 8 weeks post dose but levels still remained above baseline, consequently an 8 week supplementation regime was used in the later randomised controlled trial.

The largest trial to date studied the effect of administering four doses of 2.5 mg (100000 units) vitamin D₃ (Vigantol oil) versus placebo on sputum conversion in smear positive patients. The supplementation resulted in improvement in the 25-hydroxyvitamin D concentrations. However, supplementation did not significantly affect time to sputum conversion in the group, although a subgroup with rs731236 (*tt TaqI*) *VDR* polymorphism were noted to have a significantly shorter sputum conversion time [74]. Despite the fact that the dose of vitamin D should have resulted in levels >75nmol/L (30ng/ml), this was not attained in all patients receiving supplementation, raising questions about compliance or variation in dose-response in the patients. Salahuddin et al. in the SUCCINCT study did not identify any difference in sputum conversion time or TB score between vitamin D supplemented (2 doses of 15mg/ 600000 IU vitamin D₃) and placebo groups, but did however note a comparative improvement in radiological changes and BMI/ weight in the

supplemented group [68]. Another study by Wejse found no significant difference in TB score or mortality but this may have been related to the vitamin D dosing regimen / compliance as there were no significant differences in change in 25(OH)D levels between the control and intervention groups [75].

Further randomised controlled clinical trials include those by Morcos et al [76], Nursyam et al [77] and Coussens [78]. Table 1.3 summarises the results and problems associated with these studies.

Study	Population	Sample Size	Intervention	Results	Problems
Wejse <i>et al.</i> [75]	Guinea Bissau, adults	(I) 187 (C) 180	100,000 units Vitamin D ₃ at 0, 5 and 8 months.	No significant difference in TB score, mortality.	No significant difference in change of 25(OH)D levels between groups. Mean baseline serum patients 25(OH)D 77.5nmol/l (31ng/ml). Possible insufficient dosing of vitamin D.
Nursyam <i>et al.</i> [79]	Indonesia, adults	(I) 34 (C) 33	10,000 units (0.25mg) daily for 6 weeks	Significant difference in sputum conversion time at 6 weeks	Small group numbers. Follow up serum 25(OH)D not known ? sufficient supplementation.
Morcos <i>et al.</i> [76]	Egypt, children	(I) 12 (C) 12	1,000 units / day	Non-significant improvement in weight gain and clinical improvement in intervention group.	Small group numbers. ? sufficient supplementation.
Martineau <i>et al.</i> [74]	London, UK, adults	(I) 62 (C) 64	Four doses of 2.5mg Vitamin D ₃ .	No significant difference in sputum conversion time between groups.	Expected levels of 25(OH)D not obtained with supplementation. ? compliance of patients with treatment.
Coussens <i>et al.</i> [78]	London, UK, adults	(I) 44 (C) 51	Four doses of 2.5mg vitamin D ₃	Vitamin D accelerated sputum conversion (p=0.004)	
Salahuddin <i>et al.</i> [68]	South Asian	(I) 132 (C) 127	600000IU vitamin D ₃ at initiation and 1 month.	Mean weight gain 4.02 kg in vitamin D arm versus 2.61 kg in placebo arm (p < 0.05) Mean number of lung zones involved by CXR 1.35 in vitamin D arm versus 1.82 in placebo arm (p < 0.05)	Short follow up of 12 weeks, therefore unable to determine if response / benefit was sustained throughout full treatment phase.

Table 1.3 Clinical trials of supplementation of vitamin D in patients with tuberculosis.

I: intervention group; C: control group. This table summarises key clinical trials of vitamin D supplementation in tuberculosis. The intervention (vitamin D dosing regimen) and outcomes are listed for each trial, along with any potential problems / confounders.

Although the above data suggests a beneficial effect of vitamin D, clinicians must exercise caution as adverse effects could occur. In murine models cellular inflammation is lower in VDR knockout (KO) mice due to toll-receptor hypo-responsiveness [80], suggesting the importance of vitamin D / VDR in the innate immune response. In addition the chemotactic effects of increased local LL-37 production in response to vitamin D therapy could theoretically increase cell recruitment to the lung resulting in exaggerated inflammation [81].

1.3 Genetic Association

1.3.1 Basic concepts in genetic association studies

Identifying a genetic link with disease susceptibility can be done by linkage analysis (sharing of genome regions in affected relatives) or gene–disease association studies (looking at the coexistence of genetic markers with the disease).

Variation in the gene product may arise through a number of mechanisms.

Insertions or deletions (Indels) within the DNA is common and most occur in the non-coding region of the genome, having no effect on the individual phenotype. However it may result in variation of the protein product through a frameshift mutation with loss or gain of function.

Polymorphisms at the DNA level can occur. The most common form is the single nucleotide polymorphism (SNPs), when a difference in a single DNA base occurs at a frequency of >1% at a given location within the genome [82]. SNPs which lie within the coding region of the gene may be synonymous or non-synonymous. Synonymous SNPs do not result in any change in the amino acid sequence of the protein whereas non-synonymous SNPs do alter the sequence and thereby the protein product e.g. *CHRNA5* non-synonymous polymorphism and lung cancer risk. However there is increasing evidence that synonymous SNPs are associated with disease risk and treatment outcomes.

Microsatellites, which are di, tri or tetra repeats of variable length are also common and may also be useful as markers in genetic studies to identify allelic variants within a population. Variation in these regions can be evaluated with gene localisation studies, which, by screening groups of SNPs can identify specific SNPs in the presence of certain disease states.

Another concept is *Copy Number Variation (CNV)* which is the duplication or deletion of a DNA segment of 1 kilobase or larger, present at a variable copy number compared to a reference genome [84]. CNVs may or may not result in early onset, high penetrant genomic disorders such as Prader-Willi Syndrome. Instead they may be counted as ‘neutral’ in function but may be related to the onset or susceptibility to disease later in life. An example of this is CNV in the gene encoding CCL3L1, which is a HIV-1 suppressive chemokine and ligand for CCR5, a HIV-1 ligand. Those with lower levels of CCL3L1 had lower CCL3L1-CCR5 binding and therefore more open CCR5 receptors remained to potentially bind with HIV-1, thereby increasing the risk of infection [83].

Identifying a genetic link with disease susceptibility can be done by linkage studies or gene-disease association studies.

- 1) Linkage studies aim to identify a genetic marker inherited by all those with the disease. If a genetic marker lies in close proximity to the disease gene, then although recombination does not occur between the two regions, they have a tendency to be inherited together and are said to be linked due to it. Linkage analysis aims to determine if this link is present.
- 2) Gene-disease association studies aim to identify variations within a gene which may predispose to the disease in question. Such studies may be done for single genetic variations, a gene, or the whole genome (genome-wide association study; GWAS).

Linkage disequilibrium (LD) is the non- random association of alleles at two or more loci, resulting in certain combinations of alleles being more likely to occur on a chromosome than other combinations. By detecting this indirect association between the alleles, disease associations can be screened for using a reduced number of markers, with the knowledge that through LD, the whole spectrum of variants will be detected [84]. The pattern of SNPs located in close proximity and which may be inherited in blocks (due to high linkage disequilibrium), are known as haplotypes. These haplotypes can be identified by one of the SNPs, the tagSNP which then allows the resultant block to be identified on the Haplotype Map (HapMap) described below. In practice this means that relatively few polymorphisms need to be genotyped in order to identify genetic variance in that region.

The rapid pace at which genotyping technology has developed means that in many common diseases huge numbers of genetic association studies have been published. However in many cases little is

known about the role of the polymorphisms, such that functional studies of the gene and/or SNP are required. Functional studies aim to determine (a) the role of a gene and its protein product in disease and (b) how a SNP may alter this. An example of such a study by Ben-Selma et al which notes the IL23R functional polymorphism association with the severity of active pulmonary tuberculosis [85].

1.3.2 Genetic resources

The Human Genome Project was an international effort to sequence the whole human genome and this has meant that publicly available resources for genetic studies have grown rapidly in the last 10 years. Despite being able to identify every human gene, the relevance of this work will not be immediately seen and further projects / databases enable the link between the human genome and the link between health and disease states to be made.

An example of an international collaboration in this way is HapMap. The aim of this project is to produce a catalogue of common genetic variants in the form of haplotype identification using tagSNPs. This enables researchers to choose haplotype tagging SNPs for small scale work and, at the other end of the spectrum, interpret GWAS results, where large numbers of genes may be associated with disease.

In addition there are numerous databases such as those in the National Library of Medicine, including dbSNP (documenting short variations in nucleotide sequences), OMIM (concentrating on genotypic variation and phenotypic expression), Gene (database for known and predicted genes) etc.

1.3.3 Genetic association studies in TB

The number of genetic association studies which have researched host susceptibility to tuberculosis are limited and often underpowered. Twin studies have found that the concordance for tuberculosis was significantly higher among monozygotic than dizygotic twin pairs[86]. However despite later analysis of specific genetic associations including *HLA*, *NRAMP1*, *IFNG*, *NOS2A*, *SP110*, *CCL2*, *MBL*, *CD209*, *VDR* and *TLR* none have given the degree of association seen in the original twin studies. More recently review of these earlier studies suggest that intensity and duration of exposure to the bacilli has a greater influence than zygosity of the twin and the degree of exposure may in fact be directly linked to the zygosity of the twin, through environmental circumstance [87].

Gene	Cytogenetic Location	Population	Polymorphism	References
SLC11A1 (NRAMP1)	2q35	Gambia USA (paediatric) Cambodia Eastern China (severity) Korean (severity) China Gambia S. Africa Tanzania W Africa (Guinea Conkary)	INT4/ 3'UTR 274/C 3'UTR / D543N INT4 3'UTR 3'UTR 5'(GT)n promotor 5'(GT)n promotor 5'(CA) <i>n</i> 3'UTR	[88-93] [94-97]
TLR2	4q31.3	Turkey Tunisia USA (Caucasian) / Guineau Bissau (African)	R753Q Arg677Trp (heterozygote) Insertion / deletion at -196 to - 174	[98] [99] [100]
TLR9	3p21.2	USA /Guinea Bissau	rs352143, rs574386 (exon)	[100]

Gene	Cytogenetic Location	Population	Polymorphism	References
MBL	10q21.1	India (protection in F and PTB only) Italy USA (African American) India	Mutant B allele HYA/HYA (protection), LYB/LYD(susceptibility) haplotypes B allele O allele	[101] [102] [103] [104]
CISH	3p21.3	Chinese Han (promotor) Chinese Han (promotor) Mixed populations(Malawi, Gambia).	rs414171TT, rs809451GC (susceptibility) rs414171 AA (susceptibility) rs414171 (-292)	[105] [106] [107]

Gene	Cytogenetic Location	Population	Polymorphism	References
CCL2 (MCP)	17q12	Mexicans and Koreans	(rs1024611) -2518G (susceptibility)	[112]
		Ghana	-362G/C (promoter)	[113]
TIRAP	11q24.2	Africa	S180L (exon)	[114]
		China (Meta-analysis)	C539T (susceptibility)	[115]
IFNGR1	6q23.3	Africa	-1616GG and +3234TT	[116]

Table 1.4 Association studies between innate immune genes and tuberculosis.

This table summarises some of the studies of genetic association in tuberculosis susceptibility and protection. The polymorphisms studied are listed with the studies in the order that they appear in the references listed. Genes studied include SLC11A1 (solute carrier 11A1), TLR (toll like receptor), MBL (mannose binding lectin), CISH (ytokine inducible SH2 containing protein), DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin), CCL2 (chemokine (C-C motif) ligand 2), TIRAP (adaptor molecule associated with toll like receptors) and IFN γ R1 (interferon gamma receptor 1).

Two genome wide association studies (GWAS) [117, 118] differ in their conclusions, suggesting that there is variation in the susceptibility according to continent of origin [118]. Thye et al combined two populations from Ghana and the Gambia, finding in over eleven thousand individuals rs4331426 (chromosome 18q11.2) was associated with disease. Chimusa et al failed to identify this association in a GWAS of the admixed South African Coloured (SAC) population, but found SNP rs2057178 and rs11031728 had an association result with genome-wide significance (WT1, Wilms tumour 1 gene on chromosome 11). This association was also suggested after imputation of data from the 1000 Genomes Project into populations from West Africa, Indonesia and Russia in another study by Thye et al [119]. Interestingly WT1 has a role in upregulation of VDR.

The genetic association studies looking specifically at the association of the vitamin D axis on host susceptibility to tuberculosis are discussed in more detail in the thesis and the information on these studies is found within the respective sections below, outlining the currently available data.

1.4 Vitamin D Receptor (VDR) and Tuberculosis

The presence of a genetic relationship to the protectiveness of $1,25(\text{OH})_2\text{D}_3$ was identified in early studies reporting inhibition of tubercle bacilli in macrophages with supplementation [120]. Since then, allelic variants in *VDR* have been linked to regulatory pathways in bone and calcium homeostasis [121]. *VDR* polymorphisms have been extensively described in association with bone mineral density but there have been numerous studies more recently looking at its relationship with resistance or susceptibility to *Mycobacterium tuberculosis* [56, 122-126].

VDR (a nuclear hormone receptor) is activated by binding of the ligand $1,25(\text{OH})_2\text{D}_3$. The VDR then binds with another intracellular receptor (retinoid X receptor). A heterodimer is formed which enables DNA binding and the effect is usually to activate transcription for target genes, the net result being the regulation of expression of proteins involved in calcium homeostasis [127] and antimicrobial products (e.g. cathelicidin).

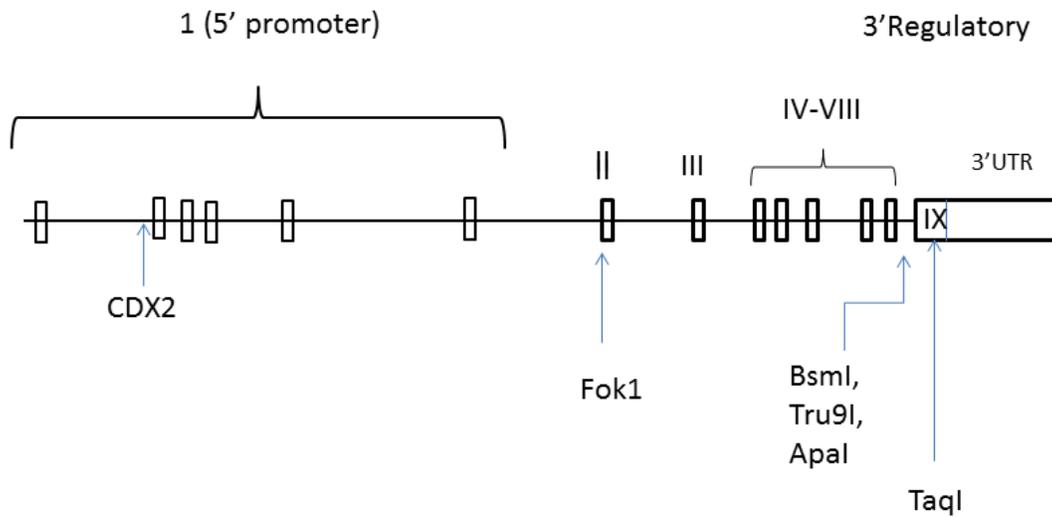


Figure 1.4 Vitamin D receptor (VDR) gene structure

VDR belongs to the transacting transcriptional regulatory factors. The non-coding 5' end of the gene contains non coding exons (1a-e), whilst exons 2-9 encode the translated product. Exons 2 and 3 are involved in DNA binding and exons 7, 8 and 9 are involved in binding to vitamin D. Restriction fragment length polymorphisms (RFLP) for BsmI, ApaI (exon 8) and TaqI (exon 9) lie at the 3'UTR (untranslated region). FokI polymorphism at exon 2 and CDX2 is found in the promoter area 1e. Tru9I is a polymorphism in the VDR intron 8 region and A-1012G polymorphism lies close to exon 1a transcription start site.

VDR is amongst the best studied genes in tuberculosis. It lies on chromosome 12 (12q13.11) and contains 11 exons spanning 75kBa. The most commonly studied *VDR* polymorphisms to date are *FokI*, *ApaI*, *TaqI* and *BsmI* (Table 1.5). These lie at the 3' un-translated region of the gene (*FokI* in exon 2, *BsmI* and *ApaI* in intron 8 and *TaqI* in exon 9). They have been shown to be associated with stability of the *VDR* mRNA [128] such that carriers of a variant may potentially have decreased ability to respond to vitamin D. In addition, Cdx-2 and A1012G exist at the 5' regulatory region and are known to affect the transcriptional activity of *VDR*.

The function of *VDR* in the regulation of antimicrobial products such as cathelicidin closely links it to the immune response to tuberculosis. A recent systematic review noted an association for *FokI* and *BsmI* with tuberculosis in Asians [125], with a more recent study also noting an association of *BsmI* variants with TB in a Turkish population [123]. However it is difficult to be sure there is a definite association of *VDR* polymorphisms with risk of TB, partly due to the small numbers of participants in the studies and partly because of confounding factors that may have not been addressed such as HIV status [96] and ethnic diversity [129]. Two subsequent studies found no significant difference in association with *VDR* polymorphisms between TB patients and healthy controls [130, 131].

Regarding the effect of *VDR* polymorphisms on TB outcome, a study by Martineau noted that when supplemented with Vitamin D, the sputum conversion time was faster in those with the *tt* genotype of the *TaqI* polymorphism [5].

Polymorphism	Apa I	Taq I	Fok I	Cdx2	BsmI	A-1012G
rs number (reference SNP)	rs7975232	rs731236	rs10735810	rs17883968	rs1544410	rs4516035
Alleles	A/C	T/C	C/T	G/A	A/G	A/G

Table 1.5 Polymorphisms of vitamin D receptor (VDR) gene

1.5 Vitamin D Binding Protein (DBP) and Tuberculosis

Vitamin D Binding Protein (DBP) is also known as group specific component of serum (Gc-globulin). The usual nomenclature for the gene is therefore *GC*.

The structure of DBP is similar to albumin. The structure of albumin and α fetoprotein is made up of three domains, each consisting of three sub-domains whereas DBP, although similar, lacks two of the sub-domains at the C-terminal. The conservation of the pattern of disulphide bonds which are responsible for the structure of each looped domain supports the evidence for their relationship [132]. The finding that α -fetoprotein bound oestrogen with high affinity [133] prompted consideration of DBP as a steroid binder.

DBP has two primary binding functions: sterol binding and G-actin binding. The ability of DBP to bind vitamin D was noted in studies of radiolabelled vitamin D₃ [29]. It is this function that allows binding and transport of both 25(OH)D₃ and 1,25(OH)₂D₃. DBP internalises vitamin D using receptors megalin and cubulin. However these only occur in some cells (e.g. renal proximal tubules) and studies have indicated that in cells with no evidence of these receptors (e.g. monocytes), the uptake of vitamin D is lower. Paradoxically the genotypes with highest affinity (GC1F-1F allele combination) appear to have a reduced response in production of cathelicidin (antimicrobial) when compared to the lower affinity genotype (GC 2-1S). This may be an evolutionary alteration conferring increased cathelicidin production in response to vitamin D stimulus in those individuals with lowest affinity for vitamin D ligands at low serum levels [134].

The second binding function of DBP is with G-actin. Following tissue injury, elevated levels of actin appear in the plasma and DBP clears this by forming DBP-actin complexes [135]. This prevents the formation of F-actin polymers. The function of these actin arrays in intracellular motility *M. marinarum* has been noted, although this is not known in *M. tb* [136]. The function of DBP in G-actin binding, related to prevention of direct pulmonary endothelial injury in a study of ARDS patients [137] could conceivably be applied to the mechanisms of lung injury in tuberculosis.

DBP also plays a role in chemotaxis by enhancing C5a activity [138-141]. It appears to have no effect on its own but in the presence of even non-chemotactic doses of C5 derived peptides, its effect on chemotaxis is noted [139]. Zhang et al identified a 20 amino acid sequence in the N terminal domain I of DBP which possessed C5a chemotactic cofactor activity, known to act as a chemotactic factor for all leucocytes [142]. DBP may therefore be relevant in recruitment of cells to sites infected with *M. Tb*.

DBP is also a precursor for macrophage activating factor (MAF) [96]. DBP is converted by glycosidases of B and T cells to MAF [143]. The ingestion activity function of macrophages (important in determining response to tuberculous infection), is enhanced by MAF.

GC is located on chromosome 4 (4q11-q13) and study of *GC* show that it is highly polymorphic with three main electrophoretic variants [29] as illustrated in table 1.6. Single nucleotide polymorphisms (SNPs) at exon 11 (rs7041 and rs4588) result in *GCI* and *GC2* variants [144] with *GCI* subdivided into *GCIS* and *GCIF*, reflecting the presence of aspartic acid or glutamic acid respectively at position 416 [145]. Alongside these there are more than one hundred and twenty other less common variants [136].

Variant	Alleles at rs7041 and rs4588	Protein composition at positions 416 and 420
<i>GC1F</i>	T/C	Asp/Thr
<i>GC1S</i>	G/C	Glu/Thr
<i>GC2</i>	T/A	Asp/Lys

Table 1.6 Haplotypes of rs7041 and rs4588 result in structurally different proteins.

The table shows the alleles present at rs7041 and rs4588 respectively (forward strand) and the resulting protein composition at positions 416 and 420 respectively.

In a study of Caucasian women by Lauridson *et al* the median plasma levels of 25(OH)D₃ differed significantly between GC phenotypes (combinations of *GC1F*, *GC1S* and *GC2*), with the highest levels being noted in GC1-1 group (also known to have the highest affinity for vitamin D ligands), intermediate levels in GC1-2 group and lowest levels in GC2-2 group [146]. However despite this, the individuals in the GC2-2 groups showed no signs of vitamin D deficiency clinically, indicating a possible reduction in required circulating vitamin D requirements in order to remain 'sufficient'.

Martineau *et al* noted that the association between *GC* genotype and susceptibility to active tuberculosis in Gujarati Asians was dependent on the vitamin D status [147]. It was noted in this multi-centre study that there was a significant association between *GC* genotype (*Gc2-2*) and tuberculosis in those with vitamin D deficiency. This was lost however in those with serum 25(OH)D₃ levels >20nmol/L [148], suggesting an interaction between genotype and vitamin level in disease risk.

Consistent with these small studies in TB a much larger GWAS in the general population has shown convincingly that SNPs in *GC* are associated with vitamin D concentrations ($p=2.0 \times 10^{-30}$) [149].

1.6 Other relevant polymorphisms in the vitamin D axis.

In addition to *GC* and *VDR* there are several other key genes which may be relevant in the cellular response to vitamin D (Figure 1.5).

DHCR7/NADSYN1 (dehydrocholesterolreductase, NAD synthetase 1)

This gene, located at 11q13.4 encodes for 7-dehydrocholesterol reductase which catalyses the conversion of 7-dehydrocholesterol to cholesterol by removing the C (7-8) bond in the B ring of sterols. Mutations in this gene could result in reduced conversion of 7-dehydrocholesterol, thereby increasing the availability of it for conversion in the skin to pre-vitamin D₃.

Although some smaller studies have found have reached differing conclusions, recently two genome wide association studies (GWAS) have found that there is an association between *DHCR7/NADSYN1* and 25(OH)D levels [150, 151].

CYP27B1

CYP27B1 is located on chromosome 12 (12q14.1) and the protein it encodes known as 1- α -hydroxylase is a member of the cytochrome p450 family. It is responsible for the hydroxylation of 25(OH)D₃ at the 1-alpha position to the active metabolite 1,25(OH)₂D₃ [152] which in turn binds to

the vitamin D receptor and regulates calcium metabolism. Vitamin D-dependent rickets type 1A (VDDR1A) is due to a mutation in *CYP27B1*.

CYP24A1

This gene encodes the enzyme responsible for degradation of 25(OH)D. It has not been found to be directly linked to vitamin D levels in GWAS but a 5-point frequent haplotype (rs2296241:rs17219315:rs2762942:rs2248137:rs2248359) was found to be associated with both 25(OH)D₃ and 1,25(OH)₂D₃ levels [153].

CYP2R1

This is just one of the enzymes responsible for the hydroxylation step to 25(OH)D at the microsomal level. Variants in the *CYP2R1* locus have been associated with vitamin D levels in GWAS [150, 151] as well as smaller studies [154].

CYP27A1

CYP27A1, found in the mitochondria, and has vitamin D 25-hydroxylase activity. The relevance of polymorphisms of this gene in relation to vitamin D has not been determined.

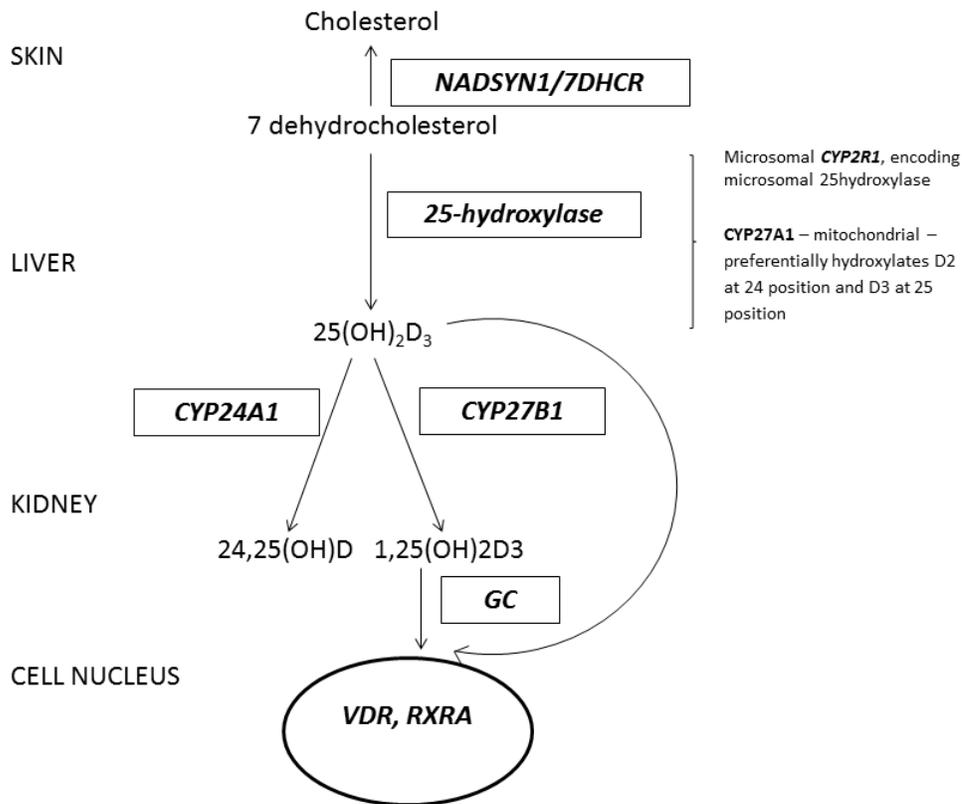


Figure 1.5 Genetic polymorphisms of key enzymes in the vitamin D axis.

The key enzymes involved in the conversion and activation of precursors to vitamin D. 7-dehydrocholesterol is hydroxylated in the liver to $25(\text{OH})\text{D}_3$ and further hydroxylated in the kidney to produce $1,25(\text{OH})_2\text{D}_3$, which is then transported into the cell by mechanisms including GC (group specific component) also known as vitamin D binding protein. Ligand bound VDR (vitamin D receptor) forms a heterodimer with RXRA (retinoid X receptor alpha) which signals to upregulate the transcription of target genes involved in antimicrobial product formation.

1.7 Immunomodulatory effects of vitamin D on circulatory blood cells

Vitamin D is known to have numerous functions in the response to infection, involving the innate and acquired immune systems and the maintenance of epithelial barrier function [155]. All of these functions can be applied specifically in the antimicrobial response to infection with M Tb. The formation of a granuloma in response to mycobacterial infection, results in a structure rich in polymorphonuclear leucocytes, mononuclear leucocytes and lymphocytes. The most important components of anti-mycobacterial activity are macrophages (innate immunity) and lymphocyte subsets (adaptive immunity), as illustrated in figure 1.6, with vitamin D exerting both inhibitory and stimulatory effects on these systems as shown in figure 1.7.

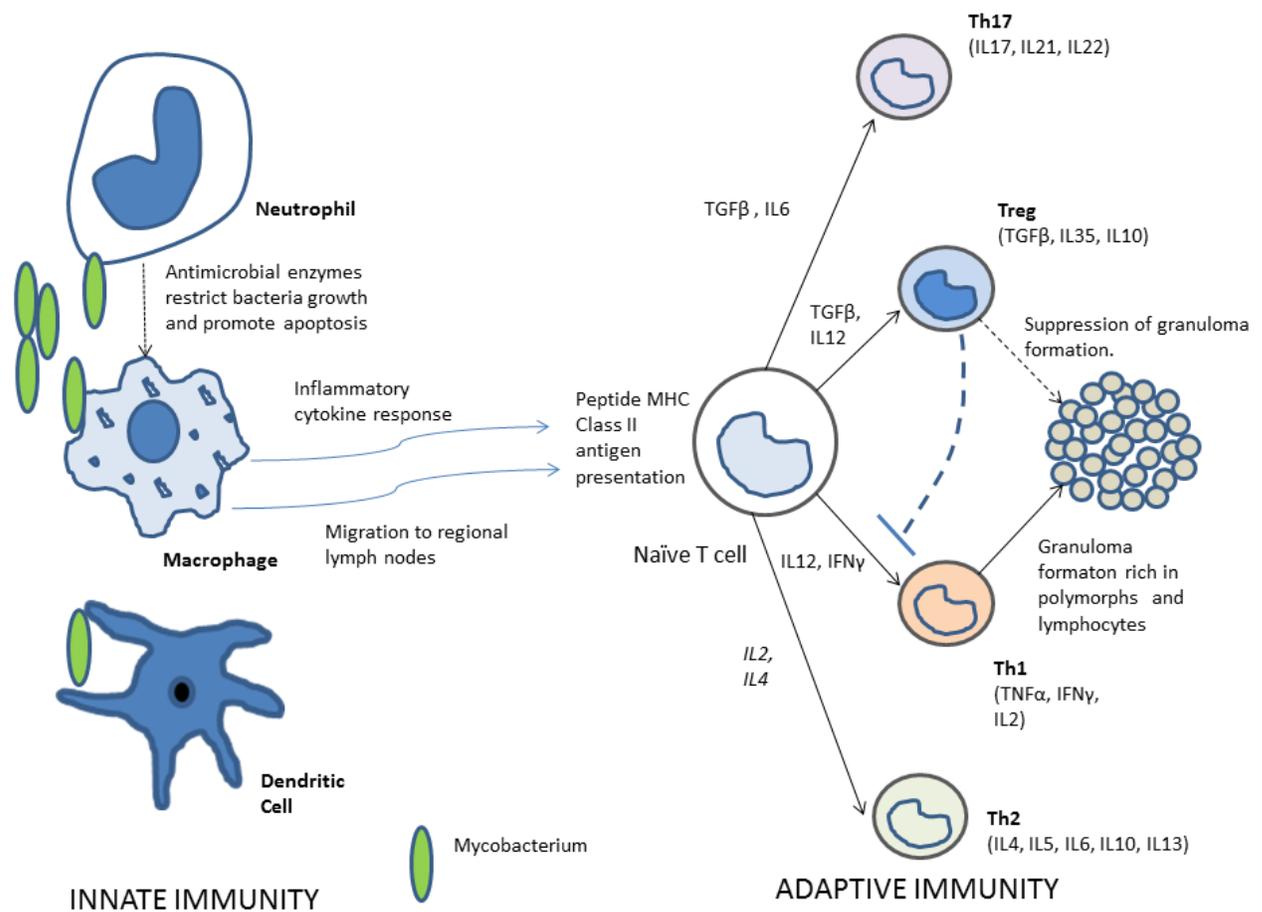


Figure 1.6 Innate and Adaptive Immune Response to Mycobacterium Tuberculosis Infection.

Mycobacterium tuberculosis uptake occurs by binding to a variety of receptors on macrophages and dendritic cells. Signalling pathways (after binding to toll like receptors) result in cell activation, cytokine and chemokine production. Binding of mycobacterial structures with toll like receptors results in activation and nuclear translocation of transcription factors such as NF-κB, leading to gene transcription and activation of the innate defence mainly by cytokine production such as TNFα, IL1β, IL12 and nitric oxide.

Neutrophils play a key role in innate defence. The production of specific cytokine such as TNFα stimulates dendritic cells and macrophage activation and differentiation.

The adaptive immune response (cell mediated response) is activated by antigen presentation of mycobacterial peptides as dendritic cells move to the draining lymph nodes. This activates naïve T cells to differentiate into various phenotypes according the cytokine profile. The naïve T cell differentiates into effector Th1, Th2, Th17 and T regulatory cells which can then migrate to the lung. A protective Th1 response results in the activation of macrophages and the production of antimicrobial products driven by IFNγ. Granuloma formation occurs, encasing the mycobacterium and is rich in polymorphs and lymphocytes. Dysregulation in this cytokine response results in breakdown of the granuloma and dissemination of infection.

The immune response is regulated by other effector responses to avoid damage from a vigorous host immune response. This is driven by IL10 production from regulatory T cells and may lead to persistence of infection.

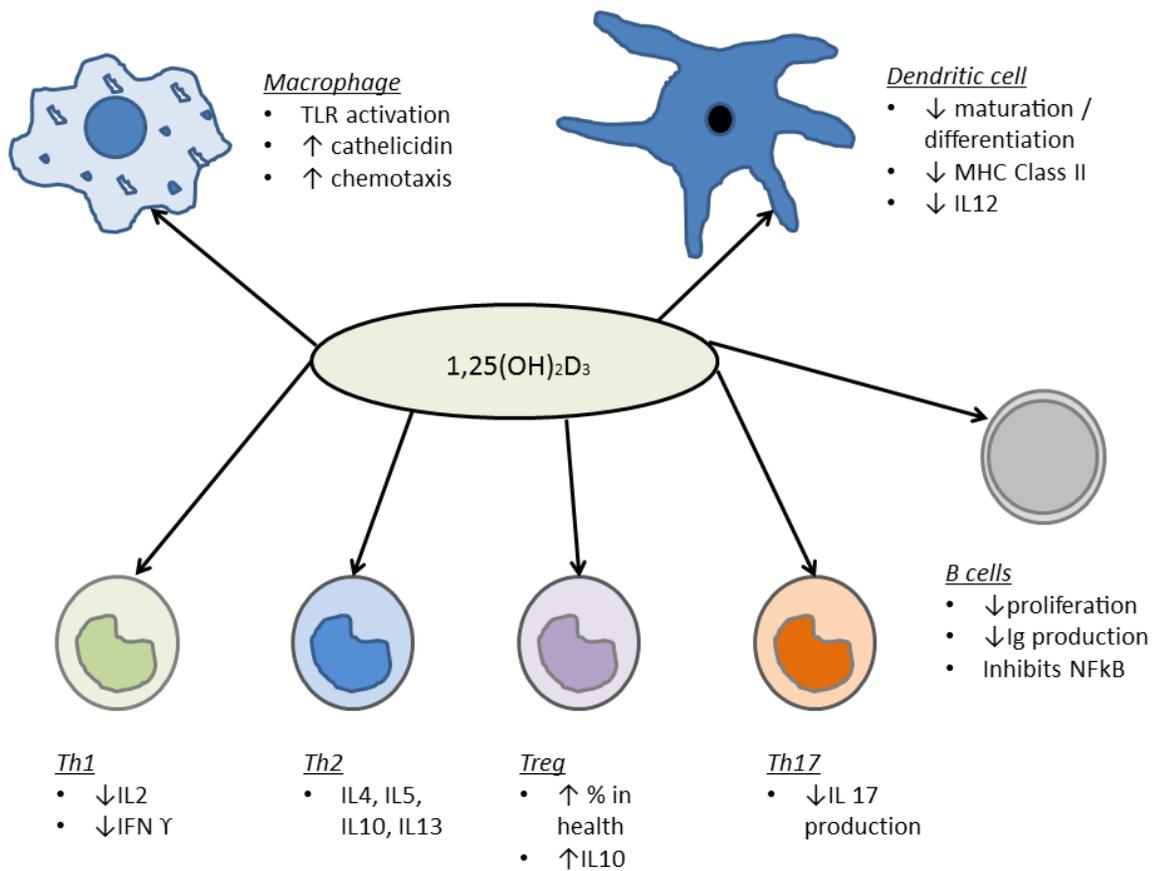


Figure 1.7 Effect of vitamin D on lymphocyte and immune regulatory cells.

An illustration of the multicomponent effect of vitamin D within the immune system, including its effect on B and T lymphocytes, macrophages and antigen presentation.

TLR, toll like receptor; MHC, major histocompatibility complex; IL, interleukin; NFkB, nuclear factor kappa beta; IFN γ , interferon gamma.

Innate Immune Response

Toll Like Receptors and Cathelicidin Production

Infection with microbial pathogens such as M Tb results in activation of toll like receptors (TLRs) which are triggered by PAMPs, which in turn results in cytokine production. Data suggests that the TLR activation results in the up-regulation of *VDR* and *CYP27B1* (1 α hydroxylase, which converts 25(OH)D₃ to the active 1,25(OH)₂D₃) expression [156]. Under the correct conditions, this then results in cathelicidin production (h-CAP, cleaved to produce LL37 in humans) which has direct anti-mycobacterial effect [157], since the human cathelicidin gene promoter contains three vitamin D response elements [158]. In addition LL37 has chemotactic activity for polymorphonuclear leucocytes and CD4+ T lymphocytes [159]. This appears to be a clinically relevant response since higher levels of LL37 correlate with increased disease burden from M.tb infection [160].

Another antibacterial protein DEF β 4 requires occupancy of not only VDRE but also nuclear factor k- β (NF-k β) response elements in the DEF β 4 gene promoter [161].

Autophagy

Recent studies have reported an association between vitamin D, cathelicidin and autophagy.

Autophagy is an essential anti-mycobacterial strategy [162] augmenting the bactericidal degradation of the mycobacterium by fusion of the autophagosome with the lysosome, thus disturbing the

evasion strategies of *M. tuberculosis*. Vitamin D ($1,25(\text{OH})_2\text{D}_3$) enhances the recruitment of cathelicidin to autophagosomes, thereby inducing autophagy [163]. It has also been shown to inhibit *M.tb* and HIV replication in co-infected macrophages via the cathelicidin dependent autophagy [164]. The production of antimicrobial peptides such as cathelicidin then creates an association between the adaptive and innate immune system, such that direct anti-mycobacterial actions act synergistically with the ability to stimulate chemotaxis of cells involved in the adaptive immune response [165].

It has also been shown that $1,25(\text{OH})_2\text{D}_3$ results in the release of superoxide from *M.tb* infected cells [166] and it has been suggested that $1,25(\text{OH})_2\text{D}_3$ suppresses the growth of *M. tuberculosis* in macrophages through the production of nitric oxide [167] in addition to promoting the anti-mycobacterial step of phagolysosome fusion [168].

Adaptive Immune Response

Dendritic Cells

Dendritic cells are vital in antigen presentation to T cells and the capability of dendritic cells to activate T lymphocytes affects the host's pathogenic response. Studies have shown that the maturation and differentiation of dendritic cells can be inhibited by the action of $1,25(\text{OH})_2\text{D}_3$ in vitro [169], resulting in down regulation of MHC Class II molecules and thereby T cell activity. The presence of maturation markers in dendritic cells is reduced after exposure to both $25(\text{OH})\text{D}_3$

and $1,25(\text{OH})_2\text{D}_3$ and the cytokine profile differs in those cells exposed to $25(\text{OH})\text{D}_3$ compared to those not exposed, specifically with a reduction in pro-inflammatory cytokines such as IL12 (vital in Th1 responses) in immature dendritic cells [170].

It has also been shown that $1,25(\text{OH})_2\text{D}_3$ inhibits the Th1 inducing capacity of myeloid dendritic cells (i.e. capability of inducing naïve CD4^+ T cells to differentiate) [171], with exposure to $1,25(\text{OH})_2\text{D}_3$ resulting in regulatory dendritic cells that are poor stimulators of T cells, inducing T cell hypo-responsiveness [172].

T cell activity

The cytokine profile produced during mycobacterial induced toll like receptor (TLR) activation determines whether a Th1 (predominantly cell-mediated response) or a Th2 (predominantly humoral) response is dominant.

Table 1.7 below illustrates the cytokine production specific to these responses.

Cytokine	TH1	TH2
IL-2	+	-
IFNγ	++	-
TNF-α	++	-
GM-CSF	++	+
IL3	++	++
IL4	-	++
IL5	-	++
IL10	-	++
IL13	-	++

Table 1.7 Cytokine production in TH1 and TH2 immune response.

Th1 (T helper1) and Th2 (T helper 2) responses produce particular cytokine combinations, resulting in enhanced ability to respond to intracellular and extracellular pathogens respectively.

The production of IL12 by macrophages induces the production of IFN γ [173]. Consistent with this, the disruption of the IL12 gene in mice results in the inability to produce IFN γ and subsequent overwhelming bacterial infection in the mice [174].

The above evidence would point towards suppression of the TH₁ response in TB, which may appear unusual, given that IFN γ is a key component in activity against intracellular pathogens, including mycobacteria. However it may be that the unwanted TH₁ effects are reduced and whilst enhancing chemotactic and phagocytic activity, 1,25(OH)₂D₃ appears to impair the production of pathogenic T cells [175]. Whilst vitamin D suppresses the TH₁ response, murine studies on graft survival have indicated that this does not overtly compromise the host's ability to fight infection [176].

T cell subtypes – regulatory T cells (Treg) and Th17 activity.

Inflammatory cytokines are important in determining induction of T cell subtypes. TGF β is required for the development of inducible T regs, whilst both TGF β and IL6 are required for the development of a newer T cell subset, Th17 [177]. Both types may be relevant to M.tb infection.

T regs are involved in immune tolerance and are characterised by the high surface expression of CD4, CD25 (IL2 receptor) and low surface expression of CD127 (IL17 receptor). They also express a transcription factor FoxP3 (forkhead box P3) and intracellularly express CTLA4+. Activated T cells are known to express VDR [178] and it has been shown that whilst 1,25(OH)₂D₃ inhibits inflammatory cytokine production such as IFN γ , IL17 and IL2, the regulatory cytokine IL10

increases in the presence of vitamin D [179]. The percentage of Tregs also increases with vitamin D supplementation in healthy individuals [180]. The relevance of these findings in relation to tuberculosis is that whilst T reg function regulates the pathological consequences of infection, their presence may also down regulate the immune response resulting in persistence of infection in the latent state.

Th17 cells are characterised by the expression of IL17A, IL17F, IL17AF, IL21, IL22 cytokines and surface expression of chemokine receptors CCR5 and CCR6 [181]. An important function of T17 cells is to mediate the recruitment of neutrophils and monocytes to sites of inflammation, through the production of IL6, IL8, G-CSF and SCF (stem cell factor). This is particularly important in extracellular infections.

1,25(OH)₂D₃ has been shown to directly reduce IL17 production and indirectly by reducing IL17 polarizing cytokines produced by dendritic cells [182], suppressing inflammatory outcomes whilst effecting a regulatory role through induction of T regs.

CD4+ Subset	Cytokines	Main Transcription Factors	Function
Th1	IL12, IFN γ	T bet	Clearance of intracellular pathogens; organ specific autoimmunity
Th2	IL4, IL2	GATA3	Clearance of extracellular pathogens; allergic inflammation.
Th17	IL6, IL 21, IL 23, TGF- β	ROR γ t	Extracellular immune response, autoimmunity.
Treg	TGF β , IL2	FoxP3	Immune tolerance, regulation of immune responses, limiting tissue damage.

Table 1.8 CD4 T helper cells, their cytokines, main transcription factors and functions.

Naïve T cells interact with an antigen – MHC complex and depending on the cytokines present in the environment, will result in differentiation into differing CD4+ lineages. The combination of cytokine signals and activation of specific transcription factors, which initiate and regulate the transcription of genes, result in expression of the characteristic phenotype.

B cell activity

B cells stimulated in the presence of 1,25(OH)₂D₃ exhibit up-regulation of VDR , whilst exposure of unstimulated B cells to 1,25(OH)₂D₃ causes inhibition of B cell proliferation, plasma cell proliferation and immunoglobulin production [183].

A recent study indicates that activating VDR using calcitriol results in impaired NF-κB (transcription factor) activation in human naïve B cells, specifically reducing p105/p50 expression [184], involved in IgE class switching. B cells are also able to upregulate IL-10, which promotes B cell differentiation, by converting pre-vitamin D₃ to calcitriol [185].

The relevance of these effects in patients with tuberculosis remains uncertain and further studies are needed.

Monocytes

Monocytes are known to be sensitive to lipopolysaccharide (LPS) and the response to this stimulation results in the production of numerous cytokines. The effects of vitamin D on monocytes has been studied and it is known to have a modulatory effect on cytokine production including IL-α, IL-6 and TNFα [186], a mechanism thought to be actioned via MAPK-1 (a mitogen activated protein kinase phosphatase), resulting in suppression of p38 (a mitogen activated protein kinase) phosphorylation and cytokine production [187]. The relevance of this effect on M.tb infected cells needs further elucidation.

1.8 Aims of the MD

The aims of the MD are based on the following hypotheses:

1. Genetic polymorphisms in the vitamin D axis determine the response to vitamin D supplementation in patients with tuberculosis.

There is growing evidence that vitamin D deficiency, genetic polymorphisms which associate with this and genetic variations that determine response to vitamin D are linked to tuberculosis. It also seems likely that supplementation with vitamin D could help in TB treatment. However, a systematic study of genes determining vitamin D levels and response to supplementation in patients with tuberculosis has not been carried out. Previous studies have selected only a few SNPs, and their role in response to supplementation has not been replicated. This MD will use an open label study of vitamin D supplementation in active TB patients to explore the role of genetic polymorphism in determining the change in vitamin D level post supplementation.

2. Baseline vitamin D and other elements of the vitamin D axis may affect clinical outcome / antimicrobial response.

The relationship of TB outcomes to vitamin D level and the interaction above will be sought in clinical trial patients. Clinical improvement will be defined by change in TB score, sputum bacteriology and radiology. Relevant cytokines indicating antimicrobial response and response to vitamin D will also be measured, as will level of DBP, since this has potential to influence interaction between DBP and vitamin D.

3. Vitamin D exerts an immune modulatory effect in vitro and ex vivo, in both health and mycobacterial disease.

In vitro work will determine the effect of vitamin D on

- a) stimulated monocytes in determining inflammatory cytokine response (IL6) and antimicrobial (LL37) production in healthy subjects, active and latent TB patients. The response pre and post in vivo supplementation as well as the mechanism of potential benefit will be explored.
- b) the effect on T reg and Th17 balance in relevant subsets of healthy subjects, active and latent TB patients exploring the mechanism by which vitamin D may be beneficial, and determining if T cell response to vitamin D is impaired in TB.

4. Genetic polymorphisms of the vitamin D axis determine the in vitro / ex vivo response to vitamin D supplementation in patients with mycobacterial infection.

Understanding the functional role of polymorphism within the vitamin D axis may aid our understanding of poor response to vitamin D clinically in previous supplementation trials. Data from the in vitro work will be compared against genotype, selecting those of most biological relevance.

CHAPTER 2

METHODS

2.1 Study Design, Outcome and Sample Size

2.1.1 Design

The study was an open label exploratory observational study, in which subjects with a clinical diagnosis of active tuberculosis were supplemented with vitamin D. All subjects received supplements, regardless of their baseline vitamin D levels and all other treatment was dictated by the clinician. The main reason not to run the study as a controlled study was based on the fact that this would have resulted in patients with severe vitamin D deficiency potentially having no supplementation for up to six months during the period of the trial. This could not be justified and therefore an observational study was planned.

2.1.2 Ethics and Medicines Health Regulatory Authority (MHRA) Approval

Since this was a trial of a food supplement it would not usually require MHRA approval, however since in this case some outcome measures were outside the usual licence indications for vitamin D we were asked by the MHRA to classify this as a phase III clinical trial of an investigational medicinal product (CTIMP).

The trial was an observational study using Vitamin D (Dekristol 100,000 units) in patients with active tuberculosis. As part of my work towards this thesis I completed all necessary steps for ethical and MHRA approval and gained permission from the Coventry and Warwickshire Research Ethics Committee (REC). The initial trial design is summarised in the flowchart below.

Amendments to the design were made at 4 time points in order to address problems with recruitment rates, and extend the in-vitro elements of the study to latent TB patients. The final trial protocol is described in section 2.2 and copies of relevant REC and MHRA documents, including consent forms, can be found in the appendices.

2.1.3 Primary outcomes and Sample Size Calculations

The primary outcome was to define the interaction between vitamin D binding protein genotype (*GC* genotype) and pre-supplementation vitamin D level in determining post-supplementation vitamin D level at visit 1. This is defined as a departure from additivity of effects when considering

genotype and starting vitamin D level as predictors of post supplementation level, and would be suggestive of a biological interaction, as predicted from DBP protein structure and function.

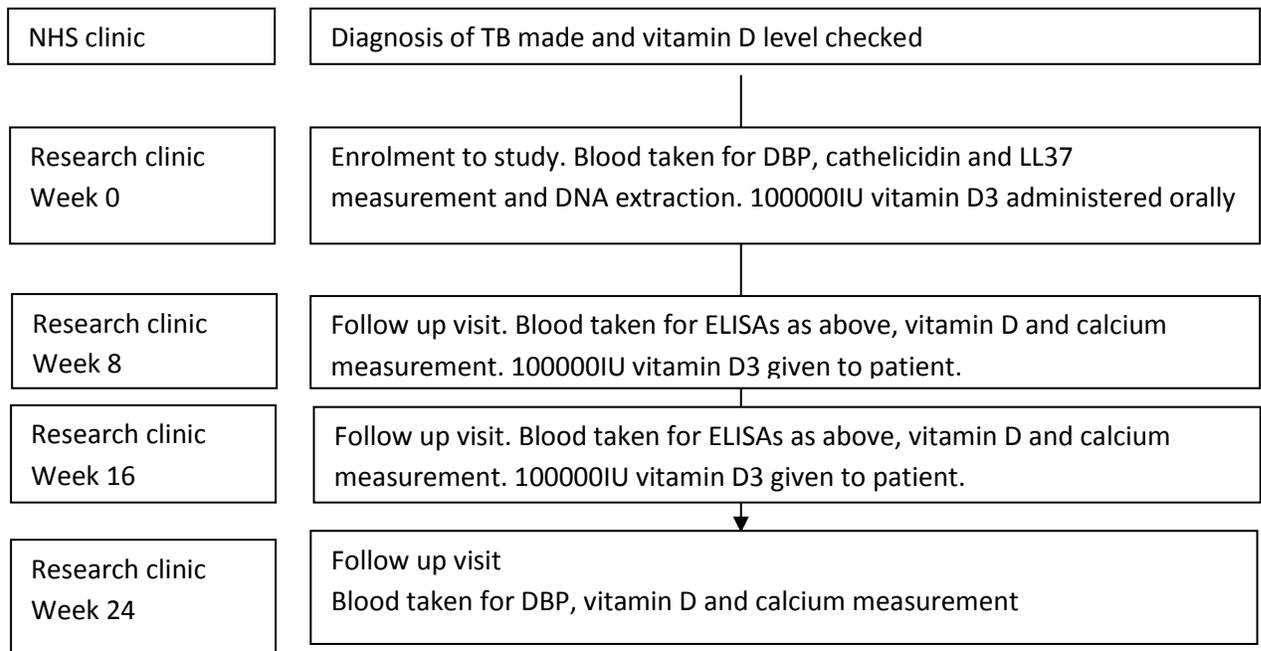
Power calculations can be unreliable in observational studies and the primary outcome was to identify if vitamin D baseline levels and DBP genotype determine post supplementation vitamin D response. Vitamin D has influences on many antimicrobial factors, which we did not have pilot data on, and we could not assume that total vitamin D response is proportional to a sole marker. Other outcomes including LL37 levels are measured in a different manner to vitamin D and therefore the powering of the study for the variety of outcomes proved difficult.

However sample size calculations for this study identified in the original study protocol were based on the minor allele frequency (risk allele) of the main electrophoretic variants.

GC genotype would be unknown at the time of enrolment hence we aimed for a total of 160 patients enrolled, to allow for inequality of genotype distribution. The minor allele frequencies of rs4588 and rs7041 vary between races such that the minor allele of rs4588 is seen in 27% of Caucasians, 32% of Gujarati Indians and 5% of black Africans. The minor allele worldwide of rs7041 is seen as the major allele in Caucasians, with a frequency of 57%, and as a minor allele in Indians (49%) and African races (18%). Initial data from Heartlands and UHB shows that 35% of registered cases in 2009 occurred in Caucasians, 50% in Indian or Pakistani patients, 12% in black Africans, with the remainder in other ethnic groups. Based on these incidence figures and mean allele frequencies for rs4588 and rs7041 a risk allele will occur in 24/56 Caucasians, 32/80 Indian/Pakistanis and 2/19 black Africans of 160 recruited, being more than adequate for comparison to the non-risk allele carriers (n=58 v n=102). We aimed to recruit 30 patients of each GC genotype of importance to the

study. These estimates also allow for drop outs of up to 40% in each arm, although current local figures suggest this will be closer to 15%, since 86% of patients complete treatment.

As the study progressed, recruitment was slower than expected and the in vitro work was added in to support the clinical study and allow for lower patient numbers than were originally planned for in the power calculations.



2.2 Clinical Methods

2.2.1 Subject Selection

Patients were selected from respiratory / tuberculosis clinics at the Heart of England Foundation Trust on a consecutive basis. Adult patients able to consent to research with a microbiologically or histologically confirmed diagnosis of active TB were included. A positive microbiological diagnosis was defined by positive microscopy smear or culture of M.tb in the setting of clinical suspicion of M.tb infection. Patients were enrolled at the start of their anti-TB therapy.

Exclusion criteria were:

1. Patients being managed with regimes outside of the standard NICE guidance [188]
2. Drug resistant M.tb
3. HIV positive
4. Known intolerance of vitamin D
5. Sarcoidosis
6. Hyperparathyroidism or nephrolithiasis
7. Taking vitamin D supplementation in the two months preceding enrolment

8. Baseline serum corrected calcium >2.65 mmol/L
9. Current haemodialysis
10. Children under 18 years, pregnant or breastfeeding individuals
11. Concomitant benzothiadiazine derivative, cardiac glycoside, carbamazepine, phenobarbital, phenytoin, primidone or long-term immunosuppressant therapy

Once recruited, patients were clinically assessed using the TB score (see below) and radiology findings. Blood was taken for extraction of serum and plasma and whole blood was frozen for DNA quantification. Each patient was given (directly observed) 100,000 units of Dekristol© (cholecalciferol) at week 0, 8 and 16. Follow up was maintained for 6 months in clinic and if treatment lasted longer than 6 months, notes were reviewed.

2.2.2 Demographic Data Collection

Demographic data was recorded including baseline information (age, address, gender, height and weight) ethnic origin, year of entry to the UK, HIV status if known (checked as routine clinical practice in all TB cases if consent obtained), smoking history, history of illicit drug abuse or alcohol misuse, co-morbidities and current medication.

2.2.3 TB Score

At the time of enrolment TB severity was assessed using the TB score, which is a composite of symptoms and signs, recognised as a predictor of subsequent mortality, with a maximum score of 13 and good inter-observer agreement [189]. The TB Score was recorded at each subsequent visit at 8, 16 and 24 weeks. An example of the TB score sheet is shown as part of the data record in Appendix 2.

2.2.4 Radiology

The severity of pulmonary disease was recorded using either chest radiography or CT thorax. Scoring systems for radiological investigations in tuberculosis are not widely used. X- rays were graded according to percentage lung involved and whether cavities were present. This is known to determine sputum positivity duration [190]. CT images were available for some patients and these could be scored in the same way if necessary. CT imaging provides a more detailed view of the lung tissue and smaller lesions which are present on CT may not be visible on CXR. Where possible, CXR scoring was calculated. The CXR score used was validated by Ralph et al [191], which calculates a score based on consolidation extent noted on CXR and the presence of cavitation.

CXR Score = % proportion of total lung affected + 40 (if cavitation present)

2.2.5 Blood Sampling

Blood was taken for 25(OH)D₃ level (nmol/l), calcium level, C reactive protein , full blood count, urea and electrolytes and liver function tests at 0, 8, 16 and 24 weeks. These were measured by the Heartlands Hospital Laboratories as part of their NHS assessment. In addition blood was taken for the measurement of DBP and LL37 and extraction of DNA for genotyping which was carried out at the University of Birmingham Research Laboratories. Since these tests were specific to the research trial they are described within the laboratory methods (section 2.3). Serum and plasma were stored for measurement of other potential biological markers later in the project, which were determined as initial results were generated.

2.2.6 Smear Microscopy, Culture and Histology

Samples were sent for microscopy (acid fast stain) and subsequent culture for M.tb on all patients able to expectorate. In some the diagnosis was made on bronchoscopy washings to confirm the diagnosis. Further sputum samples were sent at each subsequent visit to determine sputum conversion rates, if the patient was able to provide one. Histology sampling was carried out prior to the first clinic visit as appropriate. All such tests were part of routine NHS practice.

2.2.7 Dosing Regimen of Vitamin D

Clinic visits occurred at 0, 8, 16 and 24 weeks, and at each visit the vitamin D level (25OHD₃) checked. All subjects received 100000 units of vitamin D₃ orally (Dekristol capsules) at the baseline visit, and at 8 and 16 weeks, directly observed by the investigating team.

Vitamin D replacement at this dose results in a rise in vitamin D levels that would elevate to the sufficient range (>50nmol/l) and should also result in stable levels within the normal range over the subsequent 8 weeks, as shown elsewhere [192]. This pharmacokinetic study justifies the dose and dosing schedule chosen here.

2.3 Laboratory Methods

2.3.1 Acquisition of serum and plasma from whole blood

One serum and three plasma / EDTA vacuettes of blood (Greiner bio-one, UK) were centrifuged for 10 minutes at 500 rpm immediately after venepuncture (within 30 minutes) to avoid potential cytokine degradation. With regard to time from acquisition to centrifugation, one study showed no significant effect on levels of vitamin D between 2 and 24 hours [193] whilst another study looking

at transport of samples by post showed that although there was an alteration in results over time, these varied by just a few percent [194].

A Pasteur pipette was used to remove the supernatant (plasma / serum); 500µl aliquots were pipetted into labelled 1ml eppendorfs and put on ice to transfer to storage at -80°C. The samples for routine biochemical evaluation and vitamin D levels were transported in a specimen carrier to the base hospital.

2.3.2 DNA Extraction

The following methods describe the general methods used for extraction and quantification of DNA. Specific genotyping tests are described in the section 2.3.5.

Stage 1 - Pelleting

This method was used for all participants, where blood was collected in a single EDTA vacuette. The collection tubes were frozen at -70°C until sixteen samples were available to process together. Blood was mixed and emptied into a 50ml Falcon tube. It was then mixed with Reagent A (see Appendix 2) to lyse the cells, placed on a rotary mixer at speed 170rpm for 4 minutes and then centrifuged at 2400rpm for 4 minutes. The supernatant was discarded safely and 7.5mls of Reagent A was added to the pellet remaining. The sample was centrifuged again at 2400rpm for 4 minutes and the supernatant discarded safely without disturbing the pellet. The pellets were stored at -80°C.

Stage 2 - DNA Precipitation

The frozen pellets were defrosted for around 30 minutes. 2mls of Reagent B (Appendix 4) was added to the blood pellet and re-suspended using the Vortex to lyse it. This was then poured into a 5ml labelled centrifuge tube (Alpha Labs, UK) and 500 µl of Sodium Perchlorate 5M (Fisher Scientific, UK) was added and mixed by inversion to denature the protein. Chloroform at -20°C was then added to fill each tube and centrifuged at 3000rpm for 5 minutes. The supernatant was pipetted into a fresh labelled tube without disturbing the underlying protein layer.

To precipitate the DNA, 100% ethanol at -20°C was added to fill the tube to the top of the label and mixed by inversion. If there was no evidence of this (absence of a white swirl in the tube), it was incubated for 30 minutes at -80°C. This was then centrifuged at 3000rpm for 10 minutes and the ethanol was carefully poured off into a small blood beaker, leaving the remaining DNA pellet. 1ml of 70% ethanol was added and the tube inverted to wash the pellet. This was then centrifuged at 3000rpm for 5 minutes and the ethanol carefully poured away / blotted with tissue, leaving the remaining pellet in the tube. This was then air dried flat until the pellet turned transparent (taking approximately three hours but could safely be left overnight). 100 µl of T.E buffer (Appendix 4) was added and the tube left in the fridge for 2-3 days to re-suspend the DNA. This was then centrifuged at 3000 rpm for 5 minutes. Using a 1000µl ART reach tip (Aerosol Resistant Tips – to prevent carryover contamination), the contents of the tube was transferred into a labelled red screw top eppendorf, ensuring DNA was transferred to the tube.

2.3.3 DNA Quantitation

This was done using the Picogreen © method for determining DNA concentration.

Picogreen is a fluorochrome that selectively binds double stranded DNA (dsDNA). It is fluorescent when bound and has been shown to be a reliable method of DNA quantitation [195].

A standard curve is required using calf thymus DNA (Sigma, UK) as shown in table 2.1. A single vial of 1mg/ml calf thymus DNA was mixed with 1ml of T.E Buffer and left overnight to re-suspend. DNA dilutions were also prepared on the first day in preparation.

The stock DNA was diluted 1:100 by mixing 198 μ l of T.E buffer with 2 μ l of stock DNA to each eppendorf. They were then vortexed and left refrigerated overnight to re-suspend. On the second day, the plate was set out as shown in table 2.2. The Picogreen was also taken out of the freezer and allowed to thaw.

The standards and blanks were placed in rows A and B. 5 μ l aliquots of the 36 diluted DNA samples made on day one were added in duplicate across the plate from row C to H to fill the 72 remaining wells. Picogreen solution A was prepared in a brown 1.5ml eppendorf, adding 1194 μ l of T.E. Buffer to 6 μ l of thawed Picogreen. This was then added in 50 μ l aliquots to the standards in rows A and B. Picogreen solution B was prepared in a foil wrapped 15 ml falcon tube by adding 7580 μ l of T.E buffer and 20 μ l of Picogreen. This was then poured into a sterile trough with foil lid and using a multi-pipette with 6 tips, 95 μ l aliquots were added to samples in rows C to H.

	Calf thymus DNA	TE (μl)	Concentration after addition of Picogreen® (ng/ml)
1	4μl of 1mg/ml stock	1246	1600
2	600μl of 1	600	800 (Standard 1)
3	600μl of 2	600	400 (Standard 2)
4	600μl of 3	600	200 (Standard 3)
5	600μl of 4	600	100 (Standard 4)
6	600μl of 5	600	50 (Standard 5)
7	600μl of 6	600	25 (Standard 6)
8	600μl of 7	600	12.5 (Standard 7)
9	600μl of 8	600	6.25 (Standard 8)

Table 2.1 Dilutions of calf thymus DNA for producing the standard curve for DNA quantitation.
The 1600ng/ml standard is not needed for the standard curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLANK	800	400	200	100	50	25	12.5	6.25			
B	BLANK	800	400	200	100	50	25	12.5	6.25			
C	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
D	S7	S7	S8	S8	S9	S9	S10	S10	S11	S11	S12	S12
E	S13	S13	S14	S14	S15	S15	S16	S16	S17	S17	S18	S18
F	S19	S19	S20	S20	S21	S21	S22	S22	S23	S23	S24	S24
G	S25	S25	S26	S26	S27	S27	S28	S28	S29	S29	S30	S30
H	S31	S31	S32	S32	S33	S33	S34	S34	S35	S35	S36	S36

Table 2.2 The plate layout for Picogreen method of DNA quantification.

A1 and B1 contain 50µl of T.E buffer (used as blanks). The standards prepared as in Table 2.1 above are placed in wells A2-9 and B2-9. The plate was read at the standard fluorescein wavelength (excitation 480nm, emission 520nm) in a spectrofluorometer. The fluorescence value of the blank was subtracted from the each of the sample values, and the DNA concentration of each sample was derived from the standard curve. If the standard curve duplicates deviated by more than 20% from one another the plate was rejected and repeated with new standard curve dilutions. If individual paired sample readings deviated by more than 20% from one another they were repeated.

2.3.4 Choice of tag SNPs

Prior to genotyping individuals for specific single nucleotide polymorphisms (SNPs), it was necessary to determine which SNP primers were required to identify relevant polymorphisms in patient samples. As shown in section 1.6, key enzymes / proteins in the vitamin D axis were identified through a systematic literature search. Since the primary gene thought to influence downstream effects of vitamin D was *GC*, we chose to select tags that would cover the whole of this gene. Another relevant gene which were also identified was interleukin 6 (IL6) which is known to be produced by LPS stimulated monocytes and which has been shown to be suppressed in a dose dependent manner by vitamin D in vitro [196].

The next step involved the use of the genetics database, HapMap (www.hapmap.org), which analyses data from multiple racial groups – in earlier builds of HapMap this was only European, African and Asian populations but has now been expanded to much more specific sub-groups which include ASW: African ancestry in Southwest USA, CEU: Utah residents with Northern and Western European ancestry from the CEPH collection, CHB: Han Chinese in Beijing, China, CHD: Chinese in Metropolitan Denver, Colorado, GIH: Gujarati Indians in Houston, Texas, JPT: Japanese in Tokyo, Japan, LWK: Luhya in Webuye, Kenya, MEX: Mexican ancestry in Los Angeles, California, MKK: Maasai in Kinyawa, Kenya, TSI: Tuscans in Italy, YRI: Yoruba in Ibadan, Nigeria, although full linkage disequilibrium (LD) data is not available yet for all. For this study SNPs for the listed vitamin D related enzymes / proteins and cytokines in CEU and YRI groups for whom full LD data was available were identified. The aim was to identify links between genetic

variants and disease and the individuals response to therapeutic agents. The possibility of using genetic link to disease in this way may provide a route for individualising treatment for patients based on known genetic response.

Genetic variants lying close to each other in the gene tend to be inherited together (see also section 1.6), due to linkage disequilibrium. Thus, if a single nucleotide change occurs (SNP), then other genetic variations lying close to this SNP will always be inherited with it. This region or block is known as the haplotype and by identifying a single SNP (tagSNP), it is possible to identify the other polymorphisms present with a reasonable degree of confidence, if LD is sufficiently high.

Settings were configured to include minor allele frequencies of >0.05 and $r^2 > 0.8$ (measure of strength of LD). Nine different SNPs were identified using Hapmap for *CYP27b1*, *CYP24a1*, *GC*, *VDR*, *CYP2R1*, *NADSYN1* and *IL6*. These tagSNPs are shown in table 3.2, alongside the SNPs that they capture through LD.

2.3.5 PCR and Genotyping

What is PCR?

Once a DNA sequence has been identified, enough needs to be produced for research purposes.

This is done through a series of heating and cooling cycles. There are three main stages which are illustrated in figure 2.1)

- 1) Denaturation – this is the process whereby heating the DNA enables the double helix to be split and form separate strands.
- 2) Annealing – Cooling allows primers (specific DNA sequences) to bind to the single strand of DNA.
- 3) Extension – Further heating allows an enzyme, DNA polymerase to catalyse the production of a complementary DNA strand from the available free nucleotides present.

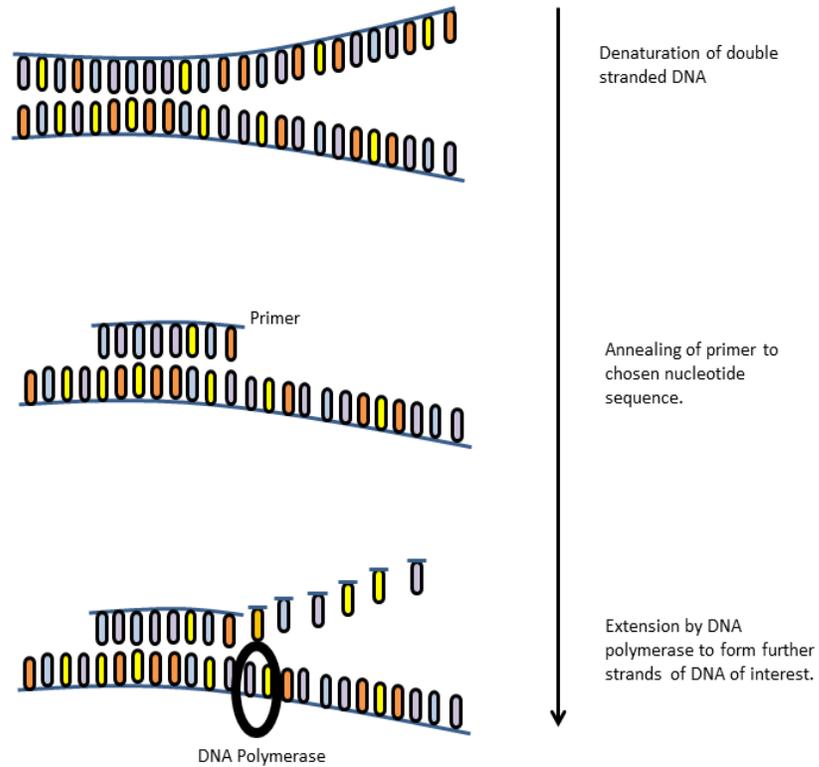


Figure 2.1 Polymerase chain reaction (PCR).

Double stranded DNA is denatured by heating to 95°C. The primer, consisting of a complementary sequence of nucleotides to the DNA in question anneals to a single DNA strand at a temperature specific for that particular sequence and kit. The complementary DNA strand then extends out from the primer, guided by DNA polymerase on further heating to 72°C for Taq polymerase. The reaction will occur more slowly at lower temperatures.

TaqMan® SNP genotyping

Taqman (Applied Biosystems® TaqMan®) genotyping technologies were used to detect polymorphisms within the genome by fluorescence. The main components of the kit required for PCR through this system include:

- *MgCl₂*- this is a source of magnesium and forms complexes with dNTPs which then bind to
- Taq (DNA) polymerase, resulting in the extension of the DNA strand of interest. Without it the extension phase of PCR will not occur.
- *Deoxynucleotide triphosphates (dNTPs)* - these are the source of free nucleotides required in the formation of new DNA strand during extension.
- *Primer* - the primers are synthetic segments of DNA that anneal or bind to a specific nucleotide sequence on the template DNA. Each primer has a fluorophore (VIC or FAM), and it is the relative expression of each that determines the genotype. The mechanism by which this occurs is shown in Figure 2.1.

The PCR reactions were set up on 384 well plates (Greiner Bio-one, UK), using 4 blanks as negative controls per plate. 1050µl of genotyping master mix (supplied ready mixed, containing dNTPs, MgCl₂ and Taq polymerase and optimised for use with TaqMan® genotyping assays by Applied Biosystems, UK), 70µl of genotyping assay at a 20x concentration (ABI, UK) and 35µl of MilliQ water were mixed in an opaque eppendorf (Alpha labs, UK), since the assay is light sensitive. For

assays supplied at a 40x concentration the amount of assay was halved and the amount of water correspondingly increased, such that the total volume was unchanged. A volume of 2.25µl of DNA at 20ng/µl was pipetted into each well of a 384 well plate, leaving 4 wells free to act as blanks, to which the same volume of MillQ water was added in order to standardise the reaction volume. 2.75µl of master mix was then added to each well. The plate was sealed with an optically clear seal (Alpha Labs, UK), and covered with foil. The plate was then centrifuged at 1000rpm for 1 minute to ensure that all reagents were at the bottom of the plate and once completed the plates were placed in a thermal cycler (MJ Research (PTC-225) UK) and PCR performed under the following conditions:

50°C	2 minutes	} 40 cycles
95°C	10 minutes	
92°C	15 seconds	
60°C	1 minute	

After completion of the PCR the plates were again covered with foil to prevent degradation of the light sensitive assay and stored at 4°C until they could be read. This step was carried out using an ABI-7900HT (ABI, UK), which is able to read the fluorescent signals emitted by the VIC and FAM markers within each assay. For each tag ABI supplied information indicating which allele the VIC and FAM markers detect, such that this can be specified prior to reading the plate. The instrument collects fluorescence data on the samples which are analysed using SDS software (version 2.2.2,

ABI, UK). The software presents the results as a scatterplot. There are four potential clusters of points, which correspond to the two homozygous genotypes, heterozygotes or no amplification. Parameters may be set to indicate cut-off values for allocation of genotypes within the software, and was set at 95% for the studies contained herein (as recommended by the manufacturers). Each plot was reviewed for the position and relative signal quality of samples where a genotype could not be allocated by the software, and a genotype assigned manually if possible. If the signal quality was <80% or the position was such that the genotype was unclear samples were reanalysed.

2.3.6 ELISA (Enzyme Linked Immunosorbent Assay)

This is a common method used to quantify cytokines, or other markers in secretions, blood or cell supernatants. Two of the main types of assay are:

1. Antibody detection ‘sandwich’ assay
2. Antigen detection ‘sandwich type’ assay.

These are illustrated in Figure 2.2. ELISAs carried out during this project included IL2, IL6, IL10, TNF α , IFN γ and LL37. Commercial ELISA kits may come in a variety of designs, with varying amounts of preparation required by the researcher or done at source by the manufacturer. Detailed methods for each are discussed below.

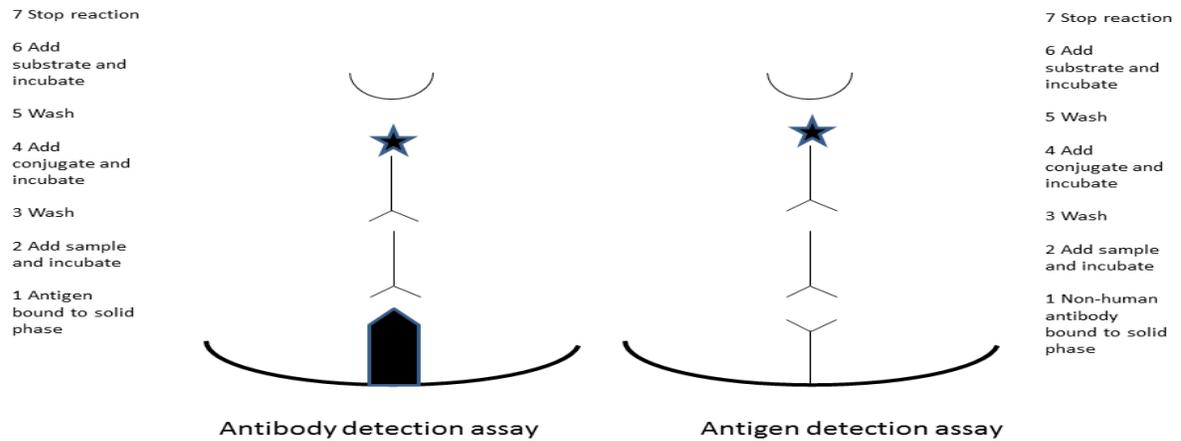


Figure 2.2 Antibody detection sandwich assay and antigen detection sandwich type assay.

The antigen or antibody is bound to the solid surface. The sample is added and incubated and if the antibody or antigen in question is present, it binds. The plate is then washed to remove any unbound antigen / antibody. A second specific antibody conjugated to an enzyme is added and incubated. A second wash removes any unbound antibody. An enzyme substrate is added and the plate is incubated again. The reaction is stopped by the addition of acid and the reaction is measured by colourimetry, the degree of colour change reflecting the amount of antigen or antibody in the sample.

DBP ELISA

The DBP ELISA (Immunodiagnostik, Germany) is a sandwich assay for Vitamin D binding assessment. Plasma samples from each of the patients in the TB study were used which had been stored at -80°C in 500µl aliquots. These were defrosted prior to commencing the assay and diluted 1:40000 with SAMPLEBUF (dilution buffer, ready to use) in the following way.

10µl Sample + 990µl SAMPLEBUF = 1:100 (Dilution I)

10µl Dilution I + 990µl SAMPLEBUF = 1:10000 (Dilution II)

250µl Dilution II + 750µl SAMPLEBUF = 1:40000 (Dilution III)

100µl of Dilution III was used at the appropriate time for analysis in each well.

The standards at 60ng/ml, 20ng/ml, 6.6ng/ml, 2.2ng/ml and 0ng/ml and CTRL (controls) were reconstituted with 500µl of deionized water. These were each allowed to dissolve for 10 minutes and mixed gently by inversion.

The conjugate (rabbit-anti-DBP, Peroxidase-labelled) was diluted 1:100 in wash buffer. 100ml of wash buffer concentrate was diluted with 900ml of deionised water (1:10 dilution) and mixed well. The remaining could then be refrigerated at 2-8°C. 100µl of standards and controls and the pre-diluted 1:40000 samples were added to the appropriate wells according to the Table 2.3 below. This was then incubated for 1 hour on a horizontal mixer at room temperature. The plate contents were decanted and washed 5 times with 250µl of diluted wash buffer. 100µl of diluted conjugate was added and incubated for 1 hour on a horizontal mixer at room temperature. The contents were decanted again and the wells washed 5 times with 250µl of diluted wash buffer. 100µl of

tetramethylbenzidine (TMB) substrate was added to each well and the plate was incubated for 10-20 minutes at room temperature. 50 μ l of stop solution (acidic solution) was then added and mixed. The absorption was read immediately on an ELISA plate reader at 450nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	2	2	10	10	18	18				
B	2.2	2.2	3	3	11	11	19	19				
C	6.6	6.6	4	4	12	12						
D	20	20	5	5	13	13						
E	60	60	6	6	14	14						
F	Control 1	Control 1	7	7	15	15						
G	Control 2	Control 2	8	8	16	16						
H	1	1	9	9	17	17						

 TB Patients
  Standards
  Control

Table 2.3 DBP ELISA plate

Standard concentrations of DBP were added to the labelled wells with control and TB patient's plasma samples added according to the colour key. The plate was then treated as per the above protocol and read on an ELISA plate reader at 450nm.

IL-6 ELISA (Duo-kit method)

This method describes the IL-6 ELISA, IL6 being an inflammatory readout for the monocyte work. This contains the components required for a sandwich ELISA designed to measure human and recombinant IL-6. The samples containing LPS were diluted to a 1:40 concentration (determined as optimal from the dose response work) and the controls, and supernatants containing 25(OH)D₃ only were not diluted.

Day 1

Separate quantities of 1L and 400ml of Phosphate Buffer Solution (PBS) were made up as per instructions. Wash Buffer was made up by mixing 0.5ml Tween with 999.5ml of PBS. The capture antibody (360µg/ml of mouse anti-human IL-6) was reconstituted with 1ml of filtered PBS. This was diluted to a working concentration of 2.0µg/ml in PBS without carrier protein. A 96 well microplate was coated with 100µl per well of the diluted capture antibody. The plate was sealed with an adhesive covering and incubated at room temperature overnight to allow the antibody to bind to the plate.

Day 2

The Reagent Diluent (1% BSA in PBS) was prepared by diluting 5ml of 10X strength reagent diluent with 45ml of distilled water. The wells were aspirated and washed three times with Wash Buffer. Excess liquid was removed using by inverting the plates and blotting against clean paper towels. The plate was then blocked by adding 300µl of Reagent Diluent to each well and incubated for 1 hour. The aspiration / washes X 3 were then repeated as above.

Standards were then prepared in 0.5ml eppendorfs as directed in Table 2.4 below.

Tube	Volume Reagent Diluent	Volume standard	Concentration (pg/ml)
1	491.73µl	8.57µl of reconstituted vial	600
2	250µl	250µl tube 1	300
3	250µl	250µl tube 2	150
4	250µl	250µl tube 3	75
5	250µl	250µl tube 4	37.5
6	250µl	250µl tube 5	18.75
7	250µl	250µl tube 6	9.375
8	250µl only		

Table 2.4 Table of standard dilutions for IL-6 Duo-kit.

100µl of the supernatant (diluted as described in paragraph 1 of this section) or standard in Reagent Diluent were added to the appropriate wells, covered with an adhesive cover and incubated for 2 hours. The plate was then aspirated / washed as above. 100µl of Detection Antibody diluted with Reagent Diluent was added to each well. The Detection Antibody (9µg/ml of biotinylated goat anti-human IL-6) was initially reconstituted with 1.0ml of reagent diluent and then diluted to a working concentration of 0.05µg/ml. This was then covered with an adhesive cover and incubated for 2 hours. The plate was then aspirated and washed as above.

100µl of the working dilution of Streptavidin HRP (1ml of streptavidin conjugated to horseradish peroxidase diluted to 1:200) was added to each well and the plate was covered and left to incubate for 20 minutes at room temperature, out of direct light. The plate was then aspirated / washed as above and 100µl of Substrate Solution (tetramethylbenzidine) was added to each well. This was left to incubate for 20 minutes at room temperature, out of direct light. 50µl of Stop Solution was then added to each well (from DBP ELISA kit) and the plate was gently tapped to ensure thorough mixing. The plate was read at wavelength 450nm, subtracting readings at 540nm to correct for optical imperfections.

Dilution determination of supernatants for IL6 ELISA

Initial IL6 assays carried out on monocyte supernatant led to results which lay above and below the standard curve. The results above the upper limit of the standard curve correlated with LPS exposure. Therefore in order to determine the correct dilution for each of the supernatants, an IL-6 assay was carried out using five of the control supernatants which included those resulting in the 3 highest and 2 lowest results on initial ELISA. These samples were control (RPMI only), 25(OH)D₃ 50nmol, LPS 1µg/ml, LPS 100ng/ml, LPS 1µg/ml + 1,25(OH)₂D₃.

	1	2	3	4	5	6	7	8	9	10	11	12
A	600		1 in 3		1 in 3							
B	300		1 in 5		1 in 5							
C	150		1 in 10		1 in 10							
D	75		1 in 20		1 in 20							
E	37.5		1 in 40		1 in 40							
F	18.75		1 in 80		1 in 80							
G	9.375		1 in 100		1 in 100							
H	RD*		1 in 160		1 in 160							

	Standards & Control
	Control (RPMI)
	25D 50nmol
	LPS 1 µg/ml
	LPS 100 ng/ml
	LPS 1 µg/ml + 1-25D 50nmol

Table 2.5 IL-6 duo kit plate layout for various dilutions of monocyte supernatants.

The values of IL-6 concentrations on the ELISA plate were analysed and dilutions falling within the standard curve were chosen for subsequent work. Consequently any samples containing LPS were diluted 1: 40 and any other samples were analysed undiluted. *RD –reagent diluent.

LL-37 ELISA

This human LL-37 kit (Hycult® biotech) is a ready to use solid phase ELISA and was used in the functional work with monocytes as an antimicrobial readout. Each sample was assayed in duplicate and mean values taken. 40 ml of wash / dilution buffer A was mixed with 360ml of distilled water. 20ml of wash/ dilution buffer B was mixed with 380ml of distilled water. These solutions were combined and mixed well. This was the wash/dilution buffer used for the ELISA. The standard solution (vial 3 in the duokit) was made up by reconstituting 0.5ml of distilled water with the standard provided. This was then serially diluted in eppendorfs according to the table below to give the various concentrations required.

Tube	Volume wash/ dilution buffer	Volume standard	Concentration (ng/ml)
1		150µl vial 3	100
2	250µl	125µl tube 1	33
3	250µl	125µl tube 2	11
4	250µl	125µl tube 3	3.7
5	250µl	125µl tube 4	1.2
6	250µl	125µl tube 5	0.4
7	250µl	125µl tube 6	0.1
8	250µl		0

Table 2.6 Standard solution preparation for LL-37 ELISA.

Supernatant samples were diluted 5X with supplied wash / dilution buffer and 100µl of each sample and each standard were added to the wells. The plate was covered with an adhesive sheet and tapped to eliminate any air bubbles. The plate was incubated at room temperature for 1 hour. The contents were then removed by inversion and tapped on tissue paper. Following this it was washed with wash/dilution buffer on the plate washer. 200µl of wash buffer was then added to each well and after 20 seconds, emptied and washed as described above. This was done 3 times in total. 100µl of diluted tracer (reconstituted with 1ml of distilled water and diluted with 11ml of wash / dilution buffer) was added to each well and the tray covered with an adhesive layer and incubated for 1 hour at room temperature. The plate was then washed repetitively as above and 100µl of diluted streptavidin-peroxidase added to each well. The streptavidin-peroxidase solution was made up by reconstituting the supplied vial with 1ml of distilled water and diluting the reconstituted solution with 23ml wash / dilution buffer. The tray was covered as above and incubated for 1 hour at room temperature. It was then washed as above and 100µl of TMB substrate added to each well. The plate was then covered with a fresh adhesive cover and incubated for 20-30 minutes at room temperature, avoiding sunlight (foil cover). The reaction was stopped by adding 100µl of stop solution and covering with an adhesive cover for 20-30 minutes having mixed the solutions by swirling gently. The plate was read at 450nm using a plate reader and the mean absorbance for the duplicate standards and samples was calculated. Intra-assay coefficient of variation (cv) was <6%, inter-assay cv <4%.

2.3.7 Flow Cytometry

Fluorescence activated cell sorting (FACS) has been used in T cell analysis for this project; the process is summarised in Figure 2.3. The general principles of FACS are described here and the specific panels of markers used in section 2.3.9 and table 2.10, 2.11 and 2.12. The use of FACS enables certain cellular characteristics / properties to be identified through labelling with particular fluorophores. Both external and intracellular markers can be identified in this way, enabling identification of CD3⁺ or CD4⁺ cells for example. The labelled cells are passed into a sheath and directed in a flow chamber towards a laser beam. As the cells enter the light source, absorption occurs and a state of excitation arises and any fluorescent particles will fluoresce, according to the fluorophore labelling, light is excited and emitted at a different wavelengths, and this fluorescence in each cell can be measured on an individual basis. The measurement of each wavelength is converted to an electrical signal at the optical detectors.

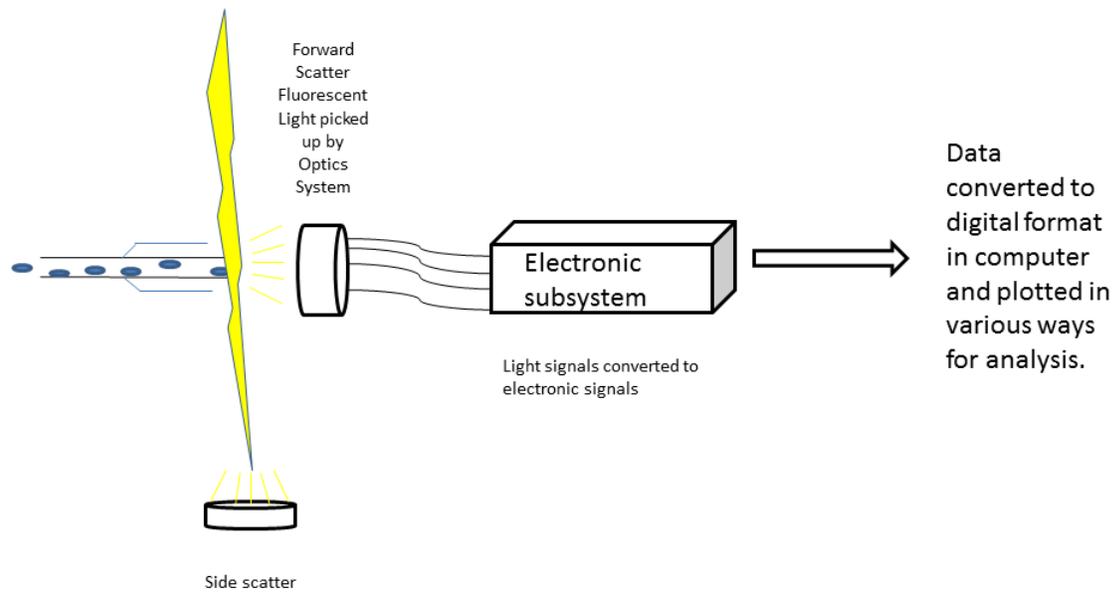


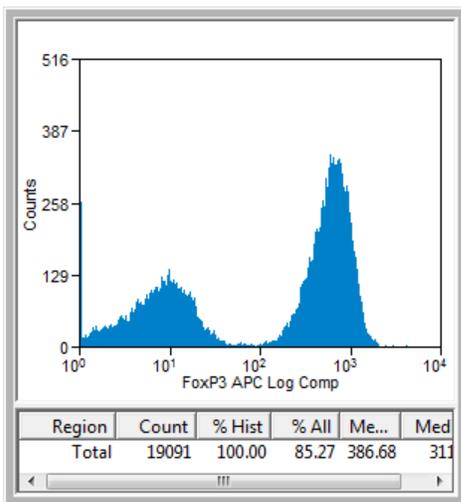
Figure 2.3 The pathway through the flow cytometer.

Cells are propelled along in a fluid suspension towards a laser beam. As the cells individually pass the laser it results excitation and emission of forward and side scatter light. The degree of forward scatter and side scatter may characterise the cells, however, fluorochromes are also used to detect specific target proteins. These fluorochromes emit light when excited by a laser with the corresponding excitation wavelength. Fluorescence from any cells that have been stained is detected by sensors and this is then converted to electrical and finally digital format to produce a variety of representative histograms / plots.

Displaying Data

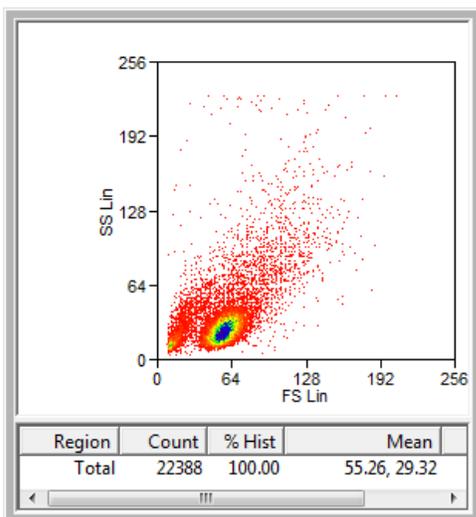
Light signals, converted to electronic signals, are produced in a digitalised format by the computer and this information can be displayed in several ways. Examples are shown below.

- Histogram



This displays the number of events for a particular measurement e.g. APC fluorescence. The greater the fluorescence intensity the further to the right the peak occurs.

- Dot and Density plots.



Dot and density plots are very similar with each dot representing a single event / cell. In density plots the colour denotes the event frequency.

- Gating

In order to analyse multiple parameters (e.g. cells which are identifiable by the presence of more than two surface markers), when two parameters are plotted against each other, regions can be marked out and this cell population is then put onto a further plot with different surface marker parameters in order to complete the analysis. This is known as gating (see figure 2.4). For example, CD3⁺ CD4⁺ cells can be gated against one another and the region of cells which are positive for both surface markers can then be marked and gated onto another plot.

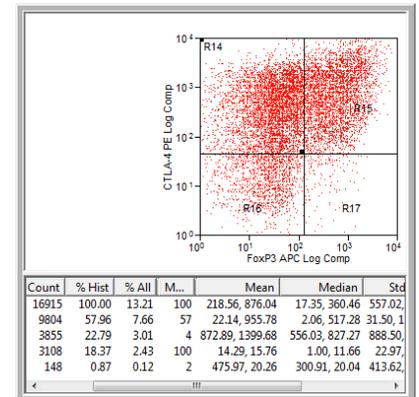
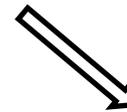
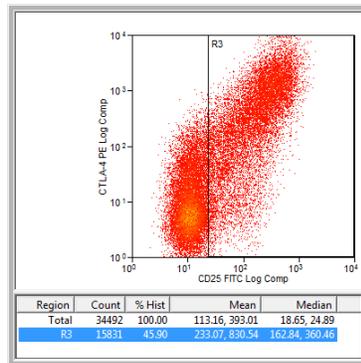
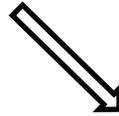
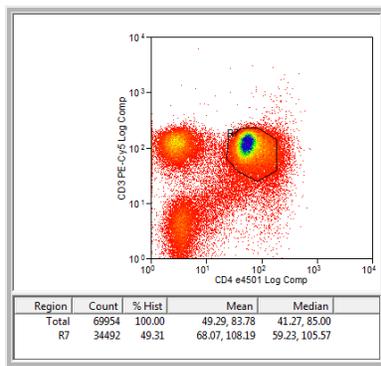


Figure 2.4 Gating dot and density plots.

This shows gating of CD3+/CD4+ cells (represented in the polygon outline) onto CD25+/CTLA4+ plot. The CD25+ cells (in region R3) are then all gated onto next plot to determine FoxP3 positive population within this group.

2.3.8 Assessing the functional effect of Vitamin D on monocytes

Monocyte Isolation

36-42 mls of blood was taken using Li-heparin vacuettes (BD Vacutainer®). The blood was poured in equal volumes into two 50ml Falcon tubes and mixed with equal volumes of 0.9% normal saline. 12 ml of Lymphoprep™ (used to create a density gradient to allow the separation of peripheral blood mononuclear cells from other white cells and red cells) was placed in several clean 50ml Falcon tubes and the 15ml of the blood / saline solution was overlaid (blood saline solution was carefully pipetted onto the surface of the lymphoprep using a 25ml automated pipette at slow speed). The tubes were centrifuged at 800g to allow separation of the cells, acceleration 1, brake 0 at 20°C for 30 minutes. Whilst the mixture was being centrifuged, RPMI culture medium was warming to room temperature. Using defrosted foetal calf serum (FCS) which provides a source of growth factors, a 10% solution of RPMI/FCS was made up by adding 45 ml of RPMI to 5 ml FCS in a 50ml Falcon tube. The Falcon tubes were removed from the centrifuge and the interphase (consisting of leucocytes) was aspirated and placed into a 50ml Falcon tube. A cell count/trypan blue exclusion test was carried out to determine total cell count. This was done by mixing 50µl interphase with 50µl of trypan blue and pipetting the mixture onto a haemocytometer. The number of cells on each grid of the haemocytometer was counted and the average value was multiplied by 20000 to obtain the total number of cells / ml

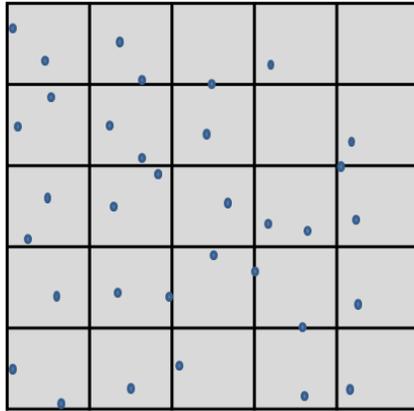


Figure 2.5 View of the haemocytometer grid under a light microscope.

The haemocytometer is used to count blood cells. The area bound by the lines is known as is the depth of the haemocytometer chamber. Any cells lying within each small square and any lying on the left or inferior border of each small square are counted. In this way, it is possible to count the cells in a specific volume and determine the concentration of cells in the fluid overall.

A differential cell count was then carried out by performing cytopspin at 200 rpm for 3 minutes. The slides were then stained using Diff Quik cellular staining kit and viewed at X40 magnification on a light microscope. The first 100 cells were counted and identified as monocytes, lymphocytes or neutrophils, allowing calculation of % monocytes of the total cell count. For example, if the differential cell count showed 10% monocytes and the total cell count was 20 million then the number of monocytes in the sample was 2 million.

0.9% saline was then added to wash the cells, filling the Falcon tube containing the interphase. This was mixed by inversion and then centrifuged at 500g for 10 minutes with the brake on. The supernatant was then discarded, leaving a pellet at the bottom of the Falcon tube.

The pellet was then resuspended using RPMI/FCS solution prepared earlier to a concentration of 1 million monocytes per ml, using the formula below to calculate this

$$\text{Volume (ml)} = \frac{\text{total number of monocytes}}{\text{desired concentration (cells /ml)}}$$

This was then added to a 24 well plate in 0.5ml aliquots and incubated at 37°C, 5% CO₂ for 24 hours. The time course was based on existing laboratory protocols and for convenience – the monocytes require enough time to adhere to the plastic culture wells (2-4 hours) whilst enabling the culture conditions to be applied at a convenient time for the operator.

The method below indicates how the conditions for LPS and vitamin D were made up. This was done following assessment of LPS dose response effect on monocytes to determine the LPS

concentration used. The optimum vitamin D and LPS concentrations were determined using the methods described in section 4.1.1 below and in Figure 4.1.

After 24 hours of monocyte incubation LPS 1 µg/ml and 100ng/ml were made up. Stock solution, strength 1mg/ml was taken and defrosted. It was then diluted to 1:1000 by mixing 5 µl of stock solution with 4995 µl of warm RPMI / 10%FCS in a bijou. 300 µl of the LPS 1 µg/ml solution (diluted as above) was taken and mixed with 2700 µl RPMI / 10%FCS (1:10 dilution). In addition the following were made:

25(OH)D₃ 50nmol

A solution of 1000nmol was initially made by diluting 1 µl of stock solution (strength 1mmol) with 999 µl of RPMI / 10%FCS. In order to dilute it to 50nmol strength in the wells, 25 µl of the 1000nmol solution was added to each required 500 µl well which contained either RPMI / 10%FCS or LPS. A 24 well diagram is shown below to give an example of the plate layout.

1.25(OH)₂D₃ 50nmol

A solution of 1000 nmol was initially made by diluting 10 µl of stock solution (100 µmol) with 990 µl of RPMI / 10%FCS in a bijou. In order to dilute it to 50nmol strength in the wells, 25 µl of the 1000nmol solution was added to each required 500 µl well which contained either RPMI / 10%FCS or LPS. A 24 well diagram is shown below to give an example of the plate layout.

Control	Control	25(OH)D 50nmol	25(OH)D 50nmol	LPS 1µg/ml PLUS 1,25D 50nmol	LPS 1µg/ml PLUS 1,25D 50nmol
LPS 100ng/ml	LPS 100ng/ml	LPS 100ng/ml PLUS 25(OH)D 50nmol	LPS 100ng/ml PLUS 25(OH)D 50nmol		
LPS 1µg/ml	LPS 1µg/ml	LPS 1µg/ml PLUS 25(OH)D 50nmol	LPS 1µg/ml PLUS 25(OH)D 50nmol		
1,25 D 50nmol	1,25D 50nmol	LPS 100ng/ml PLUS 1,25D 50nmol	LPS 100ng/ml PLUS 1,25D 50nmol		

Table 2.7 Monocyte conditions in duplicate on a 24 well culture plate following adherence isolation. Monocyte cultures were stimulated with two different concentrations of LPS in the presence or absence of 1,25(OH)₂D₃ or 25(OH)D₃.

LPS Dose Response – optimisation experiments

Two control samples were used in order to determine the suitable LPS concentration used in the monocyte culture media as an inflammatory stimulus (i.e. produce adequate stimulation of monocytes). The production of IL6 was then measured using an IL6 ELISA Duokit (method described in section 2.3.6). In addition to various LPS conditions, 25(OH)D₃ was also added to the media in separate wells at varying concentrations, to determine any additive effect on IL6 values at different LPS concentrations.

	1	2	3	4	5	6	7	8	9	10	11	12
A	600		Control		LPS 1000		Control		LPS 1000			
B	300		25D		LPS 750		25D		LPS 750			
C	150		25D + LPS 1000		LPS 500		25D + LPS 1000		LPS 500			
D	75		25D + LPS 750		LPS 250		25D + LPS 750		LPS 250			
E	37.5		25D + LPS 500		LPS 100		25D + LPS 500		LPS 100			
F	18.75		25D + LPS 250		LPS 50		25D + LPS 250		LPS 50			
G	9.375		25D + LPS 100				25D + LPS 100					
H	RD		25D + LPS 50				25D + LPS 50					



Control 1



Control 2

Standards = pg/ml

Samples containing LPS were measured at a 1:40 dilution

Samples LPS = ng/ml

All other samples i.e. controls, 25(OH)D₃ & 1,25(OH)₂D₃D were measured neat.

Vitamin D = 50nmol

Table 2.8 LPS dose response experiment : IL6 ELISA plate.

Standard solutions are placed in columns 1 and 2, with control sample 1 and 2 in the remaining wells. All conditions were tested in duplicate and any samples containing LPS were diluted 1:40 in order to achieve a measurable result on the plate reader.

Vitamin D dose response

In order to determine if there is any variability in IL6 response according to the concentration of 25(OH)D₃, a dose response IL-6 ELISA was carried out. This assessed the IL-6 response of monocytes when treated with various concentrations (10nmol, 20nmol, 50nmol, 100nmol) of 25(OH)D₃ (i.e. the effect of vitamin D on inflammatory cytokines in vitro). RPMI 1640 medium was used as the control wells.

The plate was set up using 2 control samples. The monocytes were isolated on day 1 and on day 2 each well was treated with 25(OH)D₃ at concentrations of 10nmol, 20nmol, 50nmol, 100nmol. In addition further wells were treated with combinations of LPS 100ng/ml in addition to 25(OH)D₃(same concentrations as listed above). The samples were incubated at 37°C and 5% CO₂ for 24 hours. The supernatants were then removed and IL-6 levels determined using IL-6 duokit ELISA. Standard dilutions were prepared as described in Table 2.4. An example of the plate layout is shown in the Table 2.9 below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	600		Control		25D 100nmol + LPS 100ng/ml		Control		25D 100nmol + LPS 100ng/ml			
B	300		25D 10nmol				25D 10nmol					
C	150		25D 20nmol				25D 20nmol					
D	75		25D 50nmol				25D 50nmol					
E	37.5		25D 100nmol				25D 100nmol					
F	18.75		25D 10nmol + LPS 100ng/ml				25D 10nmol + LPS 100ng/ml		Control 1  Control 2 			
G	9.375		25D 20nmol + LPS 100ng/ml				25D 20nmol + LPS 100ng/ml					
H	RD		25D 50nmol + LPS 100ng/ml				25D 50nmol + LPS 100ng/ml					

Table 2.9 Vitamin D dose response : IL6 ELISA plate.

Standards in columns 1 and 2 and various concentrations of vitamin D with and without LPS stimulation. Standards measured in pg/ml. Samples containing LPS were measured at a 1:40 dilution. All other samples i.e. controls, 25D & were measured at 1:4 concentration.

DBP genotype effect on cytokine response

Experiments to determine the effect of DBP genotype on cytokine response in plasma were carried out. Pooled plasma from each of the GC genotypes GC1S-GC1S, GC1F-GC1F, GC1S-GC2, GC1F-GC2, GC2-GC2, GC1S-GC1F from the study were obtained. At least three from each genotype group were pooled to minimise heterogeneity.

Optimisation experiments were initially carried out using control monocyte cultures in order to determine the optimal dilution of plasma with LPS in order to obtain measurable IL6 levels (i.e. those which lie in the detectable range on the IL6-duokit) from the monocyte supernatant.

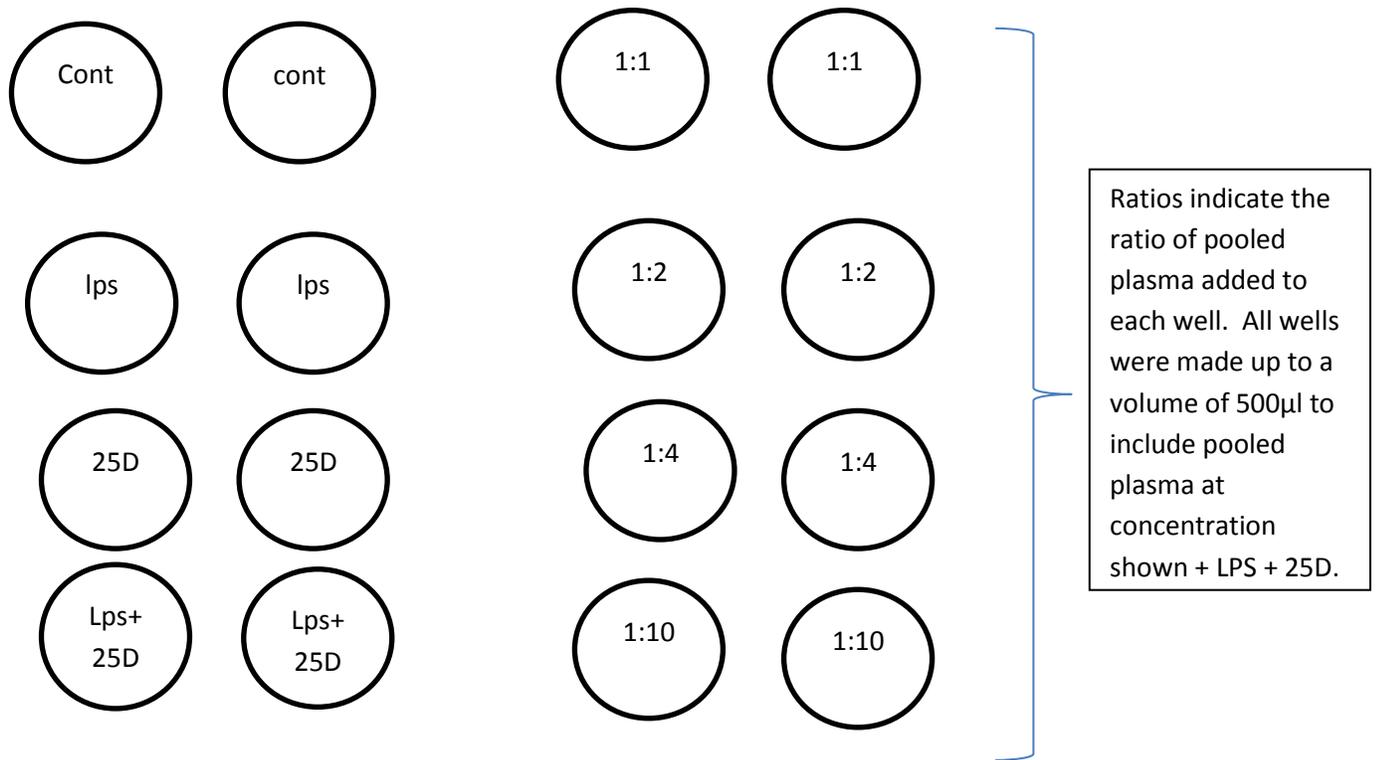


Figure 2.6 Monocyte culture plate for optimisation of IL6 response with addition of pooled plasma.

Optimisation experiments: monocyte cultures stimulated with LPS and supplemented with vitamin D were subjected to pooled plasma of a single GC genotype in order to determine the optimal dilution of plasma required for the final experiments.

Monocytes were cultured from control blood of four individuals using the method described in the monocyte isolation protocol above. The supernatant was then removed at 24 hours and culture media (RPMI) / LPS mix (volume added to wells varied in order to give a final LPS concentration of 100ng/ml once pooled plasma was added in the varying proportions). Initial experiments identified that pooled plasma in a 1:4 proportion was satisfactory / optimal to obtain adequate IL6 values on ELISA. The pooled plasma from the various GC genotypes was then added to the LPS/RPMI mix to make up to a volume of 500ul in each well including 50nmol of 25(OH)D. After a further 24 hours, the supernatant was removed and frozen. Assays were then carried out to determine concentrations of IL6, TNF α , IL1 β and GCSF using Luminex assay (RnD systems). This allowed identification of any relevant pooled plasma genotypes effect on cytokine response to vitamin D supplementation.

2.3.9 Assessing the functional effect of Vitamin D on T cell subpopulations.

PBMC Isolation

12-16 ml of blood was taken in Li-heparin vacutainers. The blood was emptied into a single 50ml Falcon tube and diluted in a 1:1 ratio with phosphate buffered solution (PBS). The blood PBS mixture was then overlaid onto 12 ml of Ficoll or lymphoprep and centrifuged for 25 minutes, 20°C, 2200rpm, acceleration 1, brake 0. This allows separation of whole blood through a density gradient.

The interphase was aspirated and placed in a new 50ml Falcon tube. This was then washed with PBS to remove any contaminating separation medium and centrifuged at 2200rpm, brake on for 10 minutes. If the solution was cloudy, the pellet remaining after aspiration was further washed at a lower speed with brake on at 1200 rpm for 5 minutes.

The PBS was then aspirated, the pellet washed again with PBS and centrifuged at 1500rpm for 5 minutes. The PBS was aspirated and the pellet was finally resuspended with 1ml of serum-free cell growing medium - SCGM (CellGenixGmbH, Germany) supplemented with 50U/ml penicillin and streptomycin (Life Technologies/Invitrogen) and then made up to a volume of 5 ml with the same media.

A cell count was performed using a haemocytometer by pipetting 7µl onto one grid of the haemocytometer and using the 4X4 large grid square in the corners, the number of cells in the 4 squares diagonally in each 4X4 was counted. The total cell count was calculated by multiplying this value by 10^4 (e.g. total counted = 239×10^4 /ml).

The cell solution was then diluted to a concentration of 1,250,000 per ml (i.e. 1.25×10^6 /ml) and a volume of 9.8ml (this allowed for 22 wells of 400µl on a 48 well plate to be filled and the remaining volume was divided up, placing 100µl in 10 clean FACS tubes for use in the next stage of the experiment as shown below.



Cell Culture and Vitamin D Supplementation.

500 μ l of cells at 0.5million cells / ml concentration were plated into a 48 well plate (Becton Dickinson, USA) and incubated at 37° C, 5%CO₂ atmosphere.

Cells were either left a) unstimulated/ untreated control b) stimulated with SEB (staphylococcal enterotoxin B) at a concentration of 1 μ g/ml (Sigma, USA) or c) stimulated with PPD (purified protein derivative) at a concentration of 1000 unit/ml (Statens serum institute). More specific M tb antigens such as ESAT6 were also attempted during initial optimisation experiments, but were not continued for this thesis due to the low numbers of activated cells.

The stimulated cells were also treated with 10nM 1,25(OH)₂D₃ or 100nM 25(OH)D₃ (Sigma, USA). Dilutions were made in medium from 100 μ M stocks.

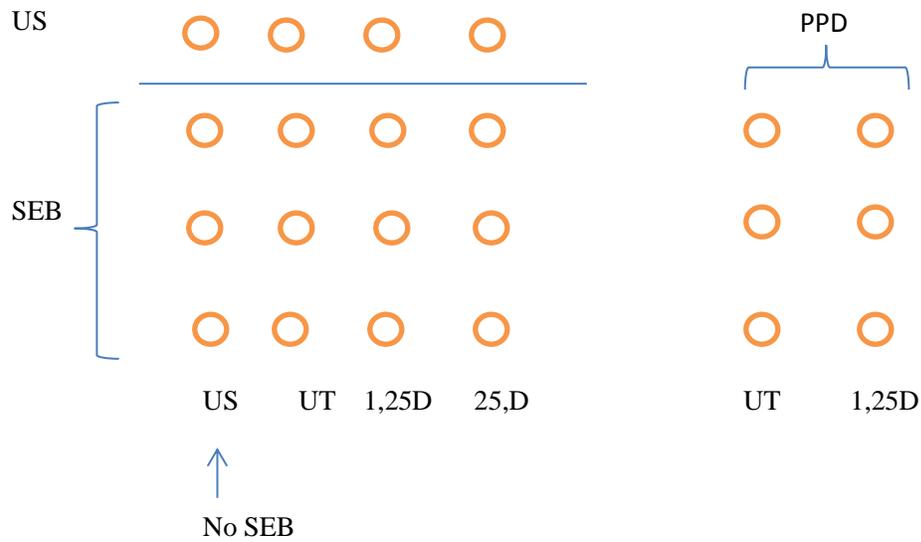


Figure 2.7 Peripheral blood mononuclear cell culture plate layout (in triplicate) : SEB / PPD stimulation and treatment with vitamin D.

Each plate was laid out to allow culture of PBMCs in the presence / absence of both SEB and PPD. The cultures were also carried out in the presence or absence of vitamin D. US (unstimulated); UT (untreated); 1,25D (10nM 1,25(OH)₂D₃); 25D (100nM 25(OH)D₃).

Antibodies for Staining Panels

The following monoclonal antibodies (mAbs) were purchased from BD Biosciences: anti-human CD4, anti-human CD69, anti-human CD25 conjugated to FITC, anti-human CD3 conjugated to PerCP, anti-human CD4 and anti-human CD8 conjugated to allophycocyanin, anti-human CD25 and anti-human CTLA-4 conjugated to PE. The following mAbs were purchased from eBioscience: anti-human IL-17 and anti-human IL-2 conjugated to PE, anti-human IFN γ , anti-human TNF α and anti-human CD4 conjugated to PB, anti-human CD127 conjugated to PeCy7, and anti-human FoxP3 conjugated to allophycocyanin. Isotype-matched controls were purchased from eBioscience or BD Biosciences as appropriate and used to account for non-specific binding. 2% Goat serum (Sigma, USA) was used to block non-specific binding. Stains were obtained from BD bioscience.

Ex vivo T reg detection by FACS

After plating out the cells as shown above, the remaining cell / media mix was distributed into FACS tubes with a volume of 100-200 μ l per tube.

Surface staining on live cells was then carried out. PBS 1ml was added to each FACS tube and centrifuged at 1500rpm for 5 minutes. The supernatant was tipped off and excess blotted onto tissue.

45 μ l of 2% goat serum / PBS to each tube (980 μ l PBS +20 μ l goat serum) was added to each tube and antibodies against surface markers CD3, CD4, CD25 and CD127 were then added to stain the live cells (table 2.10). The cells were vortexed gently and left on ice for 30 minutes.

The cells were then washed with PBS 1 ml, centrifuged at 1500 rpm for 5 minutes and the supernatant tipped off. This wash step was repeated once more and 500µl of (FoxP3) fix/perm buffer prepared by 1:3 mix of concentrate (brown bottle) 1.25ml with diluent (colourless bottle) 3.75µl was added to each tube. The tubes were then covered with foil and placed in the refrigerator overnight.

The following day the cells in the FACS tubes were washed with 1ml PBS (added directly to the 500µl of fix/perm buffer) and centrifuged at 1500rpm for 5 minutes. They were then washed once with 1X permeabilisation buffer (prepared from 10X stock by diluting with PBS, 1ml + 9ml PBS) and centrifuged again at 1500rpm. The supernatant was tipped off and the antibodies and goat serum were added as per table 2.10.

The cells were incubated in the refrigerator, covered with silver foil for 45 minutes. 1ml of permeabilization buffer was added to wash and the cells centrifuged at 1500rpm for 5 minutes and the supernatant tipped off. The cells were then washed twice more with 1ml PBS as above and resuspended in 200ul of PBS. The tubes were then covered and stored in the refrigerator until ready to FACS.

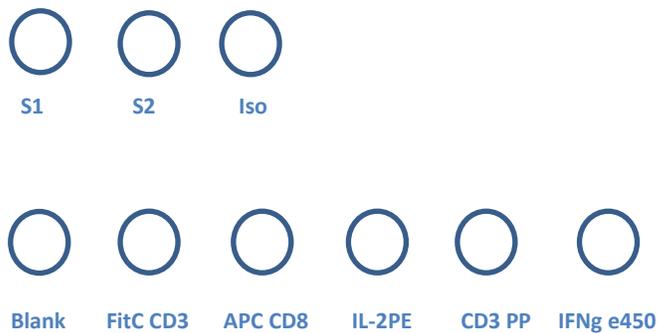
The same process was used for T reg analysis post stimulation, adding the antibodies after fixation and permeabilisation. The staining panel used is shown in Table 2.12.

Assessment of cytokine production by FACS

After 24 hours incubation, unstimulated cells were treated with PMA (Phorbol 12-Myristate 13-Acetate) at a concentration of 50ng/ml stock and ionomycin at 1µM (Sigma Aldrich, USA). After

90 minutes Brefeldin A (cytokine secretion blocking agent) was added at a concentration of 10µg/ml and incubated for a further four and a half hours at 37°C.

200µl of cells was then re-suspended and transferred into labelled FACS tubes for fixation and staining as shown below.



Each FACS tube was centrifuged at 1500rpm for 5 minutes and the supernatant tipped off. The cells were then resuspended in 500µl of 3% PFA and incubated at room temperature for 12 minutes. The FACS tubes were then centrifuged at 1500rpm for 5 minutes and the supernatant tipped off. This process was repeated, washing once with PBS 1ml and finally the 200µl of PBS was added to each tube. Cells were then washed with 0.1% saponin and antibody mastermix (as shown in table 2.11). After resting at room temperature for 30 minutes the cells were washed once more with 0.1% saponin and twice with PBS and then resuspended in 300µl of PBS.

Treg cells ex-vivo					
Stain 1		Stain 2		Isotype Control	
CD25-PE	4	CTLA-4-PE	3	IgG-PE	4
FoxP3-APC	2.5	FoxP3-APC	2.5	IgG-APC	2.5
CD3-PerCP	1.5	CD3-PerCP	1.5	CD3-PerCP	1.5
CD4-e450	0.6	CD4-e450	0.6	CD4-e450	0.6
CD127-PeCy7	1.5	CD127-PeCy7	1.5		
2% goat serum-PBS	45	2% goat serum-PBS	45	2% goat serum-PBS	45

Table 2.10 Staining panel for Treg cells ex-vivo.

For markers shown in bold in table 2.10, antibodies were added after permeabilization directly to the cell pellet.

Cytokine production ex-vivo					
Stain 1		Stain 2		Isotype Control	
CD4-FITC	3	CD4-FITC	3	CD4-FITC	3
IL-17-PE	0.6	IL-2-PE	0.6	IgG-PE	0.6
IFN γ -e450	0.8	TNF α -e450	2	IgG-e450	0.8
CD3-PerCP	1.5	CD3-PerCP	1.5	CD3-PerCP	1.5
CD8-APC	2.5	CD8-APC	2.5	CD8-APC	2.5
2% goat saponin	41.6	2% goat saponin	40.4	2% goat saponin	41.6

Table 2.11 Staining panel for cytokine production ex-vivo.

CD25, CTLA-4 and FoxP3 expression post stimulation			
Stain		Isotype Control	
CD25-FITC	2	CD25-FITC	2
CTLA-4-PE	1	IgG-PE	1
CD3-PerCP	1.5	CD3-PerCP	1.5
CD4-e450	0.6	CD4-e450	0.6
FoxP3-APC	1	IgG-APC	1
Goat serum	3	Goat serum	3

Table 2.12 Staining panel for CD25, CTLA-4 and FoxP3 expression post stimulation.

Cytokine production post stimulation

300µl of supernatant from each condition (stimulation with SEB/ PPD / Vitamin D) was pipetted into individual labelled eppendorfs and frozen at -80°C for analysis by ELISA of cytokine production including IL-2, TNF α , IFN γ (all from Ebioscience, USA) and IL-17 (R&D system, Quantikine ELISA, USA).

	IL17	IL2	IFNγ	TNFα
Un-stimulated	non-diluted	non-diluted		
SEB/untreated		1:100	1:200	1:100
SEB/1,25D₃		1:50	1:50	1:100
SEB/25D₃		1:50	1:50	1:100
PPD/untreated		1:10	1:10	1:10
PPD/1,25D₃		1:10	1:10	1:10

Table 2.13 Dilution factors for cytokine ELISAs

Dilution factors for the cytokine ELISAs indicated in the table were determined in optimisation experiments (carried out as part of a BMed Sci project). IL17 ELISAs were measured undiluted whereas IL2, IFN γ and TNF α were all diluted according to the table above.

2.4 Statistics

All statistics were carried out in SPSS version 18.0. Data normality was assessed using the Kolmogorov-Smirnov test, and for normally distributed data mean and standard error (SEM) calculated. For non-normal data median and inter-quartile range (IQR) was calculated. Mann Whitney test was used for independent groups which were not normally distributed and Wilcoxon signed ranks test was used to compare data in the same group when conditions were applied (e.g. administration of vitamin D between 0 and 8 week). Kruskal Wallis Test was used to determine if there were statistically significant differences between two or more groups of an independent variable. Correlation was assessed using Spearman's rank correlation coefficient.

CHAPTER 3

The Role of Genetic Polymorphism, baseline vitamin D and other elements of the vitamin D axis in determining response to supplementation.

3.1 Results

3.1.1 Recruitment, Exclusions and Demographic Data

Forty nine patients with positive smear for acid fast bacilli, positive culture for *Mycobacterium tuberculosis* or granulomatous changes on histopathology of a biopsy sample were recruited.

Six patients were then excluded from the study due to various reasons including absence at follow up appointment due to deportation from the country and emigration, adverse drug reaction to TB medications with decision to discontinue trial by patient, multidrug resistant TB on culture and a subsequent malignancy diagnosis. Forty three patients' vitamin D levels were therefore analysed at Week 8.

At week 16 thirty five patients remained in the study. Further exclusion of six patients was due to non-attendance (n=6), growth of *Mycobacterium kansasii* (n=1), patient decision to leave the trial (n=1). Two patient's vitamin D levels failed to produce a result in the laboratory, resulting in the analysis of thirty three patients' vitamin D levels at Week 16.

At week 24 thirty patients remained in the study. Exclusions were due to non- attendance (n=5) and a further two patients vitamin D levels were not obtained from the laboratory sample sent, resulting in vitamin D analysis of twenty eight patients.

This indicates the difficulty in this population with recruitment and adherence for the duration of the trial.

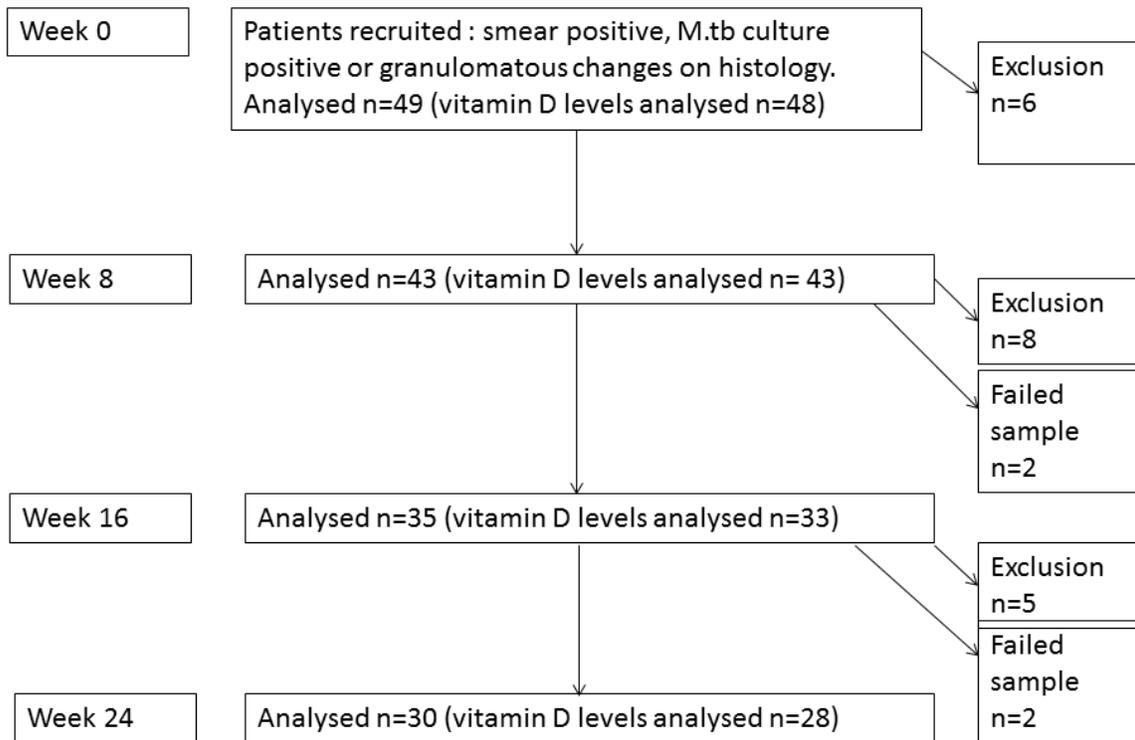


Figure 3.1 Recruitment, exclusion and dropout during the study.

This figure shows the number of patients analysed at each time interval during the study. The numbers fell from 49 initially recruited to 30, of whom only 28 patients vitamin D levels could be analysed. The main reason for this was due to dropout of patients from the study / non-attendance in clinic.

Study Population and baseline characteristics

Gender	
Male	33
Female	16
Age	30 (15.16, 21)
Ethnicity	
Caucasian	7
Indian subcontinent	30
Black African	7
Middle Eastern	1
SE Asian	1
Caribbean	3
Site of Disease	
Pulmonary	35
Extrapulmonary	14
TB Score	3.8 (0.35)
Vit D Level nmol/l	12.05 (20.86, 14.6)

Table 3.1 Demographics of the study population.

Data is presented as mean (standard error of the mean) or median (standard deviation, interquartile range) dependent on its distribution. The majority of patients were male and had pulmonary tuberculosis. The median baseline vitamin D levels were deficient at 12.05nmol/l, a finding supported by previous systematic reviews.

The majority of patients were male 33/49 (67%) with a mean age of 35 years. 61.2% of the group were from the Indian Subcontinent (India / Pakistan) with just one person each from the Yemen and Thailand.

71.4% of the group had pulmonary (pulmonary or pleural) tuberculosis (n=35) with the remainder falling into the extrapulmonary category (n=14), which included bony tuberculosis (n=2), lymph node tuberculosis (n=9), soft tissue abscesses (n=1), nasal tuberculosis (n=1) and gastrointestinal tuberculosis (n=1).

The median vitamin D level was 12.1nmol/l which is severely deficient.

The median TB score was 4.0 (maximal score of 13).

Microbiology Results

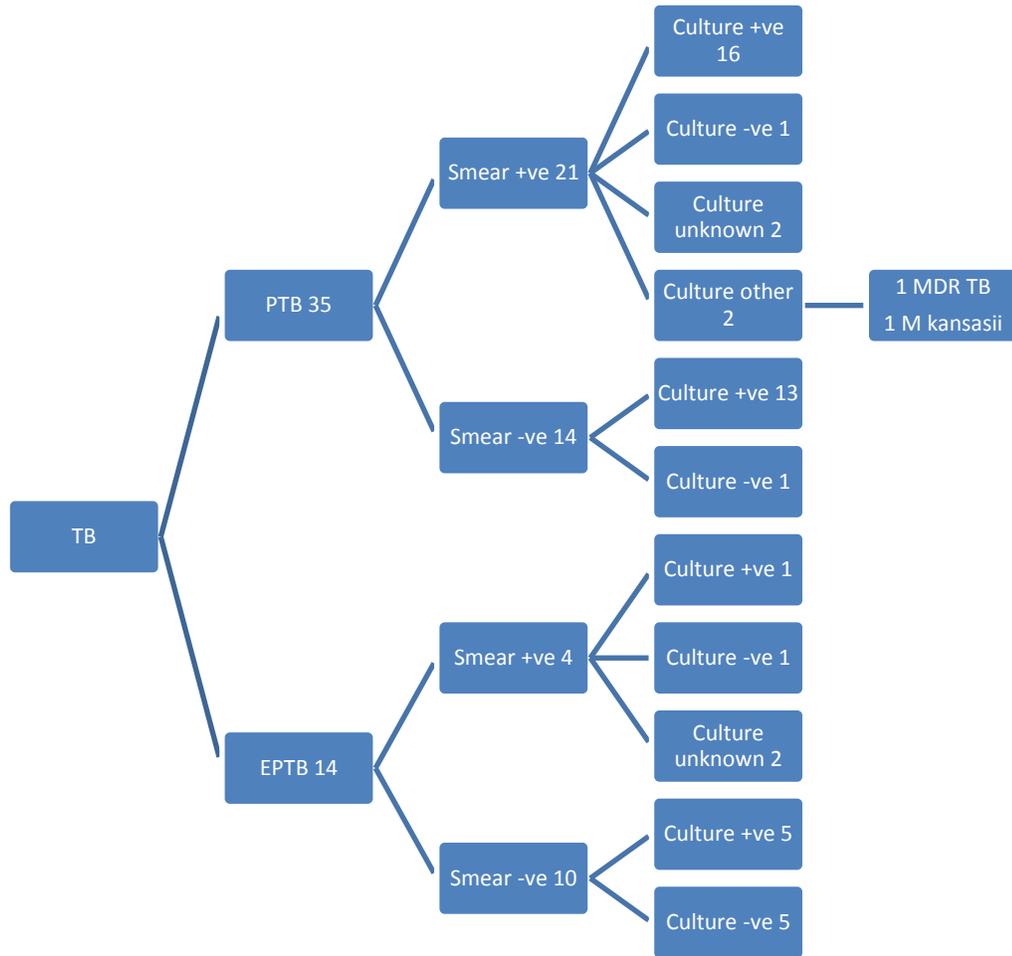


Figure 3.2 Microbiology results of the study population.

The figure shows the number of patients with pulmonary TB (PTB) and extrapulmonary TB (EPTB) and the smear and culture results for each group. The majority (31/35) of PTB patients eventually cultured positive for M.tb regardless of their initial smear status.

Figure 3.2 shows the microbiology results of the pulmonary and extrapulmonary tuberculosis groups. After the maximal culture time of ten weeks the pulmonary TB group positively cultured in 31 of the 35 patients with two of the positive cultures being due to one multi-drug resistant TB patient and one *Mycobacterium kansasii* infection (a non-tuberculous mycobacterial infection). These two individuals were excluded at this point from the trial.

In the extrapulmonary TB group (n=14), eight of whom had lymph node TB, six were culture positive for M.TB and six culture negative with two unknown results. This is not unusual given that extrapulmonary tuberculosis such as lymph node TB tends to culture less easily than cavitary pulmonary tuberculosis. Patients with smear or culture negative biopsies still remained eligible for the study however as all those who had negative microbiology were required to fulfil the inclusion criteria with histological confirmation of necrotising granulomatous changes as stated in the methods section.

As a group, there was no significant effect of baseline vitamin D levels or the severity of vitamin D deficiency on sputum conversion i.e. those with severe deficiency did not have significantly different sputum conversion rates. However the numbers were very small and therefore analysis of genotype effect was not carried out further. Neither was there any relationship between baseline vitamin D level and smear positivity at baseline.

Site of Infection and Vitamin D levels

The pulmonary and extrapulmonary groups were analysed using Mann Whitney-U test to determine if there was any difference in the baseline and subsequent vitamin D levels according to the site of disease. There was no statistically significant difference in the vitamin D levels in the pulmonary and extrapulmonary groups ($p>0.05$) at the four time intervals during the study.

Season and Baseline Vitamin D levels

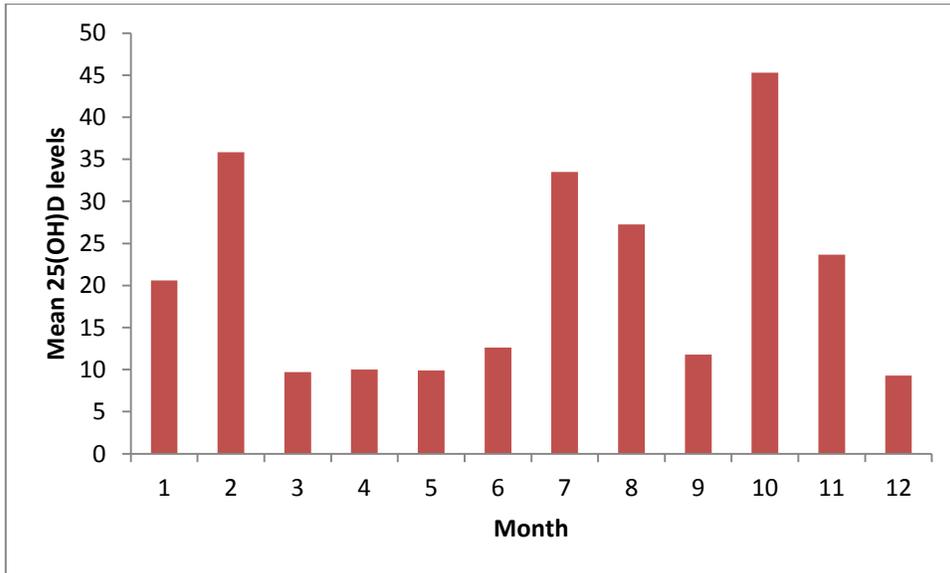


Figure 3.3 Mean baseline vitamin D levels for patients according to season of recruitment.

Although mean vitamin D levels appear to vary with month of blood draw in the above figure, using Kruskal Wallis test there is no significant difference in the vitamin D levels at baseline of the study participants ($p=0.273$) according to month of recruitment, i.e. season did not affect baseline vitamin D levels when patients were analysed within these groups.

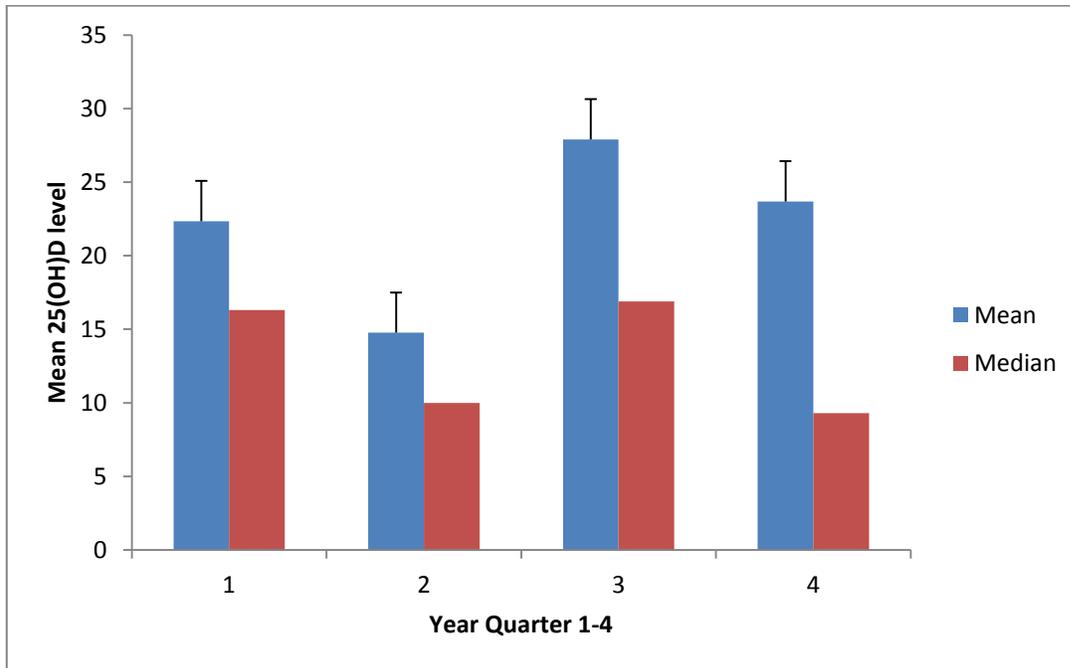


Figure 3.4 Mean and median baseline vitamin D levels according to yearly quarter of recruitment. Quarter 1 Jan-March, quarter 2 April-June, quarter 3 July-September, quarter 4 October –December.

Although baseline vitamin D levels in this study did not appear to vary with month / season of blood draw, there have been examples of studies which have shown a seasonal tendency of TB which suggest that the number of TB cases is related to hours of sunshine [197]. This would fit with peaks of TB around six months after the winter dips in sunshine.

Ethnicity and Baseline vitamin D levels

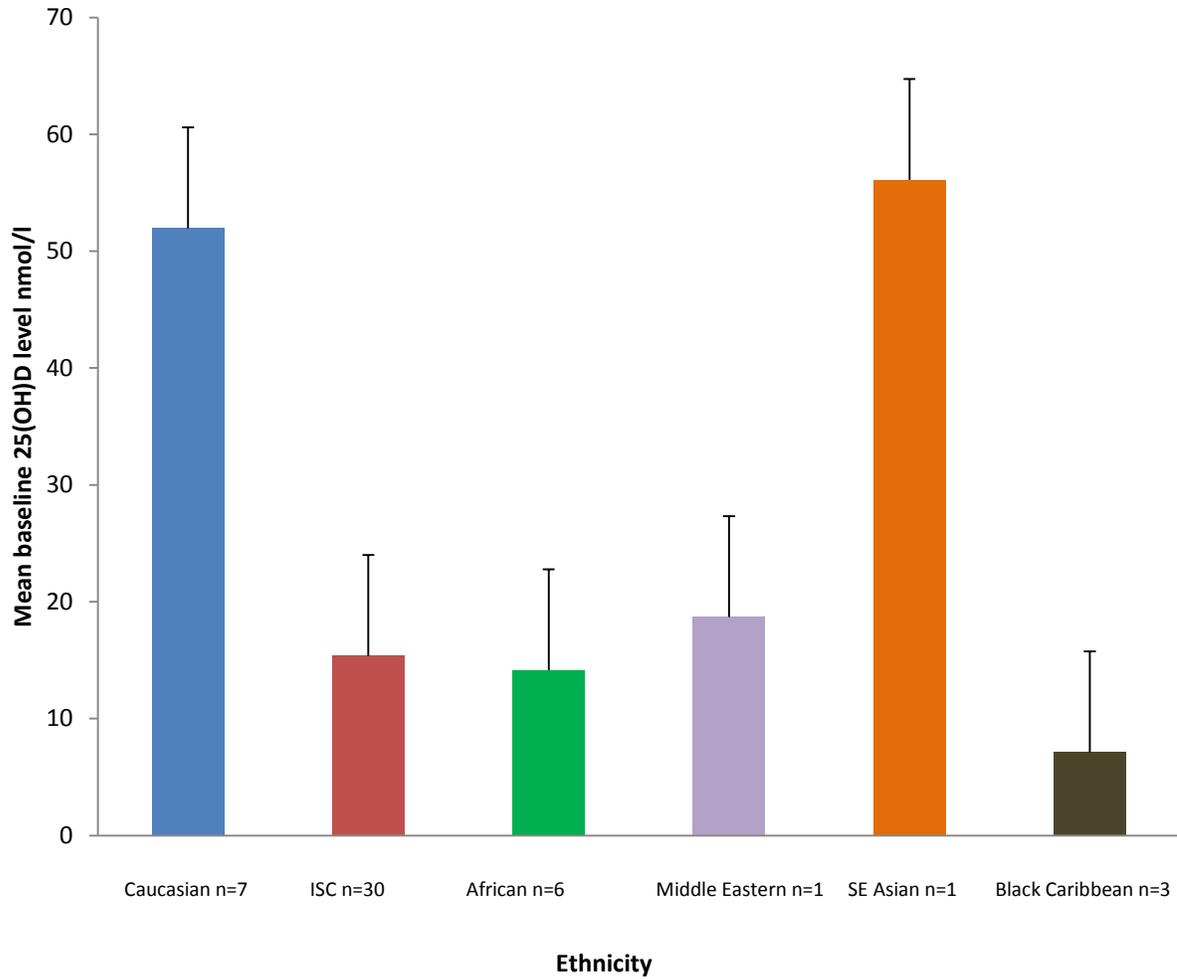


Figure 3.5 Mean baseline vitamin D levels and ethnicity.

(n=48, Standard Error of mean is shown). The largest ethnic group in the cohort included patients from the Indian Subcontinent. It was noted in post hoc analysis that there was a significant difference in vitamin D levels between different ethnicities in the cohort.

At baseline there was a significant difference in vitamin D levels between ethnic groups (Kruskal Wallis test $p=0.009$). Post-hoc analysis of the groups using Mann Whitney-U test showed that baseline 25(OH)D levels were significantly higher ($p<0.05$) in the Caucasian group compared to the Indian Subcontinent (ISC), African and Black Caribbean groups.

3.1.2 Genetic Polymorphisms and baseline vitamin D in determining response to Vitamin D supplementation

Vitamin D status

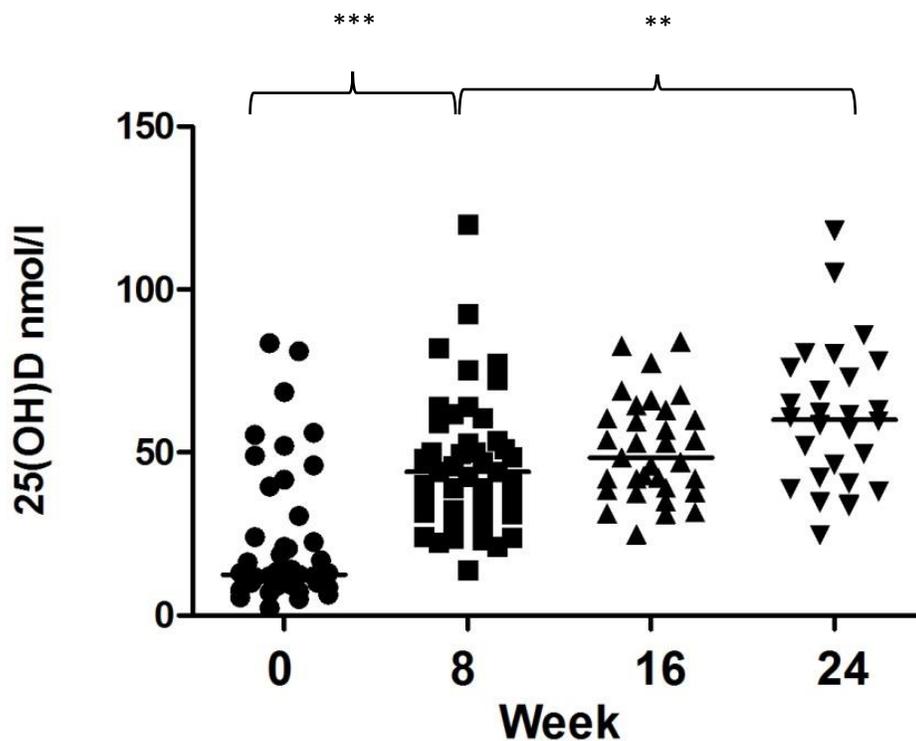


Figure 3.6 25(OH)D levels (nmol/l) at Week 0, 8, 16 and 24.

This figure illustrates vitamin D levels (and median lines) at each time interval. There is a statistically significant rise in vitamin D levels with supplementation during the study. *** $p < 0.0001$, ** $p < 0.05$. Most patients achieve sufficiency (>50 nmol/l), however it is clear that this is not the case for the entire group.

Figure 3.6 shows the vitamin D levels of individuals at Week 0, 8, 16 and 24. Wilcoxon ranks test shows that there is a significant rise in the 25(OH)D levels between weeks 0 and 8 and thereafter there is a steady rise which is also significant from baseline.

Sufficiency was determined as 25(OH)D levels ≥ 50 nmol/l. At 8 weeks 17/43 (39.5%) achieved sufficiency. At 16 weeks 17/33 (51.5%) achieved sufficiency and at 24 weeks 19/28 (68%) achieved sufficiency. Note that the numbers in the groups decline due to exclusions at the respective time periods.

There was no significant difference in baseline CRP, DBP or LL37 levels in those who had 25(OH)D levels ≤ 20 nmol and those with levels >20 nmol/l.

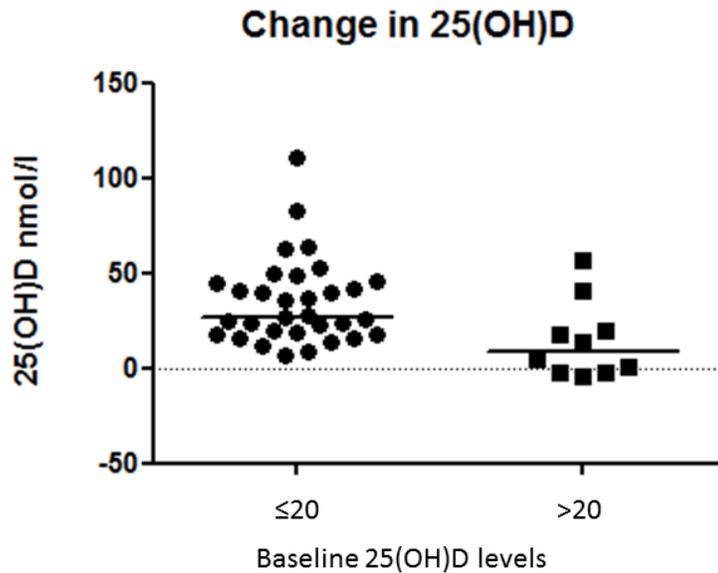


Figure 5.7 Change in 25(OH)D levels with baseline vitamin D status.

Patients with vitamin D deficiency ($< 20 \text{ nmol/l}$) had a greater rise in vitamin D levels with supplementation at week 8. i.e. those with lower levels had greater response.

There was a significantly greater rise in vitamin D levels between weeks 0 and 8 in those patients who had 25(OH)D levels $\leq 20 \text{ nmol/l}$ at baseline than those whose levels were $> 20 \text{ nmol/l}$, (** p = 0.009). This indicates that those who are severely deficient respond more vigorously to supplementation than those who are deficient.

Effects of Single Nucleotide Polymorphisms (SNPs) in the Vitamin D Axis.

The following SNPs were analysed :

GC: rs7041, rs4588, rs2070741, rs2298849, rs3755967, rs1352844, rs222035, rs2282679.

CYP27B1 rs10877012 (unable to analyse due to technical error – unable to identify VIC / FAM marker), rs4646536.

CYP2R1 rs10741657

DHCR7 (rs12785878

IL6 rs1800796

VDR rs2228570, rs1544410

From each of these SNPs, further breakdown of allele carriage was performed as per table 3.2 for further analysis.

Gene	SNP	Allele carriage/ haplotype carriage
GC	rs7041, rs4588, rs2070741, rs2298849, rs3755967, rs1352844, rs222035, rs2282679	T allele carriage, TT homozygous rs7041, CC homozygote rs4588, GC1S carriage, GC2 carriage, GC2-2 carriage, GC1S-1S carriage, C allele rs2298849, C allele rs2282679,
CYP27B1	rs10877012, rs4646536	CC homozygotes rs4646536, TT homozygotes rs 4646536
CYP2r1	rs10741657	A allele rs10741657, GG homozygote rs10741657
DHCR7	rs12785878	T allele carriage rs 12785878, GG homozygote rs 12785878
IL6	rs1800796	GG homozygote rs1800796
VDR	rs2228570, rs1544410	CC homozygote rs 2228570, T allele rs2228570, G allele rs1544410, AA homozygote rs1544410

Table 3.2 Genes, single nucleotide polymorphisms (SNPS) and allele / haplotype used for statistical analysis.

Key enzymes were identified through extensive literature searches and vitamin D / TB related SNPs were chosen to optimise the possibility of identifying relevant polymorphisms.

GC genotyping results

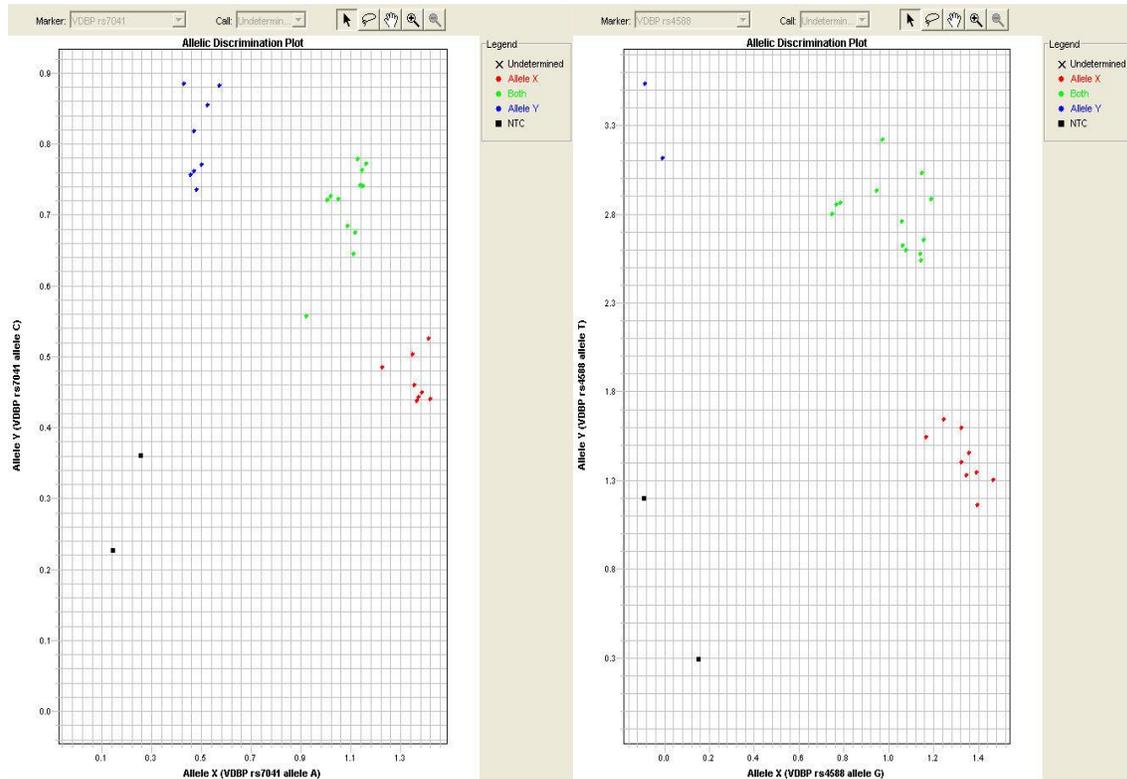


Figure 3.8 Allelic Discrimination Plots.

The above figure exemplifies the use of the allelic discrimination plots at SNPs rs7041 and rs4588. These figures are produced through TaqMan@SNP genotyping as described in section 2.3.5. PCR results in the relative expression of each fluorophore, the results of which are represented in a scatterplot. The four potential clusters of points correspond to the two homozygote genotypes or the heterozygote genotype (or no amplification). The combination of the above 2 SNPs enabled the GC genotype to be determined and the results are shown in table 3.3 below.

<u>GC haplotype</u>	<u>TB patients (n=43)</u>	<u>25(OH)D level nmol/l</u>	<u>Ethnic origin</u>
GC1F-1F	2	10.7 (1.05, -)	Caribbean
GC1S-1S	14	17.5 (25.2, 37)	Caucasian, ISC, African Middle Eastern.
GC2-1S	13	10.5 (13.4, 11.2)	Caucasian, ISC, African
GC2-1F	7	27.2 (11.7)	Caucasian, ISC, African, Caribbean.
GC2-2	4	12.88 (4.8)	Caucasian, ISC, African
GC1F-1S	3	12.1 (5.4)	ISC, African

Table 3.3 GC haplotype of TB patients with mean and median vitamin D levels.

This table shows the GC haplotype groups which were identified in the TB population. 43 of the 48 individuals were included in the study. Five of the patients were not analysed due to insufficient genetic material or failure of the assay in discriminating the allele. Data is presented as mean (standard error of the mean) or median (standard deviation, interquartile range) dependent on its distribution.

Analysis of DBP genotype using Mann Whitney showed that there was a significantly greater rise in DBP levels between weeks 0 and 8 in rs7041 TT homozygotes ($p=0.048$). There were no other correlations found for any of the other SNPs or genotypes analysed.

Further analysis of the DBP and CRP levels showed no correlation between these measures at week 0 or 8 and there was no significant correlation between change in DBP or CRP between week 0 and week 8, indicating that resulting changes in levels are not reflective of an interaction between the two e.g. change in inflammatory protein production with change in inflammatory response.

Haplotype analysis of DBP levels and mean levels for the groups with adequate numbers for analysis are shown in Figure 3.9 below.

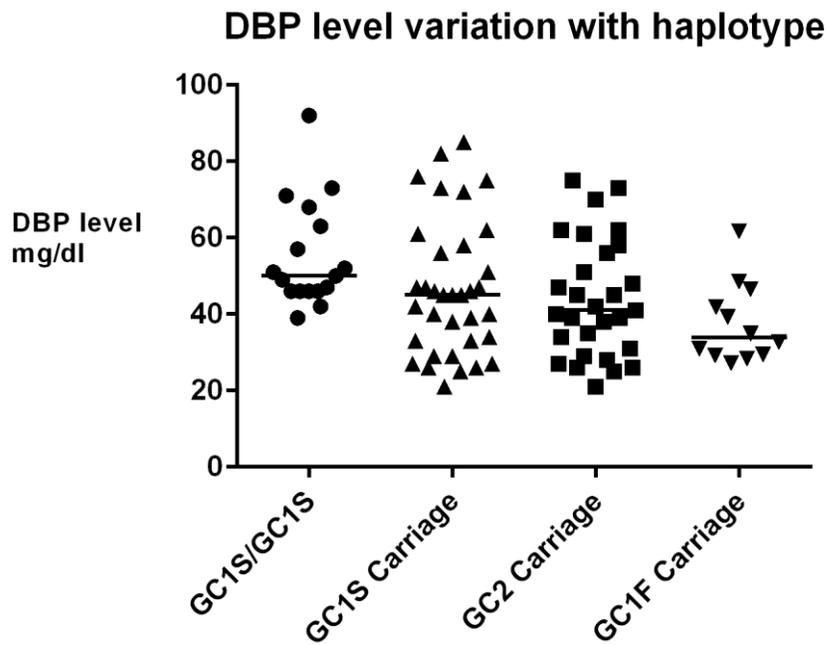


Figure 3.9 Vitamin D binding protein level variation with haplotype at Week 0.
 $p=0.02$ analysed with one way Anova. Haplotype of DBP appears to affect the DBP levels. This may reflect that genotypes with the greatest affinity for vitamin D require lower amounts of the protein to produce the same effect in terms of vitamin D transportation, compared to those genotypes with lower vitamin D affinity. GC1F is known to have greater affinity for vitamin D than either GC1S or GC2.

For GC (rs7041, rs4588, rs2070741, rs2298849, rs3755967, rs1352844, rs222035, rs2282679), Mann Whitney U tests were used to determine if there is any association of the above SNPs with baseline vitamin D levels. There was no significant difference in baseline vitamin D levels and carriage of GC1s-GC1s, GC2 or GC1s.

Response to vitamin D appears to vary with genotype. Carriage of CC allele of GC rs 4588 (p=0.048) was also found to have a significant association with change / increase in levels between 0 and 16 weeks but this was not maintained at week 24, possibly due to a reduction in numbers analysed at this time point.

Further analysis of GC genotype and vitamin D levels during the study are summarised in the table below. This analysed the carriage of 3 or more major alleles combinations (named WT+) from rs7041/rs4588 combination and whether this had any significant influence on vitamin D levels at each time interval when compared to those who carried <3 major alleles (WT-) in their genetic specification of GC.

Vit D SNP	Baseline (n=)	Baseline 25(OH)D median (mean)	Week 8 (n=)	Week 8 25(OH)D	Week 16 (n=)	Week 16 25(OH)D	Week 24 (n=)	Week 24 25(OH)D
<u>Rs7041</u>	17	12.1 (25.8)	15	44 (44.4)	10	53.4 (54.2)	10	53.4 (57.7)
Major homozygote (GG)								
Heterozygote	17	12.1 (23.9)	16	41.8 (48.4)	13	42 (49.6)	12	60.9 (62.4)
Minor homozygote (TT)	12	11.8 (21.2)	9	42.5 (45.7)	8	45 (44.5)	4	57.6 (55.6)
<u>Rs 4588</u>	20	12.6 (23.7)	18	43.3 (47.9)	13	53.8 (57.2)*	11	57.2 (62.1)
Major homozygote (CC)								
Heterozygote	19	11.0 (20.0)	17	41.0 (41.1)	14	42.5 (44.6)	12	59.0 (57.6)
Minor homozygote (AA)	4	14.5 (12.9)	3	35.5 (37.0)	2	34.8 (35.0)	1	63.1 (63.1)
WT+	19	13.0 (17.9)	17	39.1 (40.8)	12	53.0 (53.0)	11	49.5 (59.7)
WT-	24	12.6(14.8)	21	39.0 (42.2)	17	47.1 (45.9)	13	63.1 (62.5)

Table 3.4 Median and (mean) values for 25(OH)D according to GC genotype rs7041 and rs4588 at various time intervals.

Wild type + (carriage of ≥ 3 major alleles in the rs4588 and rs5041 allelic combination) or wild type - (carriage of < 3 major alleles) is shown with the corresponding median (mean) vitamin D levels.

WT wild type.

*P=0.014 (carriage of CC homozygote rs 4588 results in greater vitamin D level).

VDR (rs2228570, rs1544410).

Carriage of rs2228570 T allele (VDR Fok1) was associated with a significantly higher level of vitamin D at baseline (week 0) (mean rank 25.83 vs 17.57, $p=0.029$) i.e, **baseline vitamin D levels appears to vary with genotype.**

Response to vitamin D appears to vary with VDR genotype. VDR rs2228570 CC homozygotes had greater change in vitamin D levels between 0 and 8 weeks ($p=0.026$ Mann Whitney).

Other SNPs (CYP27B1 rs4646536, CYP2R1 rs10741657, DHCR7 rs12785878 and IL6 rs1800796).

No other allele carriage in the various SNPs listed was associated with baseline vitamin D levels. However, **response to vitamin D appears to vary with genotype.** With regard to the effect of genotype on change in vitamin D with supplementation, DHCR7 rs12785878 GG homozygote had greater change in vitamin D levels between 0 and 8 weeks ($p=0.029$ Mann Whitney).

A negative correlation (i.e. non carriage of the following genotypes was associated with greater interval vitamin D change) was noted at 8 and 16 weeks with carriage of cyp27b1 rs4646536 CC homozygote and at 8 weeks with carriage of rs2228570 T allele as did DHCR7 rs12785878 T allele carriage at 8 weeks ($p=0.029$).

However there does not appear to be a relationship between genotype and achieving vitamin D sufficiency (>50nmol/l) when measured at 8 and 16 weeks.

Due to small numbers of patients reaching the final 24 week time point, analysis of genotype and vitamin D levels have not been done in this group. For the same reason, further analysis of severe vitamin D deficiency was not further analysed against genotype. The results are likely to be affected by the small numbers.

3.1.3 Effect of Baseline Vitamin D and Other Elements of the Vitamin D Axis on Clinical Outcome / Antimicrobial Response.

Vitamin D Binding Protein Levels

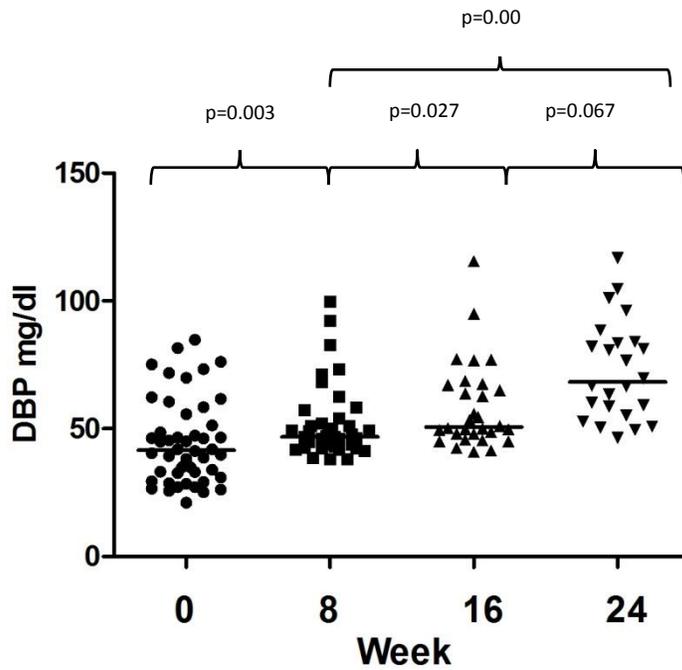


Figure 3.10 Vitamin D binding protein levels (mg/dl) at Week 0, 8, 16 and 24.

Vitamin D binding protein levels increase at each time interval during the study, although no direct correlation was noted with vitamin D levels during the study i.e. the availability of vitamin D does not significantly affect the circulating DBP levels.

The levels of vitamin D binding protein rose between weeks 0 and 24. Using Wilcoxon Ranks test it can be seen that there is a significant difference in levels of DBP at each time interval. This does not reach significance between weeks 16 and 24, but does so between weeks 0 and 24 and it is clear that there is a steady increase nevertheless.

Using both Kruskal Wallis and Mann Whitney tests, given that the DBP data had a non-normal distribution, it was determined that neither age, gender or ethnicity affect DBP levels and therefore multivariate analyses were not further performed.

LL37 levels

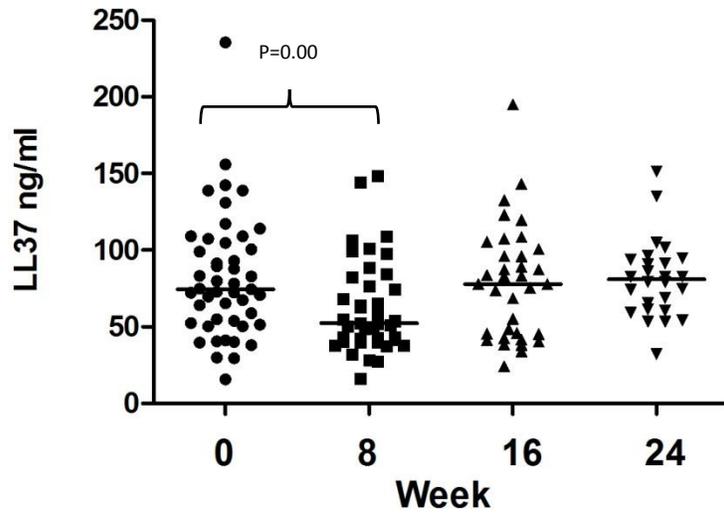


Figure 3.11 LL37 levels at Weeks 0 (n=49), 8 (n=38), 16 (n=34) and 24 (n=26) in TB patients. Levels of LL37 fell significantly by week 8, with a return to baseline levels thereafter. There is no correlation with vitamin D levels in the study. Total systemic LL37 levels however, may not reflect localised autocrine levels which may be a more important reflection of antimicrobial activity.

There was a statistically significant fall in the LL37 levels between week 0 and 8 but this did not persist at weeks 16 and 24 and the levels returned to a levels comparable with the baseline. The Wilcoxon Ranks test takes into account the dropouts during the study.

It may be expected to see a fall in the LL37 levels in the initial treatment period as the inflammatory component lessens with treatment but the reason for the return to baseline levels is not clear.

Analysis of baseline LL37 and vitamin D levels show that there is no correlation between these parameters. This concurs with previous findings by Yamschchikov [64].

Neither was a relationship identified between LL37 levels and severity of vitamin D deficiency i.e. in those patients with levels of 25 (OH)D₃ <20nmol/l, the LL37 levels were not statistically different from those with 25(OH)D₃ levels ≥20nmol/l (Mann Whitney test).

Further analysis of LL37 levels and other variables showed that there was no relationship between smear positivity, gender, TB score (Mann Whitney) or ethnicity (Kruskal Wallis test) at baseline and LL37 levels.

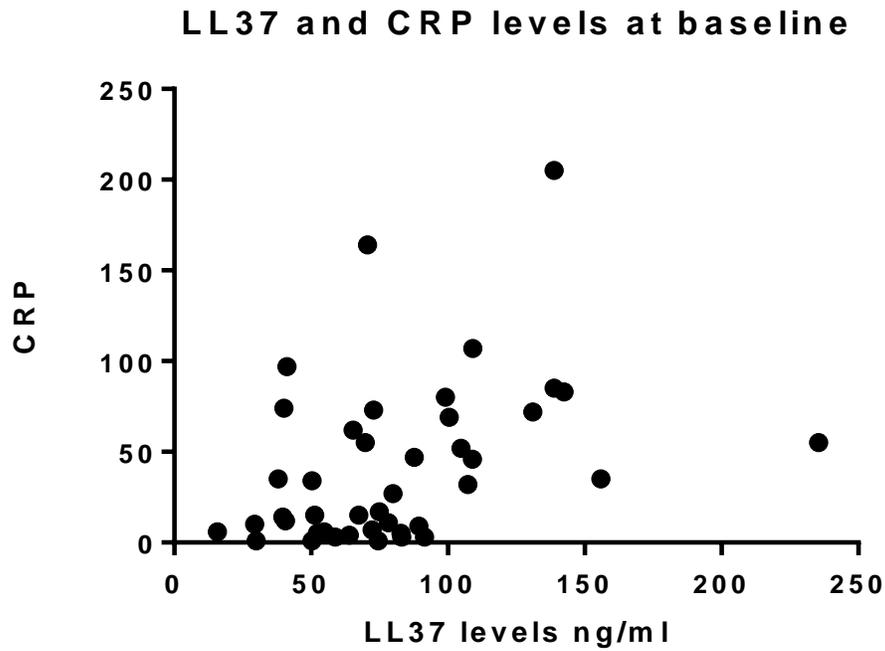


Figure 3.13 Baseline LL37 and CRP levels

There is a positive correlation ($p=0.06$) between LL37 and CRP level at baseline. Rising CRP is reflected by greater LL37 levels, which may be a result of disease activity at baseline, prior to TB treatment. However the relationship persists to week 16.

Baseline LL37 levels appeared to have a positive correlation with baseline CRP levels ($p=0.06$) and this relationship appears to continue at weeks 8 ($p=0.024$) and at week 16 ($p=0.006$) but is lost at week 24. There was also a correlation with total white cell count (WCC) at week 0, (Figure 3.14), however this relationship was again lost at weeks 8-24. This may indicate that the greatest levels of inflammation prior to TB therapy results in a rise in all inflammatory markers which would be expected. However, it would also be expected that as inflammation reduces with treatment, all markers would do so, this does not appear to be the case however, with LL37 declining by week 8 but rising again to levels similar to baseline for the remainder of the study time.

LL37 levels and total WCC at baseline

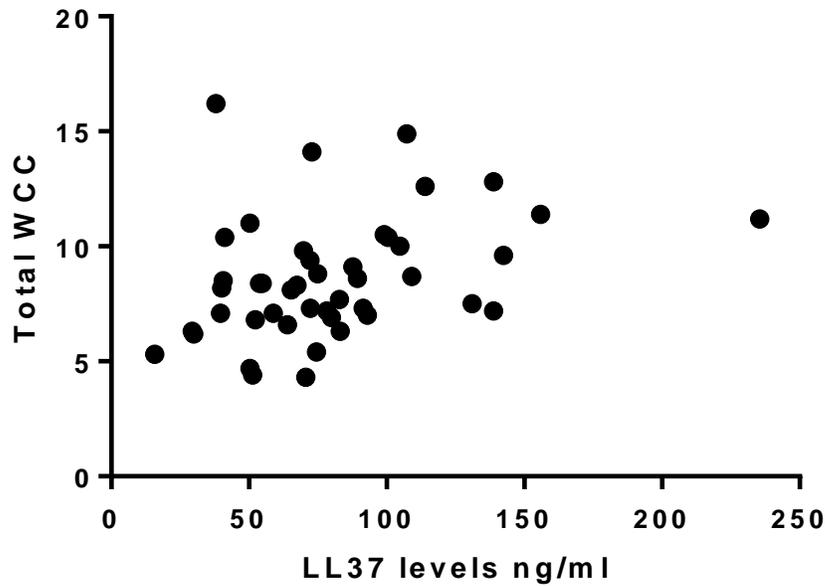


Figure 3.14 Baseline LL37 level and total white cell count.

There is a significant correlation between the LL37 levels and total white cell count at baseline. (Spearman test $p=0.004$). White cells are a source of LL37 and may reflect the conditions of inflammation. Consistent findings are shown below in Figure 3.15 which show further correlations of LL37 with lymphocyte / monocyte ratios in the study population.

The positive correlation between LL37 levels and total WCC persists at week 8 ($p=0.007$) but is lost at weeks 16 and 24. The correlation does not follow for total lymphocyte count but there is a statistically significant negative correlation between the lymphocyte / monocyte ratio and LL37 levels ($p=0.006$) (Figure 3.15), with greater LL37 levels correlating to lower L/M ratio.

Lymphocyte / Monocyte ratio and LL37 levels at baseline.

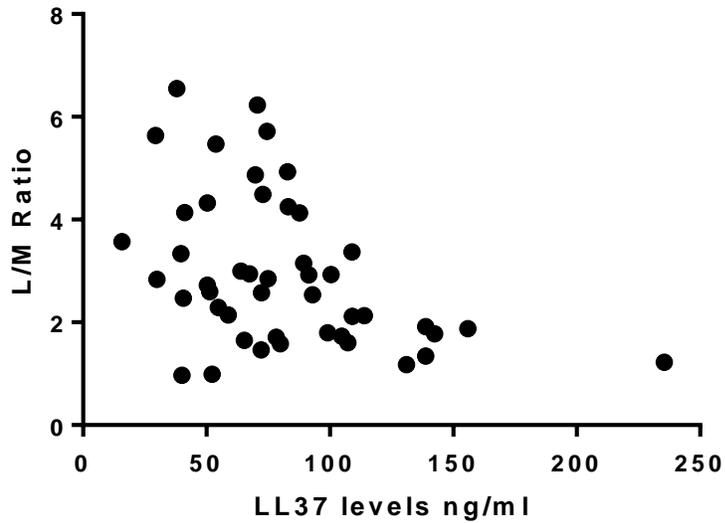


Figure 3.15 LL37 levels and lymphocyte / monocyte ratio at baseline.

($p=0.006$ Spearman Correlation test at baseline). There is a weakly negative correlation between LL37 levels and lymphocyte / monocyte ratio at baseline with $r_s = -0.398$. Rising lymphocyte monocyte ratio levels during the study are shown below in figure 3.18 and reflect a recovery in lymphopenia and decline in monocytosis with treatment.

TB Score

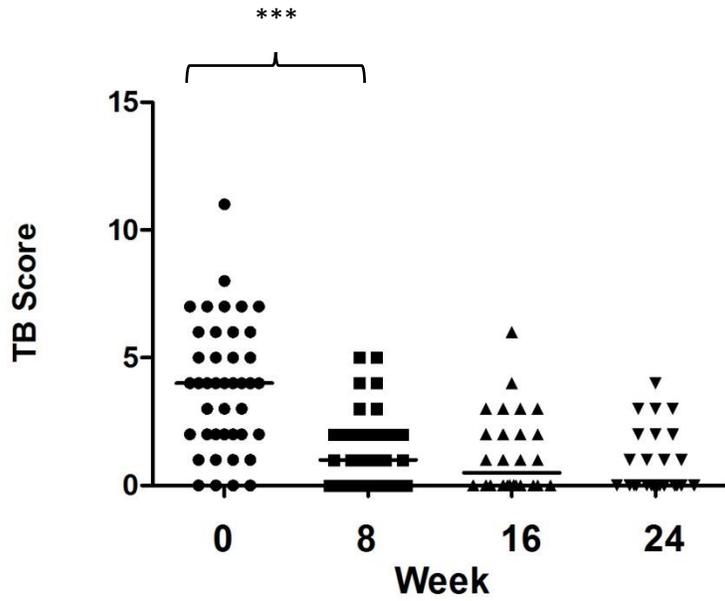


Figure 3.16 The TB score (maximum 13) at Week 0 to 24.

*** $p < 0.0001$ There is a significant reduction in TB score (TB score 4 at week 0, TB score 0.5 at weeks 8). Further breakdown of the cohort showed that those with extra-pulmonary disease had lower TB score than those with pulmonary disease (median 2.0 vs median 5.0).

There is a significant reduction in the TB score between Week 0 and 8 (median 4.0 at week 0, median 0.5 at week 8) and remains at a similar level thereafter. The median TB score at baseline appears to be associated with a greater inflammatory process and patients with TB Score ≥ 4 have a significantly higher CRP value (Mann Whitney U test $p < 0.005$). The TB score is significantly lower in patients with extrapulmonary TB (median 2.00) than those with pulmonary TB (median 5.00) ($p = 0.00$).

The relationship between vitamin D levels and TB score was analysed. No significant correlation was found between baseline vitamin D levels and TB score < 4 (i.e. those with less severe disease do not appear to have significantly different vitamin D levels to others). Those with severe deficiency i.e. $< 20 \text{ nmol/l}$ did not have statistically significant different change in TB scores between baseline and each of the interval measurements at weeks 8, 16 and 24 ($p > 0.05$).

In calculating the TB score, the MUAC (Mean Upper Arm Circumference) and BMI was calculated.

Using Mann Whitney Tests, it was calculated that the MUAC had no relationship to vitamin D levels at baseline. The numbers of patients with MUAC < 220 was only 8/48 with results and so with a small cohort the results may be unreliable. Previous studies have identified a relationship between BMI and vitamin D levels. In the same way BMI was calculated and grouped into those with a BMI < 16 or < 18 . However no relationship with baseline vitamin D results was identified. MUAC and BMI correlated to each other using Pearson test $p = 0.01$.

Radiology Score

No significant difference was found in CXR score in severe vs non severe vitamin D deficiency i.e. those with levels $<20\text{nmol/l}$ vs those with levels $>20\text{nmol/l}$ $p=0.066$. No statistically sig difference between high and low TB score (<4 or ≥ 4) on CXR score ($p=0.34$), even when the group was split in pulmonary and extrapulmonary disease.

No sig correlation was identified between CXR score and baseline vitamin D, LL37 or CRP using Spearman's rho test for the non-parametric data. Neither was baseline TB score noted to have a significant correlation with CXR score, although it was approaching significance ($p=0.073$).

Other Markers in the Circulating Blood

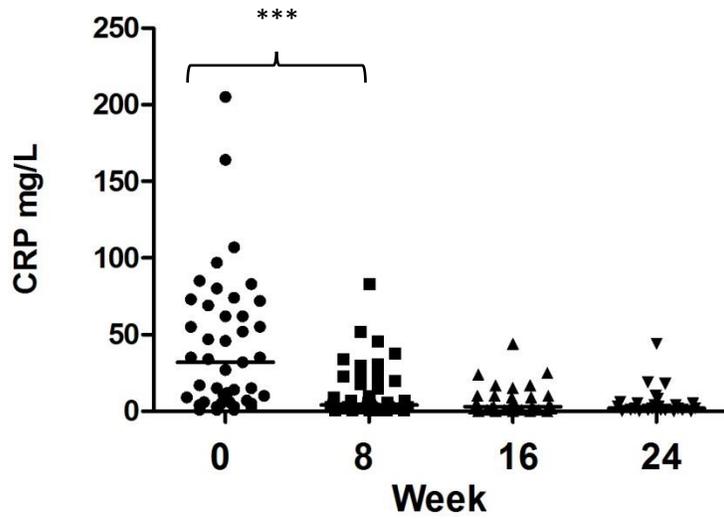


Figure 3.17 CRP levels with median values between week 0 and 24

As treatment and vitamin D supplementation progressed throughout the study, the CRP levels declined, fitting with a reduction in inflammatory stimuli from pathogenic organisms.

CRP levels show a significant reduction between week 0 and 8 (see figure 3.17) with a steady reduction towards zero thereafter (***) $p < 0.0001$).

The stimulus for CRP is likely to be IL6 (secreted by T cells and macrophages), IL1 (produced by macrophages, monocytes, fibroblasts and dendritic cells, resulting in increased expression of adhesion factors to help with tissue transmigration) and TNF α (produced by activated macrophages). Thus as these decline and the stimulus lessens, CRP will also fall.

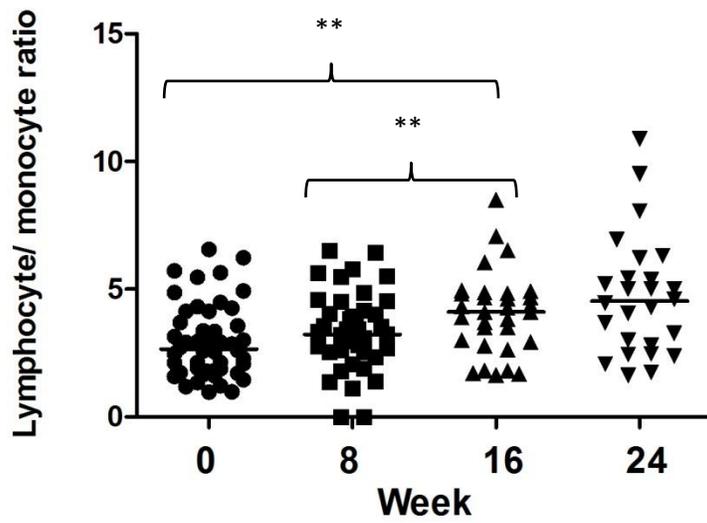


Figure 3.18 Lymphocyte / monocyte ratio with treatment.

** $p < 0.05$. Lymphopenia and a monocytois is commonly found with M.tb infection and as treatment progresses, the lymphopenia recovers and monocyte numbers decline to normal levels. This correlates with previous work showing monocyte / lymphocyte ratios are greatest in those infected with tuberculosis compared to controls and it is expected that with treatment, the levels will return to normal post treatment.

During the trial the lymphocyte / monocyte ratio rise at each interval (see figure 3.18).

There was no association between lymphocyte / monocyte ratio (L/M) and vitamin D levels or TB score. However L/M ratio <3 is associated with significantly higher levels of LL37. The reason for this is unclear, perhaps LL37 drives tissue transmigration of lymphocytes, thereby reducing numbers.

A statistically significant rise in lymphocyte count is noted between week 0 and 8 (see figure 3.19)

**p<0.05 (0.043) but this is not incrementally statistically significant at other time intervals.

Twelve (25%) of the patients were lymphopenic (<1.1x 10⁹/L) at baseline.

The converse is true for monocyte count with a gradual reduction in levels (significant between week 0-8. This reflects the reduction in monocytosis with treatment.

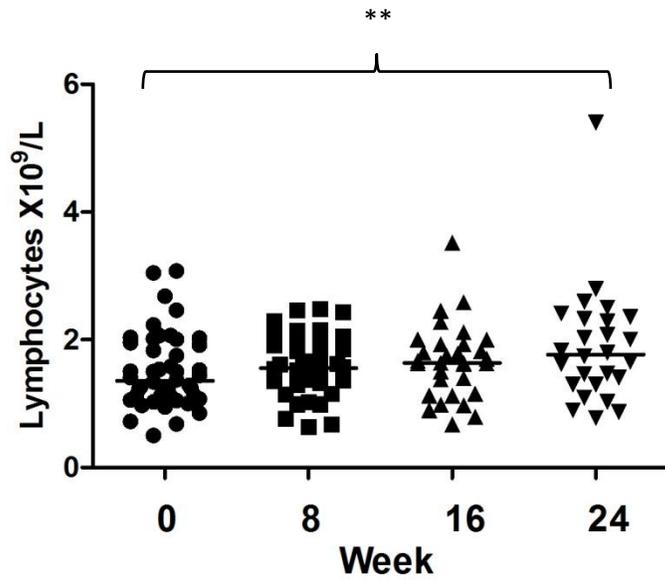


Figure 3.19 Lymphocyte levels with median values during treatment.

Lymphopenia in tuberculosis is commonly seen and levels tend towards normality with treatment. There is a significant increase in levels between weeks 0 and 8 which remains as such throughout the study with no further significant rises lymphocyte counts, although it can be seen that median values do increase at each time interval.

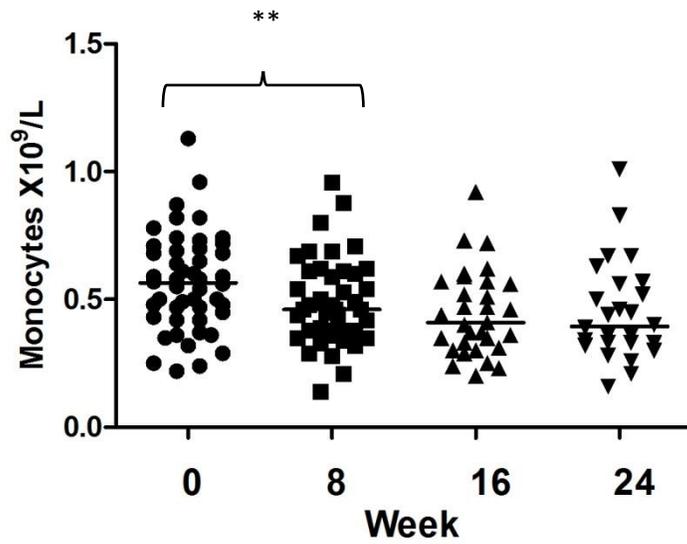


Figure 3.20 Monocyte levels with median values during treatment.

Monocyte levels are essentially a reflection of the lymphocyte response to treatment as illustrated in figure 3.19. As treatment progresses median monocyte counts decline. Monocytosis is a common finding in active tuberculosis. **p<0.05

3.2 Discussion

3.2.1 Recruitment, Exclusions and Demographic Data

The study finally recruited forty nine patients for the main clinical trial and this was fewer than the one hundred patients I had initially aimed for. Several factors contributed to this figure, including delays in obtaining final MHRA approval, pharmacy administration and availability of suitable patients during the remaining recruiting period. Maintaining study participants in the study proved to be a challenge and this reflected the reality of the TB population served, in that they are frequently mobile, some leading chaotic lifestyles. Although some of the patients did not attend for the trial follow up, every effort was made to ensure that despite this, they maintained their antituberculous therapy using a team of dedicated nurses who provide home or work visits to ensure that this is the case. The loss to follow up rates for patients with drug sensitive TB in 2013 for the West Midlands was 4% [18]. This compares to the study populations failure to complete the trial protocol at 43% (28/49 patients). This figure would not be reflective of the final completion rates in the TB patients on active treatment and indeed, the figure for the West Midlands is 85.9% completion in 2013 for drug sensitive disease which meets national standards and may reflect either loss to follow up or difficulties with the clinical management / treatment of the patient.

The reason for the higher loss to follow up rates in this study was partly because study sampling and recruitment was only carried out at the designated clinics. Although some leeway was given for

collection of samples either side of the allocated time intervals, we did not collect from patients' residence / outside clinic times and this may be something which, if taken into account may allow for additional recruitment in further studies.

Despite these challenges the results of the main clinical trial and the results from the functional work which was carried out concurrently have given some interesting findings on which to build future work.

The ethnicity of the population studied is reflective of the diversity of ethnicities in Birmingham. 61.2% of the group were from the Indian Subcontinent (India / Pakistan) and this is similar to national figures (table 5.1) from Public Health England [198].

Country of birth	Number of cases	Percentage of cases*	Median time since entry UK (IQR)**
India	1,858	30.7	4 (2 - 13)
Pakistan	1,091	18.0	7 (2 - 21)
Somalia	380	6.3	8 (3 - 12)
Bangladesh	276	4.6	7 (2 - 23)
Nepal	216	3.6	2 (1 - 5)
Nigeria	184	3.0	5 (1 - 10)
Philippines	136	2.3	5 (2 - 9)
Zimbabwe	132	2.2	9 (7 - 11)
Kenya	98	1.6	13 (6 - 40)
Sri Lanka	98	1.6	7 (2 - 15)
Eritrea	87	1.4	4 (2 - 5)
Romania	78	1.3	2 (0 - 4)
Afghanistan	75	1.2	5 (2 - 10)
Poland	71	1.2	5 (2 - 7)
Others (each <1%)	1,265	21.1	5 (1 - 13)
Total*	6,045	100	6 (2 -13)

Table 3.5 Most frequent countries of birth for non-UK born tuberculosis cases and time since entry to the UK to tuberculosis diagnosis, UK, 2013

The age and gender of the population described was characteristic of the types of patients which attend the tuberculosis clinics in Birmingham, frequently a younger population, with the majority of older patients having reactivation of tuberculosis which may have been contracted many years earlier.

Culture results for TB patients are essential, in order to identify the organism and treat appropriately according to drug sensitivity results. The West Midlands rates for culture confirmation of pulmonary tuberculosis in 2013 were 64.9% (figure 3.21) below shows the rates of culture confirmation for the west midlands). This compares to the local rate within the study of 31/35 (89%) for a positive culture of any mycobacteria and 29/35 (83%) positive culture of drug sensitive M.tb. However these rates will inevitably be higher given that patients were only recruited if there was microbiological (smear positive) or histological evidence of TB at the initial stage.

Proportion of pulmonary TB cases that were culture confirmed – West Midlands PHE centre

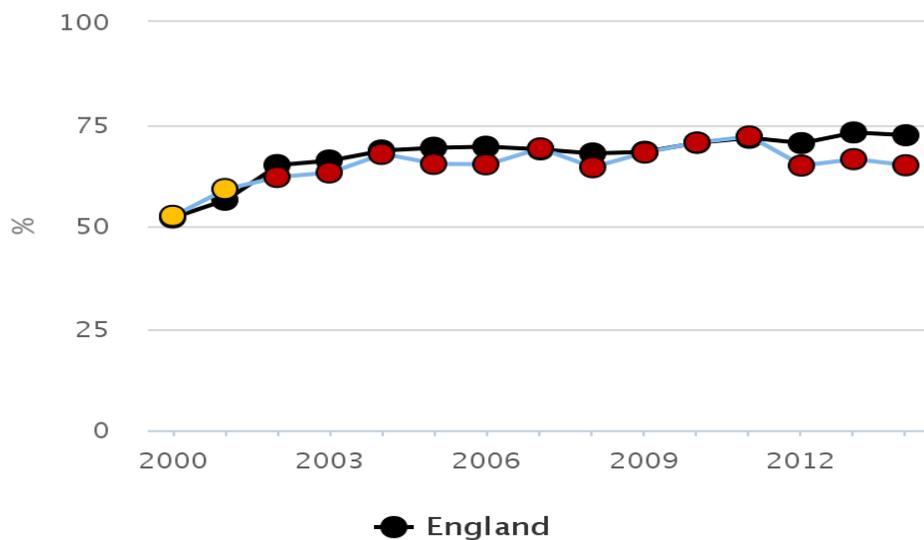


Figure 3.21 Rates of culture confirmation in pulmonary TB in the West Midlands.

The legend shows in black circles the rates for England, and superimposed the rates for the West Midlands (yellow circles identifying similar rates to England, red identifying worse rates than England).

3.2.2 Genetic Polymorphisms and baseline vitamin D in determining response to Vitamin D supplementation.

Aim 1 of this MD was to ascertain the role of polymorphism in the vitamin D axis genes in determining response to vitamin D in tuberculosis patients. In doing so I studied the effect of the baseline vitamin D level and effect on the response to supplementation, and whether polymorphisms in the vitamin D axis has any relationship to the baseline and response vitamin D levels in vivo.

Additionally on this theme, the response of monocytes to vitamin D supplementation has been ascertained, including the genetic polymorphism effect on this in vitro response.

Supplementation with vitamin D clearly results in a significant rise in vitamin D compared to baseline. However, the degree of response in achieving sufficiency was unsatisfactory with only 68% of those remaining in the study achieving levels ≥ 50 nmol. Previous studies such as those by Martineau and Wejse have had similar problems with failure to achieve sufficiency or a significant rise with oral supplementation [75] [5].

The degree of response appears to be affected by the baseline vitamin D level and this concurs with previous findings by Didrickson et al where those with the lowest baseline serum 25(OH)D (without regard to genotype) had the highest increase after supplementation. Other studies also identified similar findings [199-201].

The relevance of this may be that in individuals from regions with high sun exposure or with ethnicities which are prone to vitamin D deficiency, to a greater or lesser degree, the

supplementation regime may be adjusted accordingly. However, it is likely that both those who are severely deficient or insufficient will need adequate doses to obtain sufficiency regardless of their initial response to supplementation. It has been difficult in previous studies to attain sufficiency with some of the study dosing regimens and this may be due to concerns regarding over supplementation and its theoretical risks.

There appears to be a relationship between both baseline vitamin D levels, change / response to supplementation and genotype. Baseline vitamin D values appear to be affected by rs2228570 T allele carriage (Fok1), which showed that carriage of the T allele resulted in higher vitamin D levels. Further work is required to determine whether this is maintained in a larger trial. However a systematic review from 2010 [202] noted that two of three studies examining rs10735810 (now merged to rs2228570) found a significant relationship with 25OHD concentrations. Two studies reported significant associations [203, 204] with the minor allele (T allele) associated with higher concentrations of 25OHD. Other studies do not corroborate this [205, 206] and clearly larger studies are required. Other studies have varied results with regard to VDR polymorphisms and findings include correlation of vitamin D levels with Bsm1 SNPs [207] and Taq1 SNPS [206].

Change in vitamin D levels / response to supplementation appears to have a genetic relationship and the findings that the change in vitamin D at 0-8 weeks in DHCR7 rs12785878 GG homozygote and VDR rs2228570 CC homozygotes carriers and 0-16 weeks in CC allele of GC rs 4588 carriers would suggest this. The finding regarding DHCR7 rs12785878 concurs with the finding in a recent GWAS that genotypic variation in the DHCR7 gene accounts for 1-4% variation in 25(OH)D level concentrations [208]. However the other findings regarding GCrs4588 and VDR rs 2228570 have not been corroborated in GWAS.

In order to determine if specific independent variables affected prediction of baseline vitamin D levels, analysis of ethnicity, age and season of blood draw was analysed. This is discussed in section 3.1.1 of the results.

Ethnicity was found to be related to the vitamin D status with baseline 25(OH)D levels that were significantly higher in the Caucasian group compared to the Indian Subcontinent (ISC), African and Black Caribbean groups.

Previous studies have confirmed the above finding, Mitchell et al noting higher vitamin D levels in Caucasians [209] and Ekeroma et al noting lower levels in ethnicities with darker skin compared to those of European descent [210].

3.2.3 Effect of Baseline Vitamin D and Other Elements of the Vitamin D Axis on Clinical Outcome / Antimicrobial Response.

The second aim of this study was to identify any relationship of TB outcomes to vitamin D level. In determining whether disease activity is affected by vitamin D levels at baseline and with supplementation, several parameters were looked at including TB score, sputum smear positivity, radiology score, inflammatory markers and cytokine levels.

Relationship between baseline vitamin D levels and TB score

A previous study by Wejse showed no significant effect of vitamin D supplementation on TB score compared to the placebo group [68, 211].

This study showed that there was a significant reduction in TB score over time. Whether this was due to vitamin D supplementation or solely related to treatment with anti-tuberculous therapy is difficult to say without a placebo controlled trial. However, it was possible to look at the relationship between vitamin D baseline and TB score and the effect of change of vitamin D levels with supplementation on the TB score. By analysing the baseline vitamin D score, including those with severe deficiency it was possible to conclude that in this study there does not appear to be any correlation between the baseline or change in vitamin D level and the TB score / change in TB score at week 8. This correlates with findings by Wejse et al and Salahuddin et al that a reduction in TB score did not differ between patients in the vitamin D treatment or placebo arms [211, 212]. However, more recently Junaid et al noted that those with profound vitamin D deficiency was associated with delayed sputum conversion and bilateral vs unilateral disease [213].

Unsurprisingly those patients with extrapulmonary tuberculosis had lower TB scores at baseline (2.0 vs 5.0) which may reflect the variation in immune responses between the two subsets, with a greater cytopathogenic response in pulmonary disease, resulting in an increase in systemic signs and symptoms. Recent work by doubling in serum concentration of 25(OH)D confers a significantly reduced risk of extrapulmonary disease which is interesting and suggests that greater vitamin D levels in fact reduce the likelihood of dissemination outside the lungs which

The results from analysis of the TB score and vitamin D deficiency at baseline suggest that there is no correlation between the severity of vitamin D deficiency at baseline and the change in TB score with time, i.e. being severely deficient (<20nmol/l) did not appear to affect their overall response to treatment. This may be because they were all supplemented and this therefore counteracted the effect of deficiency. However it appears that even accounting for change in vitamin D over time with supplementation, no relationship with TB score could be identified.

In identifying whether MUAC or BMI was related to baseline vitamin D levels, there was no statistically significant relationship identified in this observational study. However the numbers are small and a larger cohort may yield further findings. A previous study by Baradaran et al shows that BMI is not associated with vitamin D levels (age adjusted) [214], however a systematic review and meta-analysis in 2013 showed that there was an overall significant inverse, but weak, association between serum 25(OH)D levels and BMI with a significant heterogeneity between the studies [215]

Sputum conversion.

It was not possible to accurately analyse whether sputum smear conversion was related to baseline vitamin D due to the small numbers (single figures) of pulmonary TB patients who were able to provide sputum samples subsequent to baseline analysis and the fact that many of the pulmonary TB patients were smear negative but culture positive at onset, so smear positivity could not be monitored. A larger cohort would have been needed to accurately assess this, although studies looking at this have been conflicting, with Salahuddin et al noted no significant relationship between

vitamin D supplementation and smear conversion whilst Nursyam et al and Martineau et al noted that sputum smear conversion is speeded up by vitamin D supplementation [74, 77] and Junaid et al noted profound vitamin D deficiency was associated with delayed sputum conversion [213].

Radiology Score.

The radiology / CXR score was calculated using an equation, validated by Ralph [190] et al.

Various markers were analysed against CXR score, with no significant differences or correlations with baseline inflammatory markers, vitamin D severity or clinical severity markers such as TB score.

Ralph et al noted that CXR score was significantly associated with sputum smear grade at diagnosis and therefore representative of the severity of pulmonary disease. This was shown to be more accurate at determining 2 month smear status by using both % consolidation as well as a weighting for cavitation than % consolidation alone. However numbers in this study may well have contributed to the negative findings.

Relationship of Inflammatory Markers and Cytokine Response to Vitamin D supplementation.

DBP levels

DBP levels rose incrementally throughout the study at each time interval. Although both vitamin D and DBP levels rise during the trial, there does not appear to be a direct correlation between the two, i.e. the availability of vitamin D does not significantly affect the circulating DBP. It may be expected that as vitamin D levels rise, DBP levels will fall, however the turnover of DBP is high and the snapshot of DBP levels may not be reflective of its activity. A study by Winters et al showed that DBP was not an important determinant of circulating vitamin D levels [216]. Additionally, the free vitamin D may not reflect that which is bound to DBP and therefore remains unmeasured, with GC genotype affecting the degree of affinity for vitamin D. For example TT allele is likely to produce GC1F variant which has been shown to have greater affinity for vitamin D, and as such may existing DBP may reach saturation more quickly, resulting in a subsequent increase in production via a positive feedback loop.

Another possible explanation for the rise in DBP may be that conversion to macrophage activating factor (MAF) which occurs in the acute inflammatory response, declines as treatment effect occurs and inflammation regresses with improving clinical condition.

The finding that DBP haplotype appears to have a direct effect on DBP levels is an interesting one and has previously been discussed in work by Coussens et al [217] which found GC1S1S individuals had greater DBP total levels compared to GC1F1F. One explanation for this may be that

the haplotypes with greatest affinity for vitamin D are required in lesser amounts to provide the same effect in terms of vitamin D transportation intracellularly.

LL37 levels

LL37 levels dropped significantly between week 0 and 8.

Interestingly analysis of baseline levels of LL37, the antimicrobial protein related to vitamin D metabolites, shows that there is no correlation with baseline vitamin D levels. This concurs with previous findings by Yamschchikov [64], however conflicts with other findings by Jeng et al which found a correlation between serum vitamin D levels and systemic levels of LL37 [218]. One reason for the finding in this study may be that localised autocrine levels (also shown to have a relation to vitamin D) may be a more important measurement and may not be reflected in the total systemic values of LL37 as detailed by Adams et al with hCAP (the precursor to LL37) [219]. Further related cell work is discussed later in this thesis.

The study highlighted that LL37 had a positive correlation with both total white cell count and monocyte / lymphocyte ratio. The findings may reflect the fact that white cells are a source of LL37 [220], with rising counts in a condition of inflammation, resulting in elevated LL37 levels. The reason that monocyte / lymphocyte ratio was considered was because work by Naranbhai and Wang that hazards of tuberculosis correlates to particularly low or high M/L ratios [221] and median monocyte to lymphocyte ration was greater in TB infection compared with controls, and recovered

post treatment to normal levels [222]. This suggests that LL37 levels seem to be related to immune activation, although no correlation could be identified with disease severity in the form of TB score.

The initial decline in LL37 levels may include the fact that treatment of the mycobacterial infection with antimicrobial therapy results in a reduction in some mononuclear cells such as neutrophils and monocytes (although lymphopenia usually corrects), thus possibly leading to an overall reduction in LL37. However, with no healthy controls to compare, it is difficult to determine if this response is purely due to treatment or due to the effect of vitamin D. Interestingly Coussens et al found that vitamin D accelerated treatment induced decreases in LL37 (rather than just decreasing the total LL37 levels) [223]. Previous studies noted that vitamin D induced the production of antimicrobial proteins, but the findings by Coussens that antituberculous therapy suppressed LL37 levels but that the levels were further suppressed by vitamin D are similar to the findings at Week 0-8 in this study.

Indeed LL37 may not be the ideal substance to measure and previous work by Martineau may suggest that measurement of hCAP18 gene may be a better indicator of antimicrobial activity [224]. Vitamin D has also been found to suppress hCAP18 as well as M.tb and these would be reasonable explanations for the pattern of LL37 expression between weeks 0 and 8.

Despite the initial drop in LL37 levels, they return to baseline levels during the remainder of the study period and this raises the question: which effect has the greatest impact – the antituberculous therapy or the vitamin D treatment on LL37? An explanation for the latter rise in levels may be that once the initial suppression of LL37 producing cells occurs with antituberculous therapy, vitamin D upregulates the response in other immune regulating cells such as lymphocytes, which tend to recover with M.tb treatment, thereby resulting in total LL37 similar to pre-treatment levels.

Further work in this area would be to measure hCAP gene protein as part of the analysis in order to see if this correlates with the LL37 picture. Analysis of the LL37 levels at weeks 2,4 and 6 may have been useful in order to determine whether the levels at week 8 are purely spurious results or whether there is indeed a gradual decline in the initial stages of treatment.

Lymphocyte / Monocyte ratio

Monocyte and lymphocyte analysis was reviewed as part of the inflammatory response analysis. Previous studies have looked at total monocyte and lymphocyte counts and lymphocyte / monocyte ratios in association with risk and susceptibility of disease.

The lymphocyte / monocyte ratio rises at each time interval during the study and this is reflected with statistically significant rises in total lymphocyte count along with a decline in total monocyte counts. These findings correlate with work by Coussens et al which notes that with antituberculous therapy monocytes declines and lymphopenia improves, whilst vitamin D accelerates both.

Previous studies in this field have shown that hazards of tuberculosis correlates to particularly low or high M/L ratios (5th or >95th centile) in HIV infected patients even with adjustment for CD4+ T cell counts [221]. A recent study by Wang et al showed that median monocyte to lymphocyte ration was greater in TB infection compared with controls, and recovered post treatment to normal levels [222], whilst an earlier study by Sabin et al in rabbits showed that high M/L index resulted in low resistance to disease experimentally [225]. Again, these findings correlate with the increasing L/M ratio found in this study.

No association was found between lymphocyte / monocyte ratio and vitamin D levels or TB score. However, an L/M ratio <3 was associated with significantly higher levels of LL37. This is probably reflective of the disease process occurring with a relative monocytosis in early active disease, and high LL37 levels reflecting the disease activity.

Chapter 4

The Role of Vitamin D and genetic polymorphisms on immune modulation and response to vitamin D in vitro / ex vivo in health and mycobacterial disease.

The aim of this chapter is to determine if vitamin D exerts an immune modulatory effect in vitro and ex vivo, in both health and mycobacterial disease. The results included were obtained from cell work on both monocytes and T cells which was carried out as part of this study. Initially a greater degree of clinical work was envisaged. However due to patient recruitment and retention the decision was made to add further functional work to the study, in order to maximise data pertaining to the aims, endeavouring to ensure that despite lower than expected recruitment rates, the patient population studied would still enable additional cell work to be completed.

The most important components of anti-mycobacterial activity are macrophages (innate immunity) and lymphocyte subsets (adaptive immunity), with vitamin D exerting both inhibitory and stimulatory effects on these systems.

As discussed in Section 1.7, vitamin D is known to have numerous functions on both the innate and adaptive immune response, including upregulation of antimicrobial products, upregulation of the

TH2 response and regulatory T cell function and down regulation of the TH1 response. This is illustrated in Figure 1.6.

4.1 Results

4.1.1 The Immune Modulatory Effect of Vitamin D in Vitro and ex Vivo, in health and Mycobacterial Disease

Functional Effects of Vitamin D and DBP on Monocytes

Optimisation experiments

In order to determine the optimal LPS concentration to use in the monocyte culture media as an inflammatory stimulus (i.e. produce adequate stimulation of monocytes) optimisation experiments were carried out with various concentrations of LPS. The production of IL6 was then measured using an IL6 ELISA Duokit as described in the methods section. Additionally, the optimal 25(OH)D₃ was also added to the media in separate wells at varying concentrations, to determine IL6 suppressive effect.

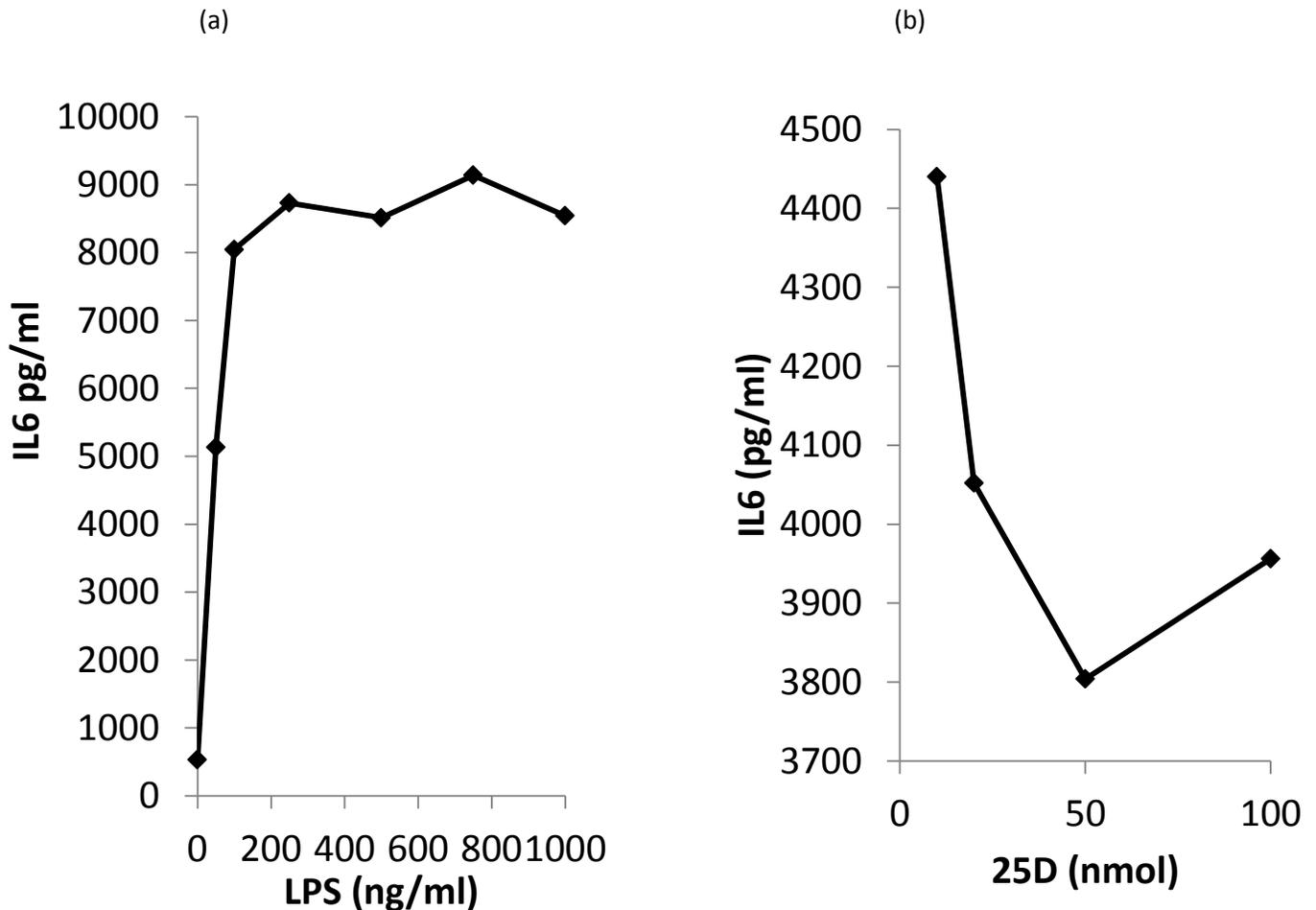


Figure 4.1 Optimisation of (a) LPS concentration and (b) 25(OH)D₃ concentration.

(a) Dose response curve (n=2 repeats) showing IL6 production with increasing LPS concentration by monocytes. This experiment used concentrations of LPS (0,50,100, 250, 500, 1000 ng/ml) in the monocyte culture media as an inflammatory stimulus to determine adequate stimulation of monocytes. The production of IL6 was then measured using an IL6 ELISA Duokit at different LPS concentrations. The optimal concentration of LPS which produced maximal stimulation was 100ng/ml LPS, after which the levels of IL6 did not significantly increase.

(b) In addition to the various LPS conditions, 25(OH)D₃ was also added to the media in separate wells at concentrations (10, 20, 50 and 100nmol) to determine any additive effect on IL6 values (n=2 repeats). This showed maximal cytokine (IL6) suppression with 50nmol of 25(OH)D₃.

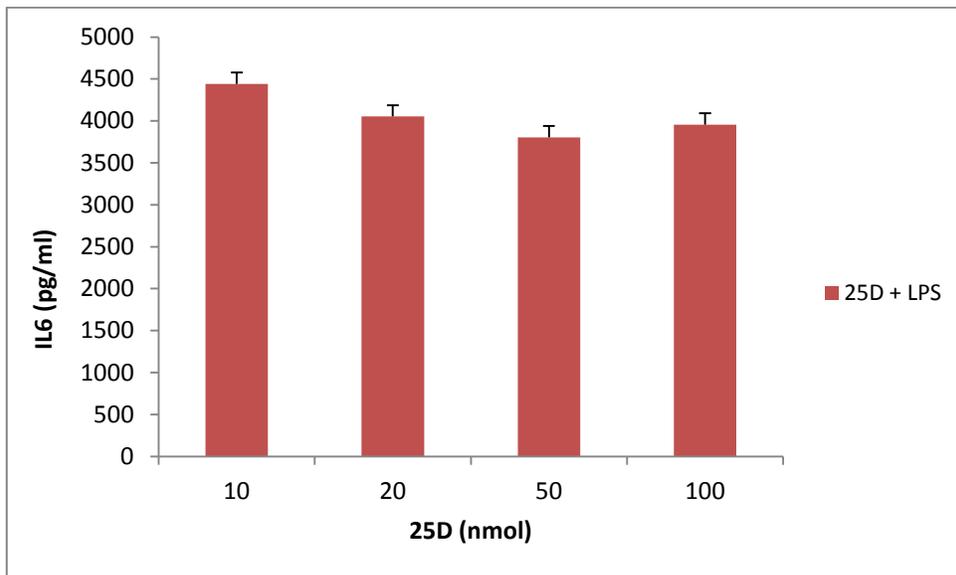
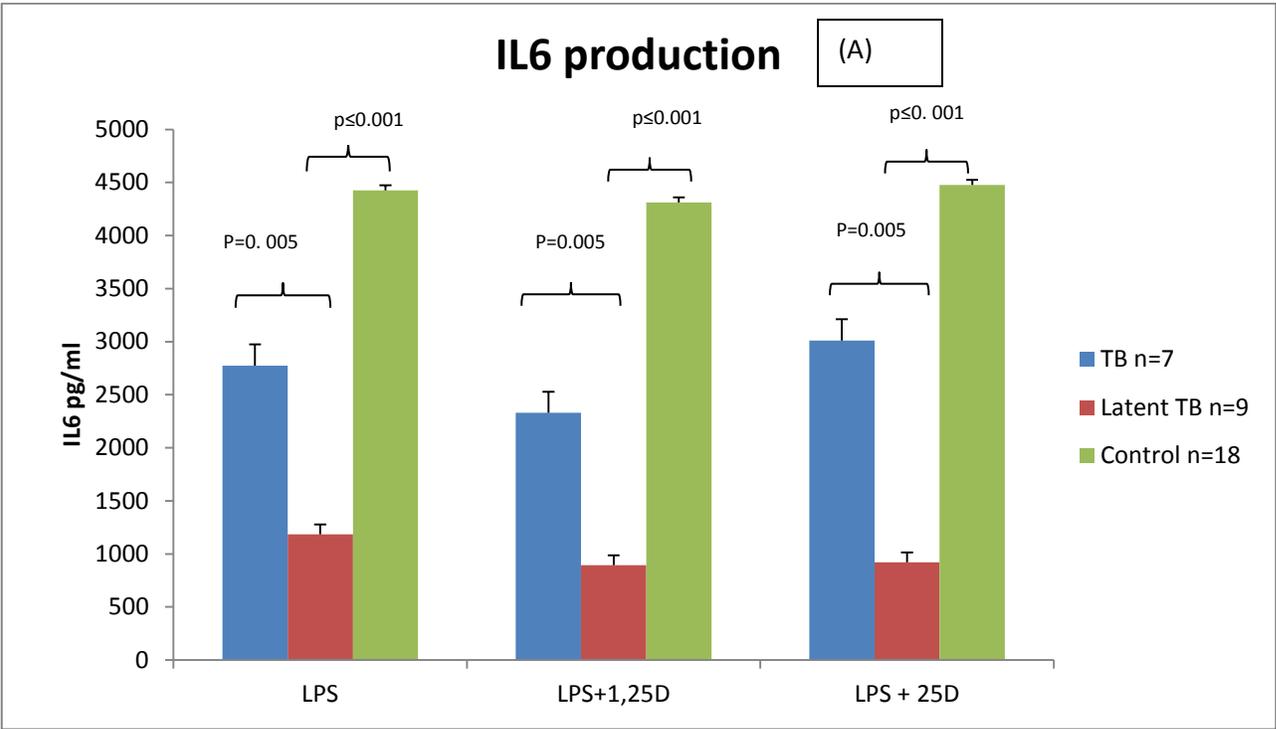


Figure 4.2 Mean (with standard error) IL6 production in the presence of LPS when monocytes were cultured with various concentrations of 25(OH)D₃ (n=2).

Comparison of controls and TB patients' monocyte function

Monocytes of TB, latent TB patients and a control group were cultured for 48 hours as described in the methods section and stimulated with LPS and subsequently cultured with 25(OH)D₃. The IL6 production was measured in the supernatant of the cultures by ELISA and the response compared.



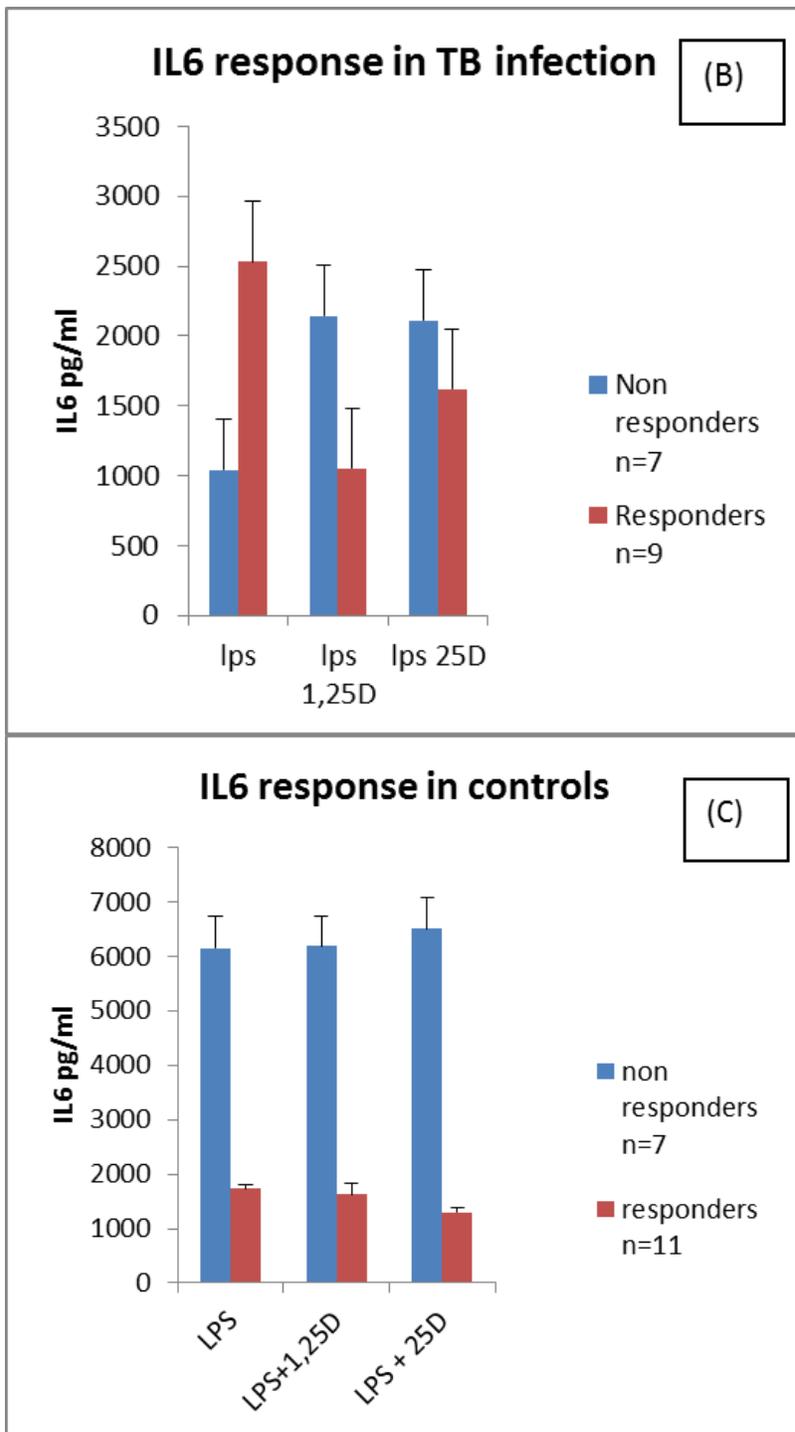


Figure 4.3 Monocyte mean IL6 production in (A) active, latent and control populations with LPS stimulation in the presence and absence of vitamin D. (B) Active and latent populations divided according to response to vitamin D (Response is a drop of >10% in the monocyte supernatant IL6 value with the addition of vitamin D); (C) Control population divided according to response to vitamin D.

Using Wilcoxon Ranks test, analysing each group individually (control, latent and active disease), and comparing the IL6 production with LPS stimulation alone or with LPS in combination with vitamin D, there is no significant difference in the IL6 production with or without vitamin D in either form ($p > 0.05$). This is shown graphically in (A).

The IL6 production in the three groups however, varied significantly, regardless of subsequent vitamin D response, on initial stimulation with LPS (A). Using Wilcoxon-Mann Whitney tests there is a significant difference between the active and latent IL6 production ($p < 0.007$). This significant difference is also seen between the controls and latent group ($p \leq 0.001$). However, the significance is lost between the active and control groups ($p = 0.824-1.0$). Despite the appearance of a difference in the mean on the graph, statistically this was not borne out when the groups were analysed.

Graph (B) shows that when the TB infected (latent and active) patient were analysed together, and then sub-categorised into responders ($>10\%$ reduction in IL6 with supplementation of vitamin D) or non-responders, it was noted that neither the responders nor non responders had a significant difference in IL6 production when stimulated with LPS. When the monocytes were cultured along with $25(\text{OH})\text{D}_3$ the mean IL6 production was lower in the responders compared to the non-responders, however it did not reach statistical significance. This was also the case for the response to $1,25(\text{OH})_2\text{D}_3$. This does however show that individuals have large variations in their IL6 production in response to vitamin D. This may be multifactorial but the possibility of an underlying genetic predisposition is raised. The standard deviations for the subsets were large and therefore the mean is not wholly representative of the statistical data.

Graph C shows the IL6 response in the control group which when analysed using Mann Whitney U, shows no significant difference in IL6 production with LPS stimulation between responders and non-responders, although the graph appears to show a trend. When 25(OH)D₃ is added with the LPS, there is a difference which almost reaches significance (p=0.052) between responders and non-responders in this control group. When 1,25(OH)₂D₃ is added there is no statistically significant difference between IL6 response in responders vs non responders.

Further analysis of the genetic relationship of a variety of SNPs are analysed further in this thesis to determine if there are any significant associations between genotype and response to vitamin D in monocyte culture. In addition to this the section below (comparison of monocyte response to vitamin D in the presence of pooled plasma of varying DBP genotypes) looks at the exposure of monocytes to a variety of DBP genotypes through pooled plasma to see if there is any further supporting evidence for the possibility of variation in immune response to vitamin D being associated with genetic predisposition.

Comparison of monocyte response to vitamin D in the presence of pooled plasma of varying DBP genotypes.

In light of the variability seen in initial monocyte vitamin D work, which I hypothesised might be due to genetic variation, further experiments were undertaken using DBP genetic variants.

Pooled plasma was taken from tuberculosis patients, pre- treatment and added to monocyte cultures taken from control volunteers. All wells contained LPS 100ng/ml and 25(OH)D₃ 50nmol. The plasma for each genotype was pooled from at least three patients per genotype to try and reduce

heterogeneity. The monocytes were cultured with each and the cytokine response (GCSF, IL1- β , IL6 and TNF α) was measured using Luminex assay (R+D systems). See Methods Section 2.3.8

DBP genotype effect on cytokine response.

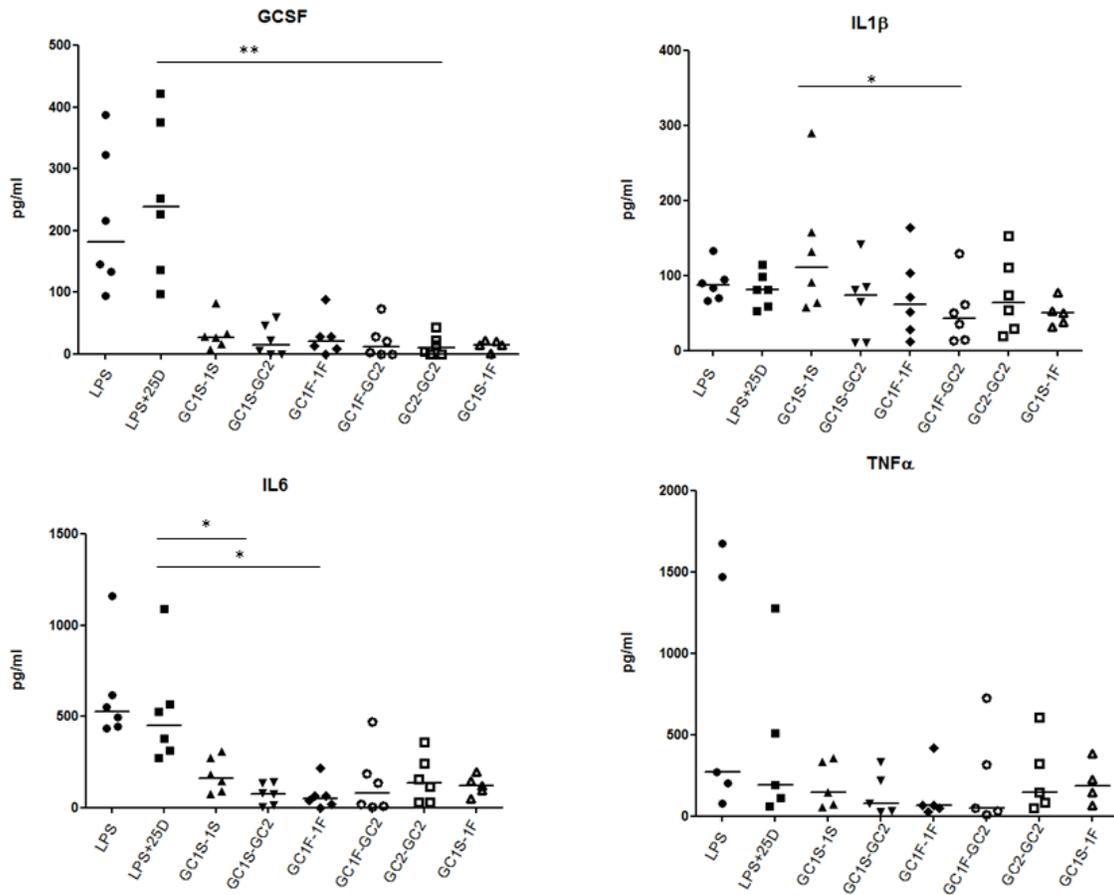


Figure 4.4 Effect of pooled plasma with differing DBP genotypes on cytokine response.

This figure shows the effect of the addition of pooled plasma on monocyte cytokine response. There is a reduction in cytokine response in all cases with the addition of plasma with statistical significance in all except TNFα. Varying vitamin D binding protein genotypes also appear to affect the degree of cytokine response. * p<0.05 **p<0.01

Analysis of the data was carried out using Kruskal Wallis tests.

The addition of pooled plasma resulted in a reduction in cytokine production which was significant in GCSF and IL6. There was a significant drop in IL6 cytokine production on addition of pooled plasma when compared to control (LPS +25(OH)D₃) in GC1s1s, GC1sGC2 and GC1f1f. There was no significant difference in response between GC2 and non GC2 carriers.

For GCSF there was no significant change in cytokine production with addition of vitamin D to culture (p=0.225) but there was a significant difference in GCSF production with lower levels (p<0.05) in all but GC1sGC1f genotype (although this nearly reached significance p= 0.068 and may have been affected by the loss of a single result in the group for analysis). There was no difference in response between GC2 carriage and non GC2 carriage.

For IL1 β there was no significant change in cytokine production with addition of vitamin D to culture. There was a significant difference in cytokine production between the genotypes shown in figure 4.4. There was no significant difference in response between GC2 carriage and non GC2 carriage.

The cytokine response variation with DBP genotype could be due to the amount of 25(OH)D₃ available to the monocytes and therefore its action on suppression of cytokine levels as demonstrated in previous experiments. The small numbers and the possible variation in vitamin D levels in the pooled plasma may have had an effect on the reliability of this experiment and a larger study on this subject may be useful. In addition, the use of a control group with added heat inactivated protein would be useful to determine if there was any non-specific effect of the addition of more protein to the cultures. Further discussion around this can be found in chapter 4.3.2, *monocyte genetics*.

T cell analysis in controls and latent TB.

Optimisation of T reg analysis ex vivo

The selection of T reg markers, antigen concentration optimisation and optimal time points for T reg analysis was carried out as part of a BMedSci Project by Jin Jin Zhang. This was done prior to commencing analysis of blood from latent TB patients and the following section on optimisation includes her findings and figures.

Optimisation of Treg cell detection by Flow cytometry

Flow cytometry was used to detect Treg cells and using differing combinations of fluorophore stains, the T reg cell markers were identified (see figure 4.5). Strength of signal was weakest in CD25-FITC and therefore this was not selected. CD25-PE and CD25-PeCy7 both gave strong signals, but CD127-PeCy7 was the clearest stain for this marker, thus it was decided to use CD25-PE and CD127-PeCy7 in combination. FoxP3 was brighter on APC than e450, thus FoxP3-APC was chosen. CTLA-4 detection in the APC channel was poor relative to PE, thus CTLA-4-PE was chosen. The final combination of CD3-PerCP, CD4-e450, CD25-PE, CD127-PeCy7, FoxP3-APC and CTLA-PE were selected for staining Treg markers.

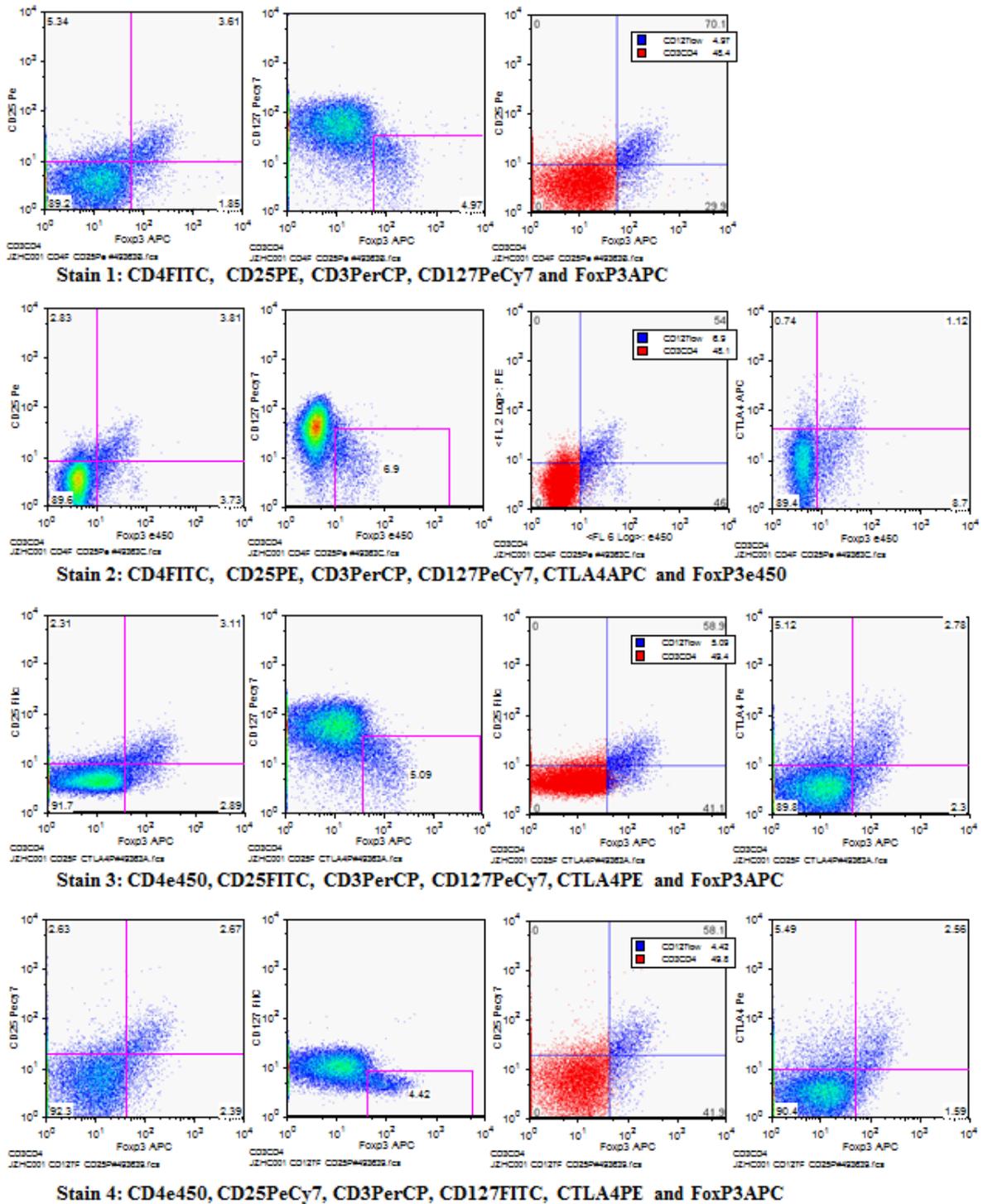


Figure 4.5 Selection of Treg markers by flow cytometry.

PBMCs stained with combinations of antibodies against Treg markers (CD3+, CD4+, CD25+, FoxP3+, CTLA-4+ and CD127 low).

Optimisation of cytokine detection by Flow cytometry

Frequencies of IL-17, IFN γ , IL-2 and TNF α producing CD4⁺ cells directly ex-vivo and following culture with vitamin D were determined by flow cytometry. Combinations of fluorophore conjugate antibodies to stain cytokines were tested to identify the ideal stain for clearest resolution with the minimum quantity of antibody stain. Cells stimulated with SEB were selected in preference to PMA and ionomycin stimulated cells due to greater cytokine production with SEB stimulation.

Fluorophores conjugate antibodies used for cytokine detection included IL-17-PE, IFN γ -e450, TNF α -PeCy7, IL-2-PE, IFN γ -APC and TNF α -e450. CD3-PerCP and CD4-APC were selected for detecting CD3⁺CD4⁺ cells. PeCy7 may break down to give signals of PE and APC, thus TNF α -PeCy7 was not selected due to the inaccurate estimation of cytokine production it may cause.

Consequently, PE and e450 conjugated to cytokine antibodies were chosen. After titrations, the best fluorophores and volume for each cytokine were determined, as shown in table 4.1.

	IL-17-PE	IL-2-PE	IFNγ-e450	TNFα-e450
Volume (μl)	0.6	0.6	0.8	2

Table 4.1 Volume of antibodies used for cytokine production staining.

Each fluorophore was assessed in titration experiments in order to determine the minimum volume required to achieve the optimal resolution on flow cytometry.

When gating on total CD3⁺CD4⁺ cells, the frequency of cytokine producing cells was low in response to SEB stimulation, therefore whether selection of activated CD3⁺CD4⁺ cells would increase the frequencies was considered. CD69 is one of the early activation markers, thus CD69 was stained alongside cytokines. The frequency of cytokine expressing cells when gating on CD3⁺CD4⁺CD69⁺ cells increased compared with CD3⁺CD4⁺ cells, as shown in figure 4.6. Therefore, CD69 co-staining was included in the cytokine detection staining panel.

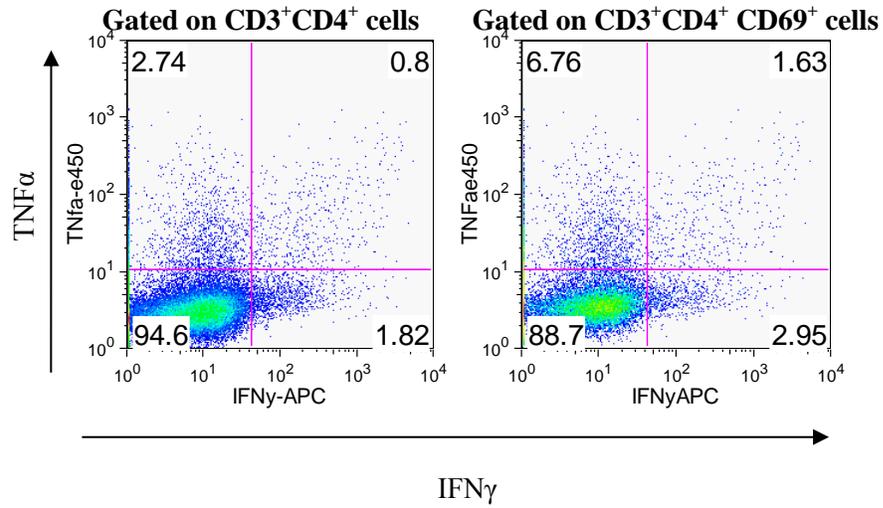


Figure 4.6 IFN γ and TNF α production by total CD3⁺CD4⁺ cells and CD3⁺CD4⁺CD69⁺ cells. PBMCs were stained with anti-CD3, anti-CD4, anti-CD69, anti- TNF α and anti- IFN γ and analysed by Flow cytometry. FACS plots show the percentages of cytokine expressing cells when gated on total CD3⁺CD4⁺ cells (left) compared to CD3⁺CD4⁺CD69⁺ cells (right).

Antigen concentration optimisation

In addition to SEB, PPD and ESAT6 were also included as antigens that play stimulatory roles, and are more specific to tuberculosis. Concentration of PPD and ESAT6 to use was initially optimised by observing the cytokine production of CD3⁺CD4⁺CD69⁺ cells in response to various concentration of antigen. The final concentration for PPD is 1000 unit/ml was used as this maximised the amount of activated cells (figure 4.7). Co-stimulatory antibodies α CD28 and α CD49d were used in combination with ESAT6, according to published methods [226, 227] and because both cross-link of T cell receptors (TcR) and co-stimulatory signals (from other molecules) are important in T cell activation and proliferation. Cytokine positive frequencies were enhanced by inclusion of co-stimulatory antibodies, although because of the low numbers of CD69⁺ cells, the estimations are subject to error. Due to the minimal number of responsive cells to ESAT6 and therefore the number of cells and amount of ESAT6 required to attain accurate estimations of cytokine frequencies by flow cytometry (figure 4.8), it was decided to continue without inclusion of ESAT6 stimulation.

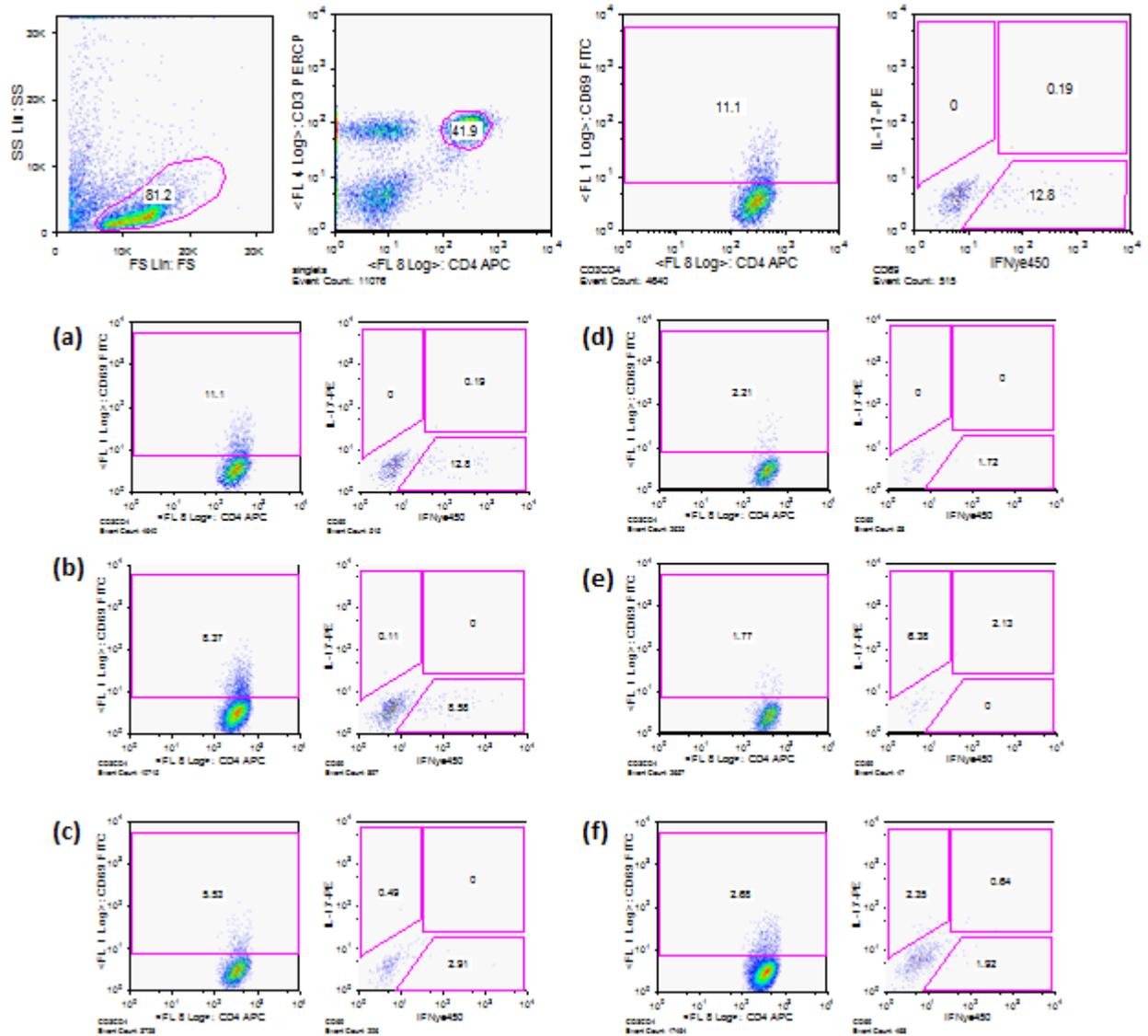


Figure 4.7 IL-17 and IFN γ production two days post stimulation by PPD.

PBMCs were stimulated with PPD at various concentrations. FACS plots from a representative experiment are shown. The top row shows gating procedures from left to right, first selecting CD3⁺CD4⁺ cells, then activated CD69⁺ and finally showing cells in 3 groups according to IL-17 and IFN γ status. In the next row only the activated and specific cell plots are shown at the following concentrations (units/ml): **(a)**1000 **(b)**100 **(c)**10 **(d)**1 **(e)**0.1 **(f)**PPD negative control.

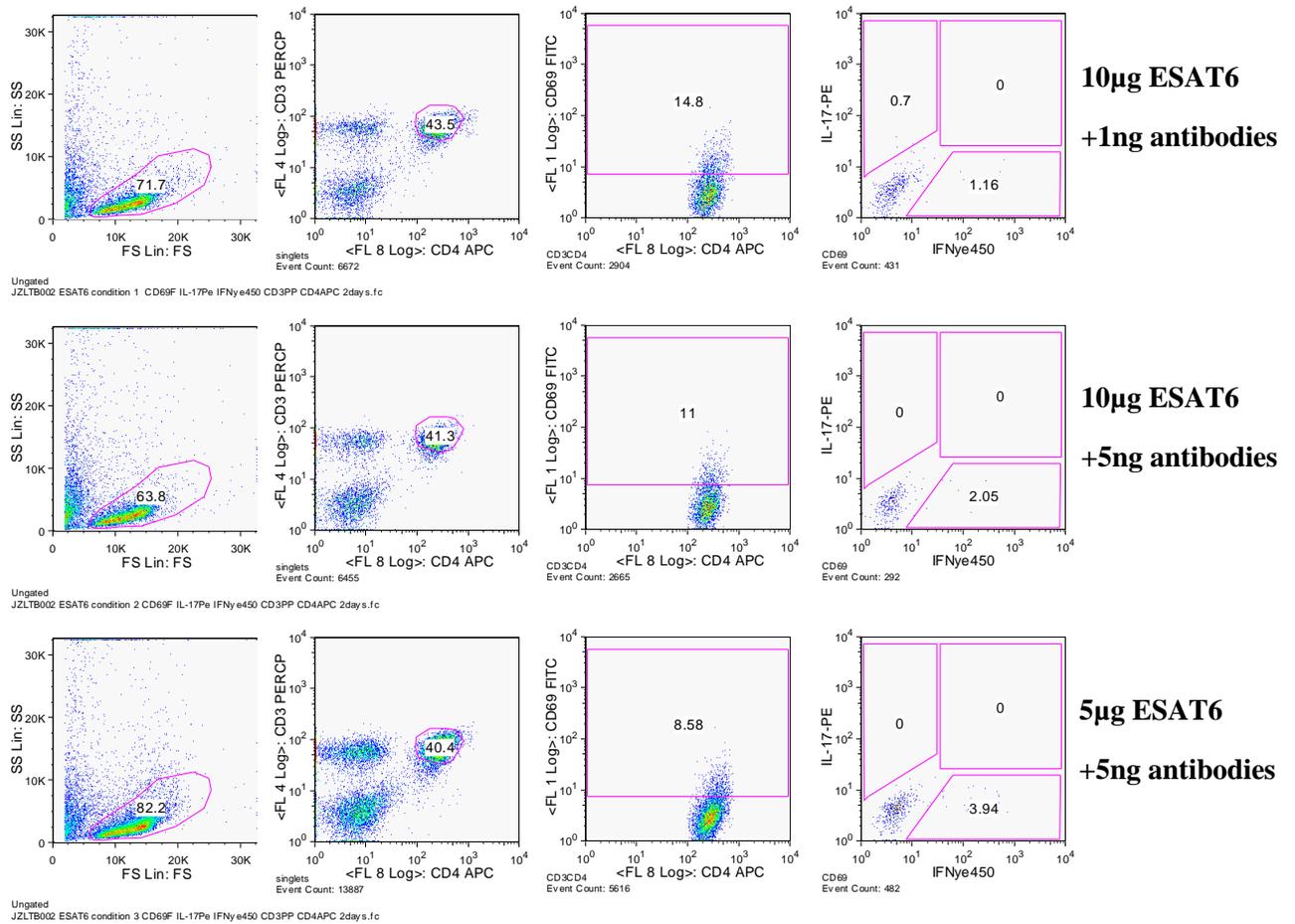


Figure 4.8 IL-17 and IFN γ production at time point of two days with various concentrations of ESAT6 stimulations.

PBMCs were stimulated with various volumes of ESAT6 in the presence of co-stimulatory antibodies α CD28 and α CD49d, IL-17 and IFN γ production two days post stimulation was measured. The top row shows 10 μ g ESAT6+1ng antibodies, gating as before, resulting in 14.8% CD69+ activated cells, and 0% IL17⁺IFN γ ⁺ double positive cells. 10 μ g ESAT6+5ng antibodies and 5 μ g ESAT6+5ng antibodies resulted in 11% and 8.58% activated cells and 0% double positive cells respectively.

Determine the optimal time point to measure the effect of SEB stimulation

The best time for detecting responses to SEB stimulation was tested by Flow cytometry.

Representative FACS plots are shown in figure 4.9. The frequency of cells expressing cytokine and their level of production was greater at two days compared to one day. The effect of vitamin D on cytokine production at two days point can be distinguished as well. Thus it had been determined that two days post stimulation is the appropriate time point to measure the effect of SEB stimulation.

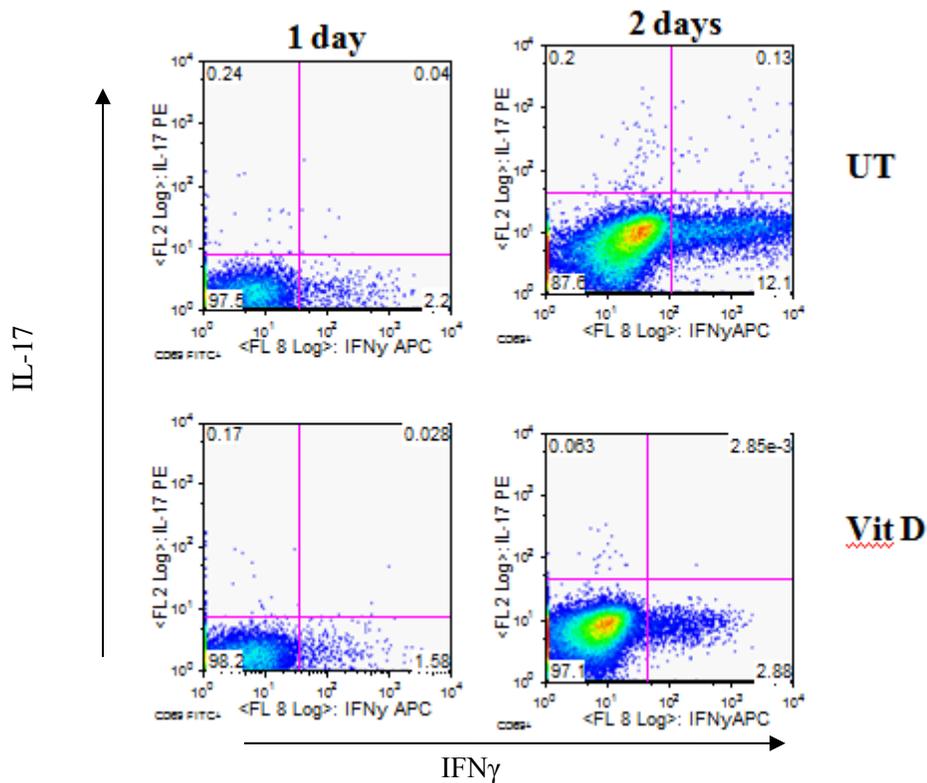


Figure 4.9 IFN γ and IL-17 expression by CD3⁺CD4⁺ T cells following SEB stimulation with SEB in the presence or absence of vitamin D.

The expression of IFN γ and IL17 in response to SEB in the presence or absence of vitamin D was compared at 1 day (left) and 2 days (right) post stimulation. For the cytokine production 1 day post stimulation, the frequencies for IFN γ ⁺IL17⁻ cell population were 2.2% and 1.58% in the absence (UT) and presence of vitamin D respectively. Whereas the frequencies for the same cell population were 12.1% and 2.88% respectively after 2 days.

Determine optimal time point to read vitamin D response

Cytokine responses to active vitamin D and T cell surface molecules, including CD25, CTLA-4 and transcription factor FoxP3 were studied to choose the best time point to detect changes response to SEB and PPD stimulation in the presence or absence of vitamin D. The results showed vitamin D effect on CTLA-4 expression can be detected at 2 days, 3 days and 4 days separately (Figure 4.10). The frequencies of CD25⁺CTLA-4⁺ expressing cells and CTLA-4 median fluorescence intensity (MFI) for CD25⁺ cells increased in the presence of vitamin D with maximal effect at day 4. Due to collection and processing time restrictions by the investigator, a shorter incubation time was needed and so the two day incubation was chosen as this gave a practical compromise.

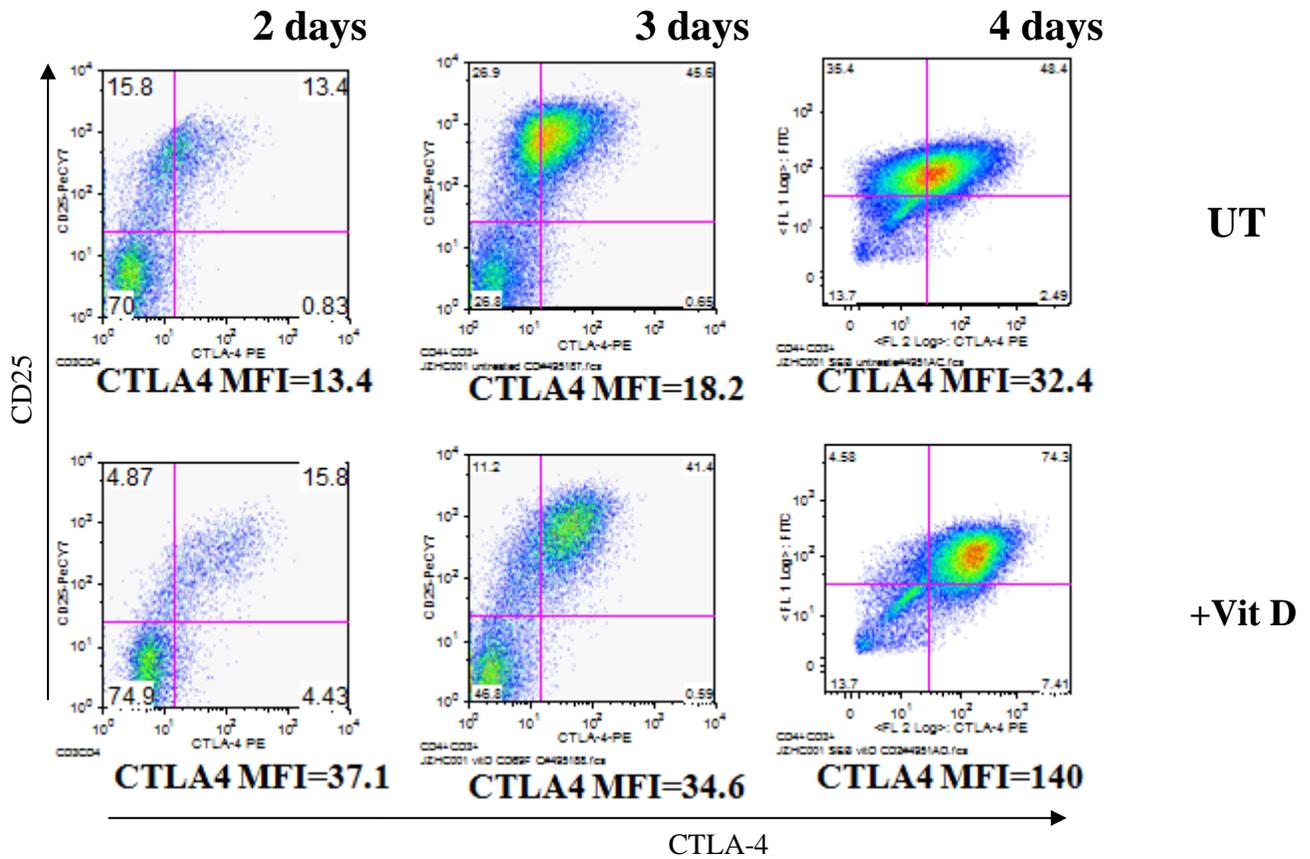


Figure 4.10 Time course analysis of CD25, CTLA-4 and FoxP3 expression by untreated (vitamin D) and 1,25(OH)₂D₃-treated PBMCs in response to SEB. PBMCs were stained with anti-CD3, anti-CD4 and anti-CD25 followed by intracellular staining of CTLA-4 and FoxP3 two days (plots on the left), three days (plots in the middle), and four days (plots on the right) post stimulation. The values of CTLA-4 median fluorescence intensity for CD25⁺ cells were recorded.

T regs in latent TB and health

Characterisation of the T reg cell used antibodies against surface markers CD3, CD4, CD25 and CD127 to stain the live cells and FoxP3 along with CTLA4 once the cells were fixed. The frequencies of FoxP3+ CD127 low CD25+ cells and FoxP3+ CD127 low CTLA4+ cells ex vivo were analysed using flow cytometry to create FACS plots. Cells expressing CD3/CD4 markers were identified, marked and gated onto further plots to identify Fox P3/CD127low cells and these were further gated to identify which were CD25+ and CTLA4+ cells. The frequencies were calculated as follows: The FoxP3+CD127 low population within the CD3+/CD4+ cells were identified and measured. Using Stain 1 (as per table 2.10 Staining panel for Treg cells ex vivo), the population within this group which were CD25+ were identified and measured as a % of the FoxP3+CD127 population. Using stain 2 (as per table 2.10) the population within the group which were CTLA4+ were identified and measured as a % FoxP3+CD127 population which is shown in Figure 4.11 below.

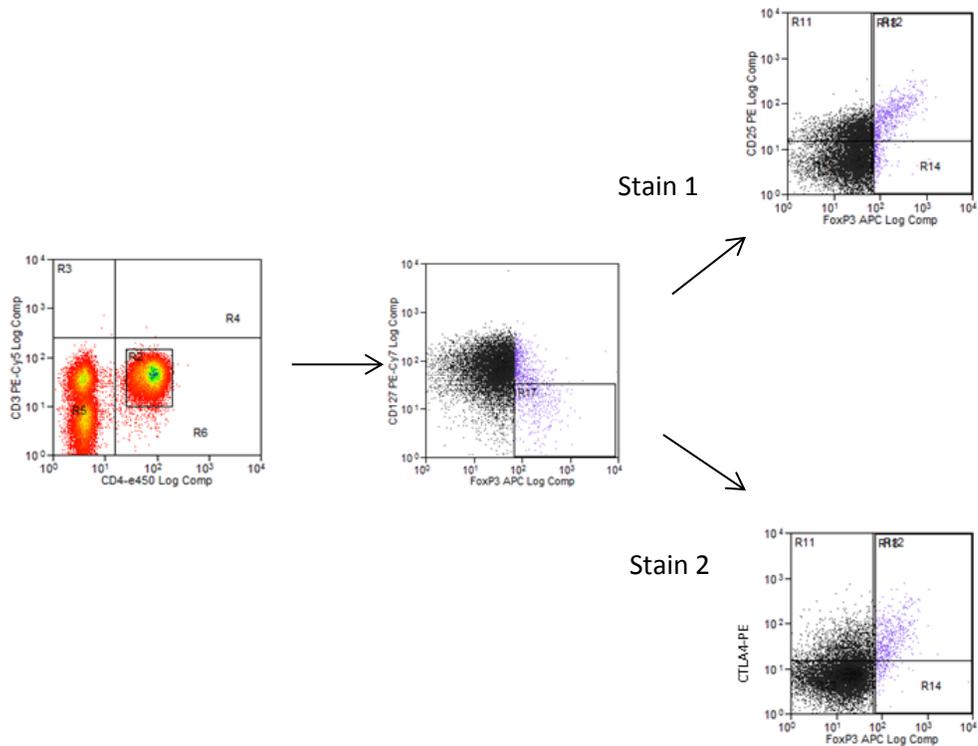


Figure 4.11 Use of FACS dot-density plots to identify characteristic markers in the identification of T reg cells (CD3/CD4, FoxP3 positivity, CD25 positivity and CD127 low expression).

This figure shows that in order to determine the frequency of regulatory T cells, it is necessary to identify the presence / absence of regulatory T cell markers. The cells are stained with anti-CD3, anti-CD4, anti-CD25, anti-CD127 followed by intracellular staining with anti-FoxP3 and anti-CTLA-4.

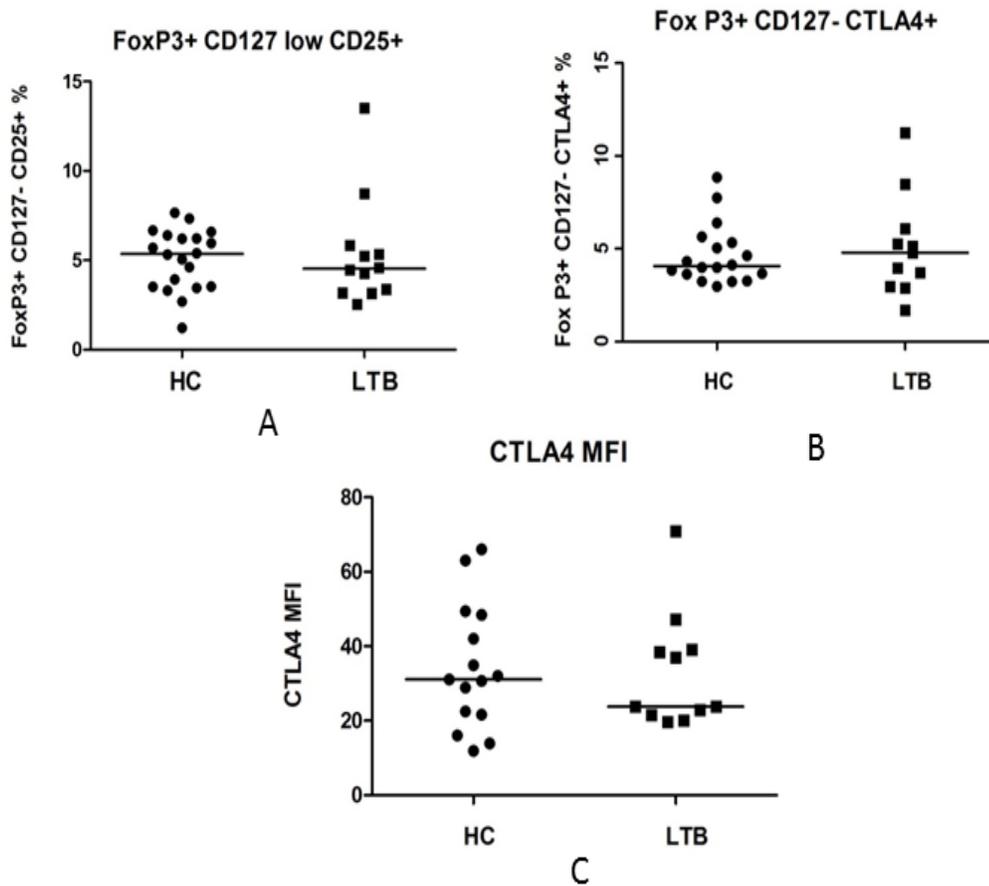


Figure 4.12 Frequency of FoxP3+ CD127 low cells, expressing (A) CD25+ (B) CTLA4+ and (C) CTLA4 MFI in both healthy (HC) and latent TB (LTB) populations.

This shows the frequency of the T reg populations (including median values), identified using the method described above for both health and latent TB. There is no significant difference in the expression of these T reg markers or T reg numbers respectively in health or latent TB. Statistical analysis using Mann Whitney U test $p > 0.05$ for (A),(B) and (C).

Cytokine production ex vivo

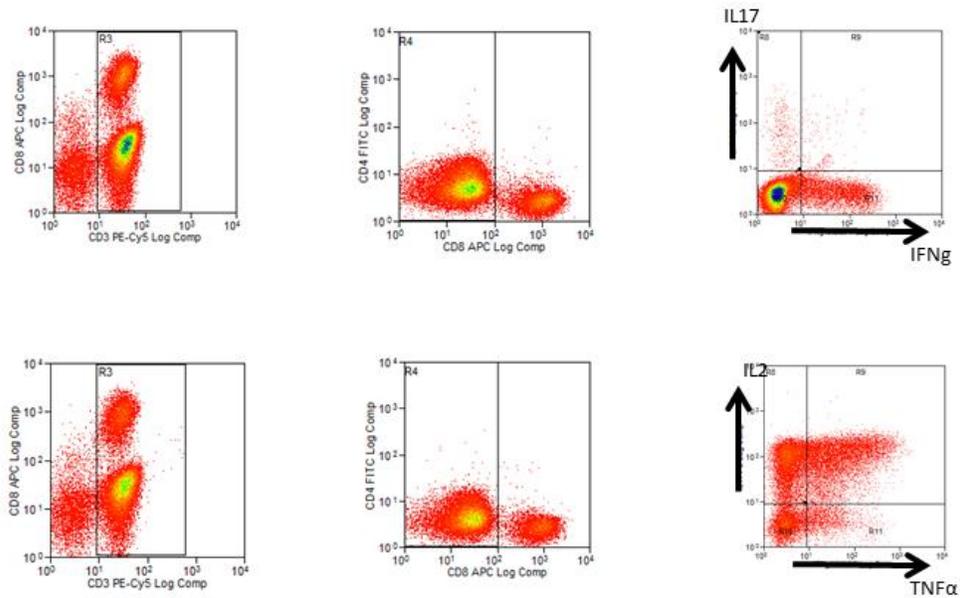
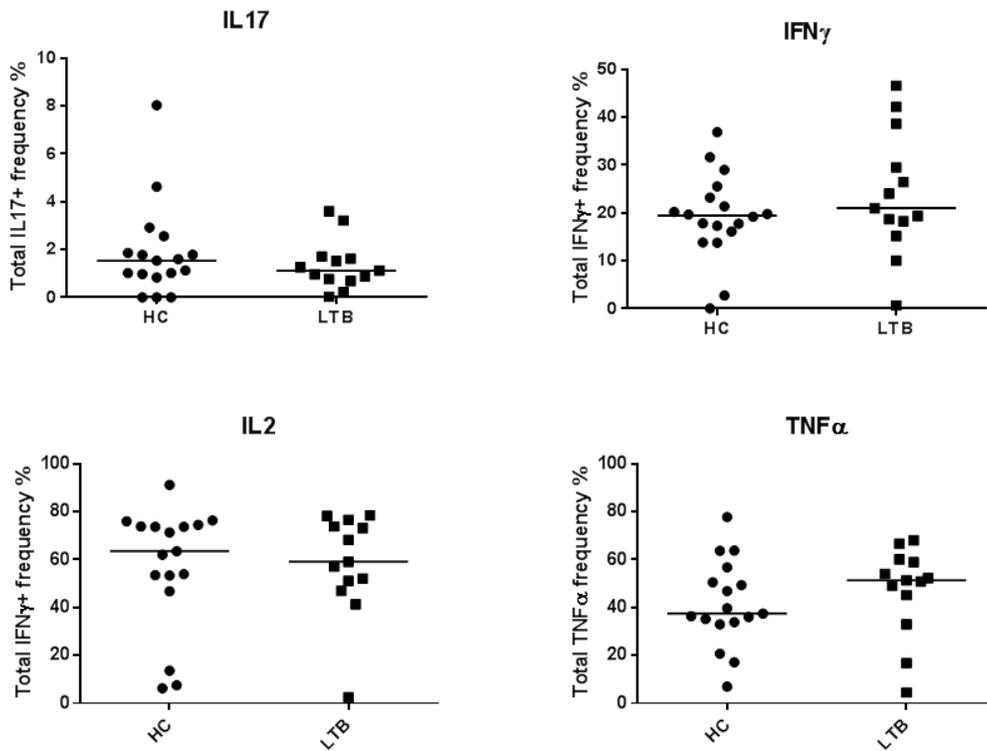


Figure 4.13 Examples of FACS plots identifying CD3⁺/CD4⁺ and CD8⁺ T cells.

Cell stimulation using PMA / ionomycin gave a non-specific response by direct intracellular activation of protein kinase C. The cytokine response (IL17, IFN γ , IL2 and TNF α) was assessed by measuring the frequency of cytokine using flow cytometry. The positive response to antibody markers were measured on the FACS plots by gating onto CD3⁺/CD4⁺/CD8⁻ cells.



Figures 4.14 Frequency of cytokine producing T cells for IL17, IFN γ , IL2 and TNF α Day 1 ex vivo in healthy controls and latent TB cases subsequent to PMA / ionomycin stimulation ex vivo.
 This figure shows the baseline cytokine frequency, as determined using FACS. This shows no significant differences between the health and latent disease groups after culturing T cells ex vivo for 24 hours.

There was no significant difference in Day 1 cytokine production from CD4+ cells ex vivo of any of the above four cytokines $p > 0.05$ using Mann Whitney U test. This suggested that the T cell response to stimulation does not differ between those in health and those with latent disease.

Even the removal of a single significant outlier did not change statistical significance.

Vitamin D - effect on T cells following antigenic stimulation

CTLA-4 and FoxP3 expression post stimulation analysis

The effect of vitamin D on the expression of T regulatory cells (identified by CTLA-4 / FoxP3 markers) was measured using flow cytometry. The effect of vitamin D on the production of cytokines including IL17, IFN γ , IL2 and TNF α was measured using ELISA. The cells were stimulated using either SEB or PPD and the effect of either 1,25(OH) $_2$ D $_3$, 25(OH)D $_3$ or both on the above parameters was recorded, with an incubation period of 48 hours before cytokines were measured.

Below is a comparison of the effect of vitamin D upon the expression of CTLA-4 and FoxP3 by SEB stimulated T cells from healthy control and latent TB infected patients.

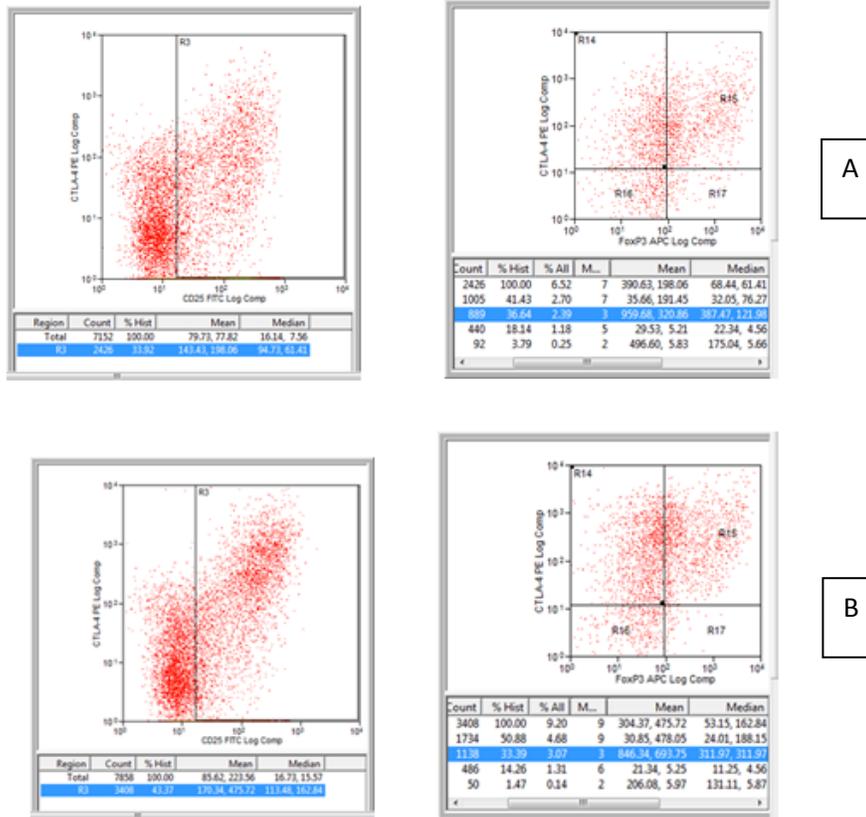


Figure 4.15 FACS plots showing SEB stimulated, untreated (A) and 25(OH)D treated (B) T reg cell marker frequencies in latent TB samples.

This gating strategy (as previously discussed in the methods section) allows evaluation of T regulatory cell marker frequencies in stimulated cells. Using gating methods for determining cell frequencies, the CD25+, CTLA4+ and FoxP3+ T cells can be isolated and further analysed in the form of the above dot / density plots.

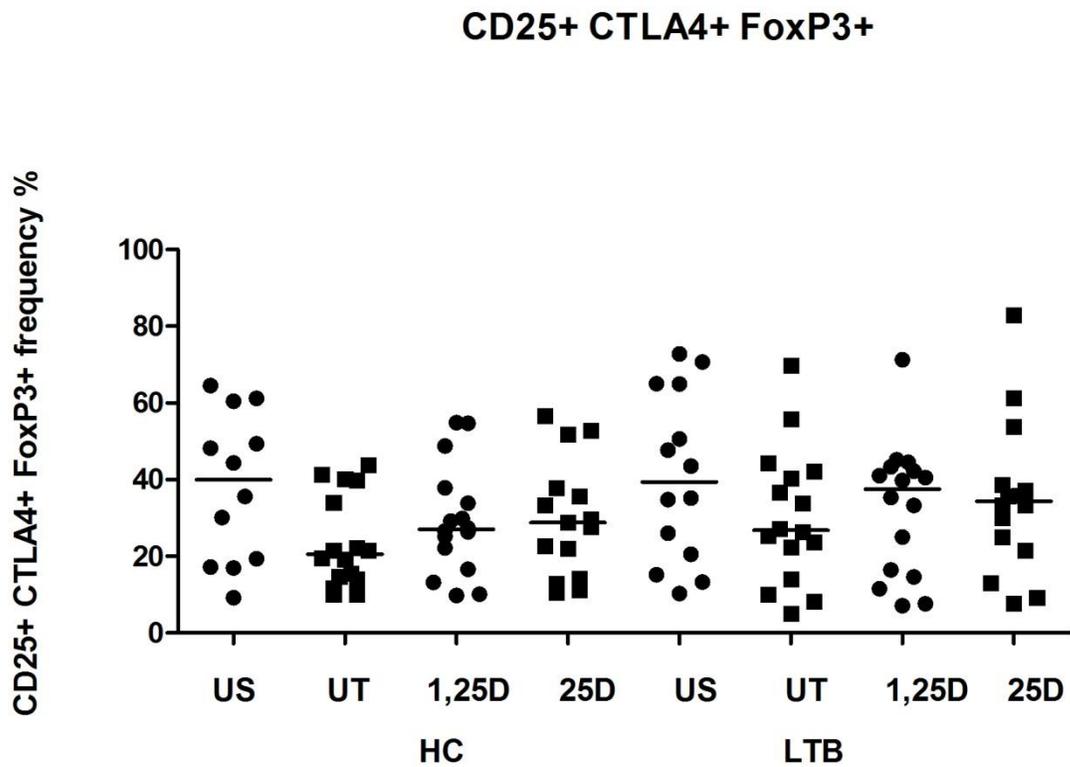


Figure 4.16 Comparison of T reg markers in SEB stimulated cells, incubated with vitamin D in health and latent TB.

Regulatory T cell marker expression rises with vitamin D supplementation in both health and latent disease, but the effect is not statistically significant ($P > 0.05$).

There is a definite albeit insignificant trend to a rise in T reg markers with vitamin D supplementation in SEB stimulated cultures in both health and latent TB. There is no significant difference in the cell response in health and latent TB.

Of note the T reg markers frequency % appears to be higher in the unstimulated population compared to the stimulated but as yet untreated cells. Perhaps the total number of T cells activated by the SEB is greater and the corresponding frequency % of CD25+ CTLA4+ FoxP3+ declines due to this, although absolute values may well be close within each group. It would be expected however that with inflammatory stimulation, the T reg population frequency might change, although this may vary with type of stimulation.

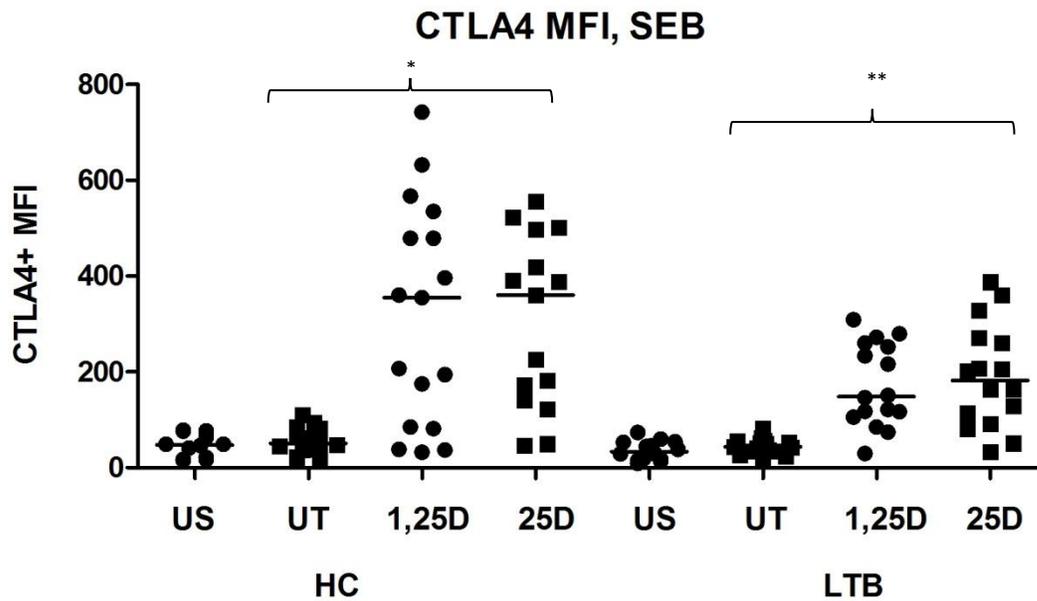


Figure 4.17 The effect of vitamin D supplementation on the CTLA4 MFI in SEB stimulated cultures, measured using flow cytometry.

There is a statistically significant rise in CTLA4+ median fluorescence intensity (MFI) in both health and latent disease with vitamin D supplementations. This is suggestive of a positive effect on regulatory T cells which express CTLA4 and on the inhibitory effect of CTLA4 on T cell priming.

Figure 4.17 shows a statistically significant rise in the CTLA4 MFI in SEB stimulated cells with both 1,25(OH)₂D₃ and 25(OH)D₃ supplementation (*p=0.001). The significant rise in CTLA4 expression with vitamin D supplementation persists in the latent group (**p=0.003), however the effect seems to be blunted in latent TB with significantly different values between health and disease in all but the vitamin D untreated (UT) group. This may indicate that vitamin D has positive effect on the differentiation of T reg cells which express CTLA4 potently. This concurs with the finding that 1,25(OH)₂D₃ was readily produced by DCs upon maturation at a level sufficient to promote an anti-inflammatory T cell phenotype, exemplified by high CTLA4 and decreased expression of IL17 and IFN γ [228].

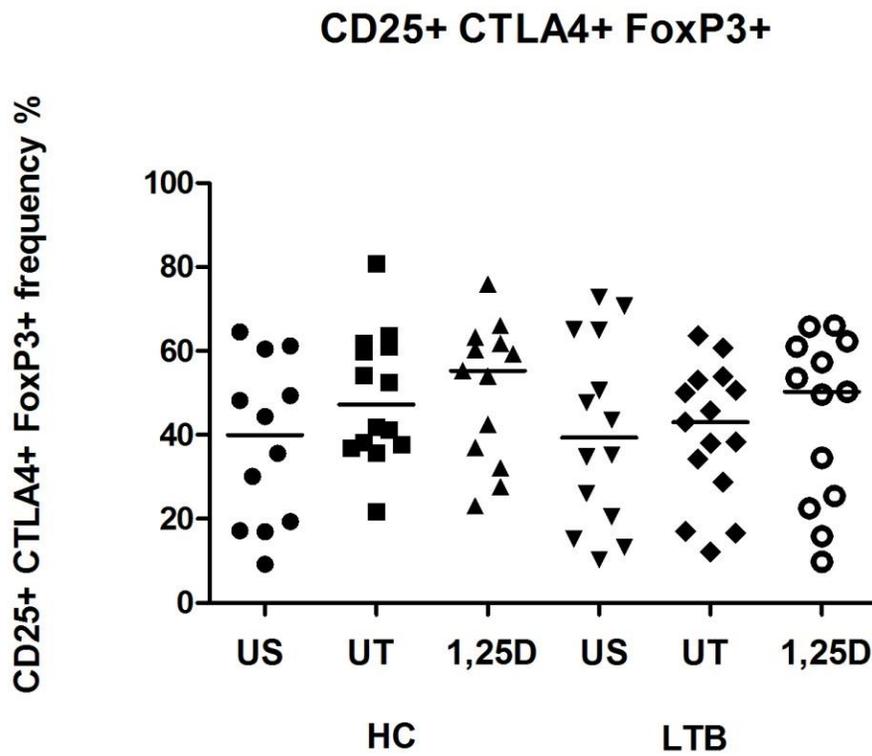


Figure 4.18 The effect of vitamin D on the frequency of Treg markers in PPD stimulated cells, measured using flow cytometry.
 CD25+ CTLA4+ FoxP3+ frequency rises with vitamin D supplementation, although this was not statistically significant. This suggests that vitamin D may have a positive effect on regulatory T cell expression.

There is no statistically significant effect of supplementation with vitamin D on the T reg cell markers in health or latent TB when cells are stimulated by PPD. There is no significant difference between the health and latent disease results of stimulation. Although there appears to be a trend towards a rise in the markers indicative of T reg cells with supplementation in the healthy controls and in latent diseases, this effect is non-significant. This may be due to the small size of samples.

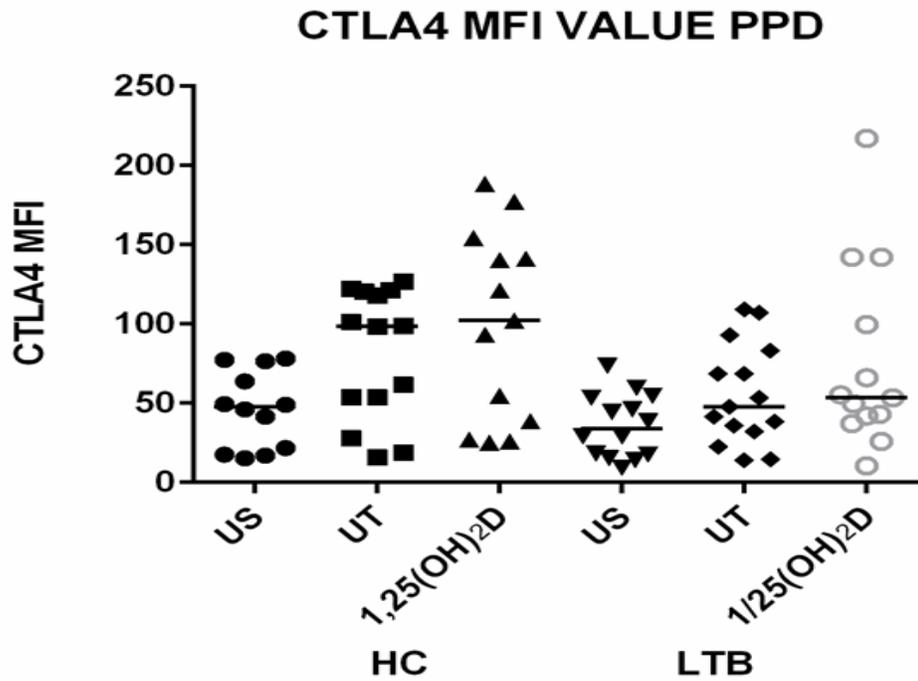


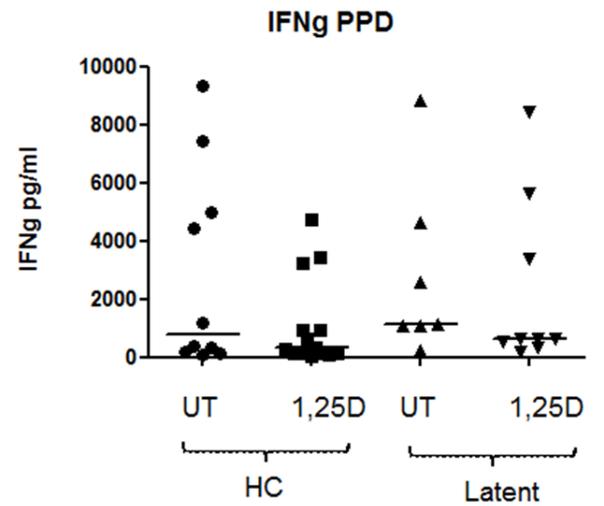
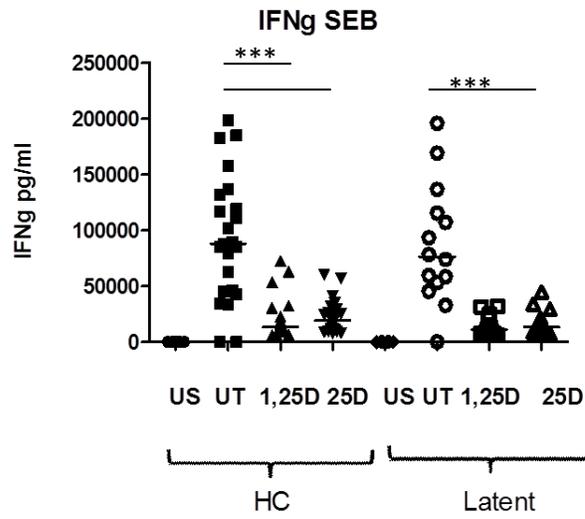
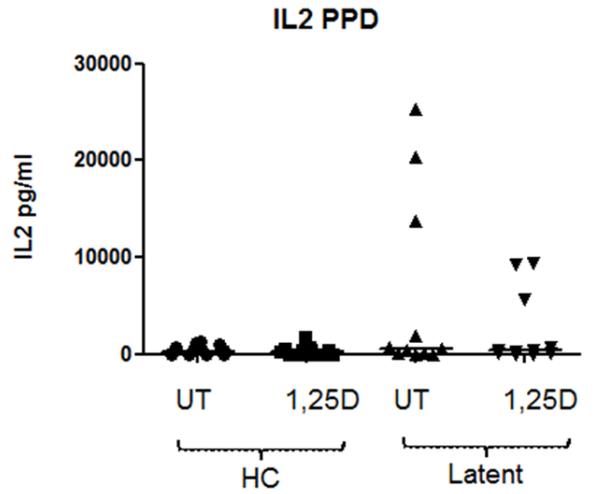
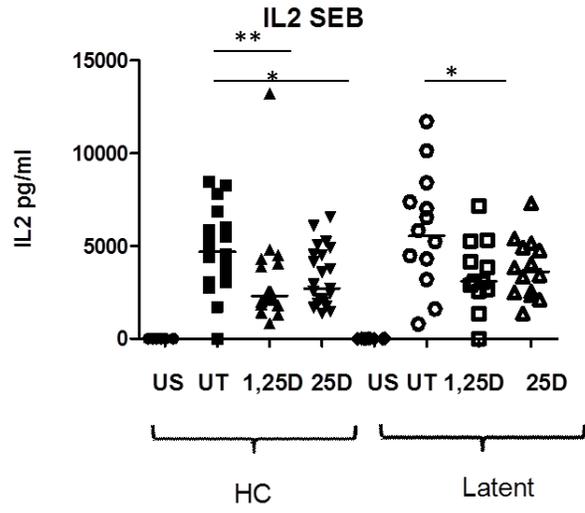
Figure 4.19 The effect of vitamin D on PPD stimulated cells in health and latent TB on CTLA4 expression on T regs.
 CTLA4 median fluorescence intensity (MFI) rises with vitamin D supplementation in both health and latent disease.

Comparing the values of CTLA4 MFI in figure 4.17 which illustrates the effect of vitamin D on SEB stimulated cells, the overall effect is significantly blunted in PPD stimulated cells. This may be due to a problem with the concentration of the PPD in producing adequate stimulation, as it appears to be the case in both the healthy control and latent groups.

The proportion of CD4+ /CD3+ / CD25+ Foxp3+ cells co expressing CTLA4 tended to be higher in the vitamin D treated cells in both healthy and latent patients but did not reach statistical significance.

The CTLA4 MFI rose in both health and latent TB with vitamin D supplementation in cells which were both SEB and PPD stimulated, which suggests there is evidence that vitamin D may effect differentiation of T reg cells which express CTLA4.

Vitamin D - effect of supplementation on cytokine activity



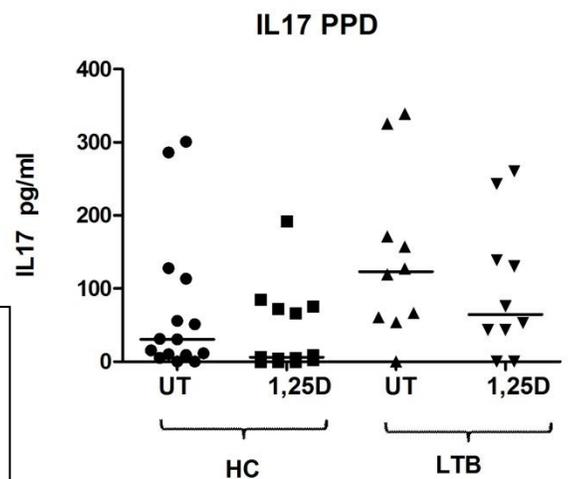
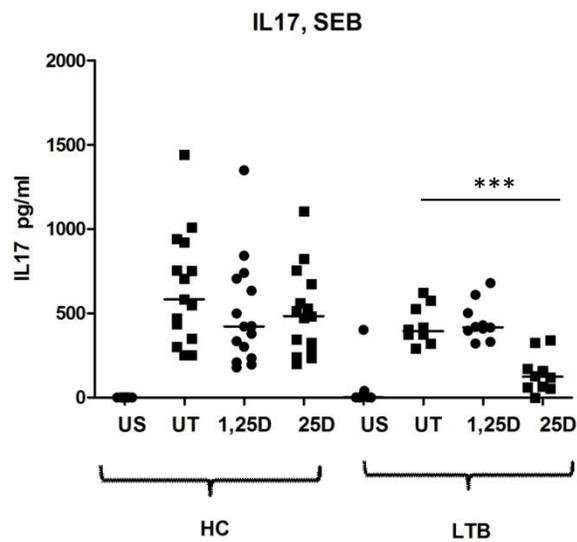
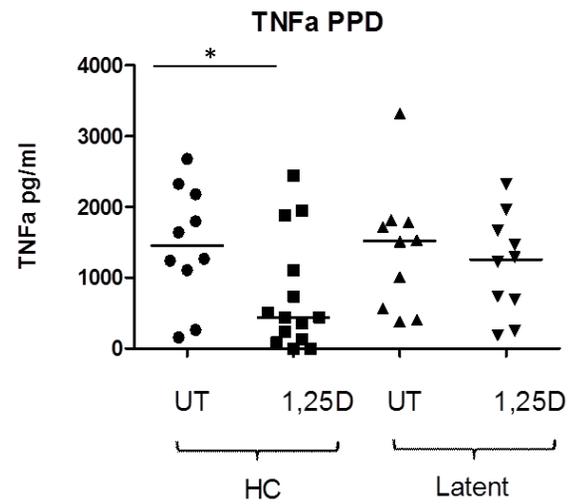
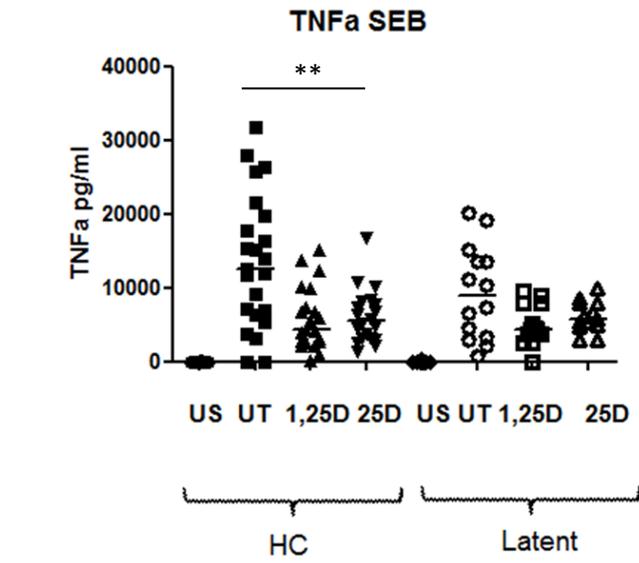


Figure 4.20 Graphs to show the effect of vitamin D on cytokine production in SEB and PPD stimulated cells in both healthy controls and latent TB population.
 *p<0.05, **P<0.005, ***p<0.001.

Figure 4.20 shows that there is a significant reduction in IL2 with both 1,25(OH)₂D₃ and 25(OH)D₃ in health and this is reflected in the latent TB response to 1,25(OH)₂D₃. The IL2 levels of PPD is much lower in the stimulated cells than in SEB stimulated cells which may indicate failure of PPD to stimulate sufficiently to result in IL2 production.

There is a reduction in interferon gamma in the SEB stimulated cells of healthy controls and latent TB patients with vitamin D and a similar trend in the PPD stimulated cells but again, the degree of cytokine production in the PPD stimulated cells is reduced.

TNFα levels in SEB stimulated cells are significantly reduced in response to both 1,25(OH)₂D₃ and 25(OH)D₃ in health and a similar non-significant trend is seen in latent disease. This trend is reflected in the PPD group, with a significant fall in TNFα levels in response to 1,25(OH)₂D₃ in the healthy group.

IL17 levels show overall reduction in levels in both the SEB and PPD groups with a significant effect seen in the SEB stimulated latent group in response to 25(OH)D₃. Again the overall cytokine levels in the PPD group is lower and this is reflected throughout the remaining cytokine values as shown in the plots, with the exception of TNFα which shows a similar cytokine response when stimulated by both SEB and PPD.

The levels of cytokines were comparable between the healthy and latent populations.

There does not appear to be a significant difference in the response to vitamin D between health and latent TB indicating that T cells of TB infected cells are unlikely to be defective in their immune response.

4.1.2 The effect of genetic polymorphisms of the vitamin D axis in determining the in vitro / ex vivo response to vitamin D supplementation in patients with mycobacterial infection

This section relates to hypothesis 4 which aims to evaluate if genetic polymorphisms of the vitamin D axis determine the in vitro / ex vivo response to vitamin D supplementation in patients with mycobacterial infection.

Monocyte Genetics

Studying the same SNPs mentioned above statistical analysis using Using Mann Whitney U and Wilcoxon tests showed that there was no significant association between any of the above SNPs and vitamin D response (measured by IL6 suppression) in monocyte supernatants.

Regarding the addition of pooled plasma of varying DBP genotypes, this resulted in a reduction in cytokine production which was significant in GCSF and IL6. There was a significant drop in IL6 cytokine production on addition of pooled plasma when compared to control (LPS +25D) in GC1s1s, GC1sGC2 and GC1f1f. There was no significant difference in response between GC2 and non GC2 carriers.

For GCSF there was no significant change in cytokine production with addition of vitamin D to culture ($p=0.225$) but there was a significant difference in GCSF production with lower levels ($p<0.05$) in all but GC1sGC1f genotype (although this nearly reached significance $p= 0.068$ and

may have been affected by the loss of a single result in the group for analysis). There was no difference in response between GC 2 carriage and non GC2 carriage.

For IL1B there was no significant change in cytokine production with addition of vitamin D to culture. There was a significant difference in cytokine production between the genotypes shown in figure 4.4. There was no significant difference in response between GC2 carriage and non GC2 carriage.

Further discussion around this is found in the discussion section.

Unfortunately due to low numbers, genetic analysis of T cell function / response to vitamin D was not carried out and this would be useful to consider in future work.

4.2 Discussion

4.2.1 The Immune Modulatory Effect of Vitamin D in Vitro and ex Vivo, in health and Mycobacterial Disease

Functional Effect of Vitamin D / DBP on Stimulated Monocytes in Health and Disease

Part of the third aim of the study was to explore the effect of vitamin D and TB on monocyte function, concentrating on the effect of vitamin D on stimulated monocytes and its effect on inflammatory cytokine (IL6) and antimicrobial (LL37) production in healthy subjects, active and

latent TB patients in order to ascertain the mechanism by which vitamin D may be beneficial, and determine if monocyte response to vitamin D is impaired in TB.

During the initial response to infection macrophages produce pro-inflammatory cytokines. To compare the effect of vitamin D on stimulated cells, mimicking the effect of an infectious stimulus, monocytes of TB, latent TB patients and a control group were cultured for 48 hours as described in the methods section and stimulated with LPS and subsequently cultured with 25(OH)D₃. The IL6 production was measured in the supernatant of the cultures by ELISA and comparisons in the response analysed.

LPS was chosen as it is commonly used as a stimulant in monocyte culture, however an alternative such as TNF α would be interesting to pursue in further experiments in cultures from TB patients and those without the disease, as this is also a potent inducer of IL6, but levels in TB patients may be variable when compared to healthy controls, thereby potentially affecting the therapeutic dose of vitamin D that an infected individual may require when compared to health. Previous study by Kent et al has illustrated this dose dependent response of IL6 to TNF α [229].

The optimisation experiments were carried out in order to determine the optimal concentrations of LPS and vitamin D to use in the monocyte culture. Both the LPS concentrations and vitamin D (25(OH)D₃) gave a dose dependent response in IL6 levels. LPS, used to stimulate an inflammatory response in the monocytes has been shown to upregulate IL6 levels previously in human lymphatic endothelium, most likely through activation of NF-k β [230]. However, the mechanism for this may vary according to the cell type analysed. Although the measurement of IL6 by ELISA is a useful tool, further experiments using IL6 mRNA levels would be of interest as previous studies have also identified a dose dependent relationship with vitamin D and the suppression of mRNA levels [231].

The optimisation experiments (Figure 4.1) showed that maximal IL6 expression was found with LPS concentrations 100 ng/ml, IL-6 levels leveling off at higher concentrations.

25(OH)D₃ levels of 50nmol gave maximal IL6 suppression and this correlates with prior work showing that vitamin D inhibits LPS-induced p38 phosphorylation in human monocytes [231] and although the cells in the study were pre-treated with vitamin D prior to LPS stimulation there still appeared to be a dose dependent response.

Vitamin D concentrations in vitro don't necessarily correlate to the circulating levels available in vivo which are considerably lower. The concept of the experiment however was to look at the effect of vitamin D on circulating cytokine levels and this could then be extrapolated into therapeutic considerations e.g. direct administration into the airways etc.

Measurement of VDR mRNA along with mRNA in other genes may be worth consideration in future experiments in order to determine the effect of vitamin D and LPS on this. Earlier experiments suggest that LPS and 1,25(OH)₂D₃ synergistically induces the VDR mRNA in THP-1 cells [232].

In this study pre-treatment with LPS was not shown to affect the IL6 response to vitamin D and therefore experiments were subsequently all carried out without pre-treatment. Previous studies have shown pre-treatment with LPS to enhance or potentiate the IL6 response even using sub-stimulatory levels of LPS prior to complete activation [233]. Others however suggest the possibility of chronic stimulation where infection can exist in a 'latent' state which, although there are no overt clinical signs, there is still activity / turnover of the bacteria at undetectable levels. This concept is further considered when the cytokine response in latent and active disease and health is observed.

IL6 is known to be a pro-inflammatory cytokine, but murine experiments have identified an anti-inflammatory role in moderating other inflammatory response such as TNF α production [234]. Hence if certain conditions were to modify the ability to produce IL6, then perhaps other cytokine responses would continue unchecked, resulting in greater pathology. Whether supplemental vitamin D would modify the IL6 response to the detriment of the individual depends on the effect of vitamin D on other pro-inflammatory cytokines in addition to IL6. This raises the consideration whether supplementing patients who are severely vitamin D deficient with TB is the correct decision, although the risks vs benefits of other detrimental effects of vitamin D deficiency also needs to be considered, or whether altering the timing of vitamin D administration until after the initial phase of TB therapy may be wise.

The monocyte experiments carried out in controls, latent and active TB patients show the variation in mean IL6 response at 48 hours between the groups with controls reaching levels of almost 4500pg/ml IL6, whilst the TB patients and latent patients reached 2700pg/ml and 1200pg/ml respectively. Consideration needs to be taken when looking at the range of results. Previous experiments have indeed shown that as time proceeds, the LPS stimulated IL6 response would be expected to decline from initial measures, but there is clearly a significant variation at identical time points between those with no disease and those with disease.

In addition, it has been previously noted that CD4⁺ lymphopenia and a reduction in CD4⁺/CD8⁺ ratio is reduced in tuberculosis infection [235], likely resulting in an imbalance in the cytokine response to chronic infection with M.tb. This may in part account for the lower baseline IL6 levels following LPS stimulation in latent and active TB compared with the control population in this study.

Within the groups of control, latent and TB patients, there were also clearly subgroups who showed significant variation in their response to vitamin D in its attenuation of the IL6 response. This has been categorised as responders and non- responders for the purpose of this study and the reasons for this are discussed further in section 4.1.1.

Graphs (A) to (C) figure 4.3 indicate the difference between the IL6 production in disease and controls with and without vitamin D. Explanation of this data is given in the results section.

It may have been interesting to ascertain the IL6 response in those patients who had been treated and received vitamin D supplementation and if their subsequent results correlated at all with the original healthy population. This was not however possible due to the small numbers recruited for the monocyte response aspect due to drop out rates / failure to attend of those who were recruited.

The effect of Vitamin D on Stimulated T Cells in Health and Disease.

Part of the third aim of the study was to explore the effect of vitamin D and TB on Treg and Th17 balance by in vitro work to look at the effect of vitamin D on stimulated T cells and its effect on relevant subsets in healthy subjects, active and latent TB patients in order to ascertain the mechanism by which vitamin D may be beneficial, and determine if T cell response to vitamin D is impaired in TB.

T reg analysis ex vivo

FoxP3⁺ T cells are a subgroup of CD4⁺ T cells. Using flow cytometry, T regs in healthy control and latent TB population were identified using the methods described in section 2.3.9. This study shows that there is no significant difference in the expression of these T reg markers or T reg numbers respectively in health or latent TB and is illustrated in figure 4.12.

The presence and activity of T regulatory cells in tuberculosis has only recently been considered in detail. It is known that T reg cells tend to accumulate in areas of infection with M.tb [236]. However latent TB differs from active TB in that there is no clear site of disease activity visible and hence the activity and location of T reg cells in this population may vastly differ. The effect of T reg cells in acute infection has been discussed in the introduction of this thesis, highlighting the theory that whilst controlling the pathogenic effects of disease, they may be responsible for persistence of infection as a secondary effect.

However previous studies have shown both an increase in peripheral T reg numbers in early infection [237] and decrease in T regulatory FoxP3⁺ cells in active tuberculosis. There is little definite information regarding latent disease and the fact that only a small proportion of those who are infected with M.tb progress to develop active disease suggests that further research is needed to determine which elements of the T regulatory cell response are key to determining progression to disease. The presence of pathogen specific Treg [238] cells is possible with the effect on the individual being secondary to a balance between cytoprotective or cytopathogenic effects of the T cells.

Murine studies by Scott-Browne et al identify increased T reg numbers in areas of disease activity / granuloma formation [239] only. Findings by Semple et al also noted higher T reg levels in active disease compared to latent disease in washings from bronchoalveolar lavage (BAL) [236].

Therefore in peripheral blood samples, one may not expect a significant difference in T reg numbers between latent and healthy populations as shown in the above experiment. This is unsurprising given the above reasons of disease inactivity / slow mycobacterial turnover in latent disease. It would be interesting to perform the same experiment with an active TB population to determine values in relation to these groups also. Unfortunately due to limited numbers recruited for this part of the study and time limitations this was not possible. It may also be worthwhile considering lymph node analysis of T reg cells in latent and healthy populations, to determine the degree of disease control which T reg cells may be responsible for in the latent population.

A small study by Singh et al comparing TB patients with healthy controls noted an expansion in the T reg population which was greater in those with higher bacillary load in their sputum (i.e. greater infectivity). The T reg population declined towards normal with treatment but stayed elevated in those with treatment / drug resistant disease, with higher levels of IL10 production as a result of disease activity thereby suppressing host protective T cell response [240].

Clearly there is an imbalance in certain situations, resulting in disease persistence which is likely to be multifactorial, however the possibility of vitamin D affecting this process is an interesting consideration and one which is discussed later in this section. The effect of vitamin D during an infective process such as TB could be considered potentially harmful if the effect of expanding the T reg population were to impact on the protective immune response in a negative way.

The measurement of CTLA4 MFI was also incorporated into the T cell analysis as shown in Figure 4.12. This is because cytotoxic T lymphocyte antigen (CTLA4) is involved in T cell tolerance as it acts as an inhibitory T cell receptor. It is always present on CD4+ FoxP3+ cells (T reg cells) and in the absence of either CTLA4 or FoxP3 devastating autoimmune disease is present. The expression of CTLA4 rises with T cell receptor activation and therefore measurement of CTLA4 is a way of determining activity of T reg cell in disease and health once activated by a stimulus and whether this varies according to severity of disease and exposure to possible immune modulators such as vitamin D. This is further discussed in the section below titled ‘CTLA4 and FoxP3 expression post stimulation analysis’.

A murine study by Jain et al showed that CTLA4 expression in activated T cells limited pathology and tissue destruction [241]. It may therefore be hypothesised that CTLA4 MFI would be higher in patients with TB, particularly in those with tissue damage secondary to infection.

This study showed no significant difference in the CTLA4 MFI in healthy controls compared to latent disease which would correlate with the findings above of no significant difference in CD4+ T reg population numbers in these two populations along with the fact that latent TB does not produce the same cellular response and tissue damage as active disease.

Cytokine Production ex vivo

The cytokine response (IL17, IFN γ , IL2 and TNF α) was assessed by measuring the frequency of cytokine using flow cytometry. This study found that there was no significant difference in cytokine production between health and latent disease from CD4+ T cells Figure 4.14.

The importance of individual cytokines in pathology of disease is highlighted in previous studies which have shown that TNF α inhibits the suppressive function of T reg cells [242], thereby potentially resulting in increased disease activity, with the use of anti-TNF α antibody having the effect of enhancing the number of FoxP3+ T reg cells [243], highlighting the importance of T reg balance in disturbed immunity resulting in disease.

FoxP3, by interacting with NF κ B will suppress the production of certain cytokines including and IFN γ , IL2, IL4. This suppression of cytokines such as IL4 allows the suppression of disease activity / progression in this way.

The findings in this study are that there is no significant difference in the day 1 ex vivo production of IL17, IFN γ , IL2 and TNF α between health and latent disease, suggesting that there is no significant difference in T cell activity between these two groups one day post stimulation. The use of disease specific stimulation using ESAT-6 or CFP-10 would be of interest to determine if the cytokine response differs with a more disease specific mycobacterial stimulus. However Marin et al reported that the number of IFN γ producing cells was not significantly different between latent disease and health (when stimulated with either CFP10 or PPD) [244]. Future work may be worthwhile to assess further longer term cultures to evaluate the memory cell activation effect on the cytokine response, as the 1 day cytokines may only reflect a single snapshot and not that of the

disease process. Culture time periods may also be affecting the cytokine responses. Marin et al also identified variability in the cytokine response between active and latent disease depending on the duration of culture, with CD4+ IFN γ levels being notably higher in the early stage of culture in active TB, and CD8+ IFN γ release being higher in the latent group [244].

In addition, the functionality of CD8+ specific T cells in the same situation may also be a useful comparator.

Studies looking cytokine response in health vs. active TB are limited in number. Few are related directly to latent disease, the majority concentrating on IFN γ assays and titres. This is likely due to the fact that currently the key diagnostic test for latent TB is to assay TB antigen specific IFN γ titres (T-spot / QFN Gold Assays).

The nature of the immune response to mycobacterial antigens is not just related to a single cytokine and measuring thus may underestimate the entirety of the immune response. Due to the polyfunctional nature of the T cell response the presence of a 'functional signature' has been considered and the detection of polyfunctional T cells (producing multiple cytokines IL2, IFN γ , TNF α) have been found to be lower in active disease [244] with lower IFN γ +IL2+ dual secreting CD8 cells in active than in latent disease, possibly conferring a protective function in latent [245]. In other situations, bifunctional IFN γ (+) TNF α (+) CD4(+) T-cells have been significantly increased in active vs latent disease. Therefore the situation is unclear as to where the balance lies, and whether there may be differing responses according to the pathology caused, i.e. response in the lungs differing to the peripheral values.

IL2, which is produced by activated cells, is known to be associated with T cell tolerance through expansion of the T reg population. Relating this to the clinical population, the current cytokine

related diagnostic tests for TB involve measurement of IFN γ titres or production following antigenic stimulation of the individuals T cells, but this unfortunately does not differentiate between active and latent disease. A previous study by Millington et al looked at IL2 response in active and treated TB [246] , showing co-dominance of IFN-gamma-only secreting and IFN- γ /IL-2 dual secreting CD4+ T cells in active disease that shifted to dominance of IFN-gamma/IL-2-secreting CD4+ T cells and newly detectable IL-2-only secreting CD4+ T cells during and after treatment. Whether IL2 assay can help to determine the disease state was the subject of a recent systematic review [247] which suggests that IL2 assay may be a useful tool in differentiating between active and latent TB . The conclusion of the review was that when there is no definite gold standard for the diagnosis of LTBI, the IL-2 release assay in addition to IGRA can improve the ability of IGRA to identify individuals with recently acquired latent TB infection. The fact that this study did not show this may be due to the fact that the IL2 assays were based on cytometric analysis of cell cultures, rather than extraction and ELISA analysis from whole blood directly. It would be useful to compare the cytometric analyses in latent and active disease compared to whole blood ELISA analysis in future work.

Finally the IL17 cytometry results are similar to the other cytokine responses in latent and health with no significant difference between the two groups. This is expected given that IL17 is increased during a pro-inflammatory response to mycobacterial infection, resulting in tissue damage. However in latent and active disease, the cytopathogenic picture is not seen as it is in active disease. Further analysis of the same key cytokines in active disease would be useful for comparison.

CTLA-4 and FoxP3 expression post stimulation analysis

This section of the study looks at the effect of stimulation on the T reg proportions and markers in response to stimulation with SEB or PPD and thereafter the response to supplemental vitamin D within the culture. In turn the CTLA4 (immune regulatory receptor) MFI is assessed under the above conditions, comparing health and latent populations response.

T reg frequency appears to rise with vitamin D treatment in both SEB and PPD stimulated T cells, although this does not reach statistical significance as shown in figure 4.16 and 4.18. Both 1,25(OH)₂D₃ and 25(OH)D₃ have this resultant effect and is likely to be explained by the fact that T cells constitutively secrete α-1hydroxylase resulting in conversion to 1,25(OH)D₃ intracellularly.

This finding concurs with previous study by Urry et al which found that in vitro frequency of FoxP3+ cells increased with 1,25(OH)₂D₃ supplementation, allowing selective expansion of this group of cells [248] and the findings of Correale et al [249] which show that culture of PBMC in the presence of vitamin D significantly increases the proportion of regulatory T cells. In contrast a study by Khoo et al [250] shows that naturally occurring T reg cell proliferation (i.e. those produced by the thymus which are not induced by antigen presenting cells) is inhibited by vitamin D, highlighting the importance of considering the effect of vitamin D in health and disease and the possible differing effects on immune suppression depending on the disease state. The finding in this study suggests that the immune response is similar in health and latent disease.

Further work of interest would be to confirm differences in active disease states in the proliferation of T regulatory cells and the effect of vitamin D on this activity in disease states. Studies such as

that by He et al [251] suggest that *M. tuberculosis* infection induces circulating CD4⁺CD25⁺FoxP3⁺. Whether vitamin D in this context is beneficial may be debatable and the possibility of inducing a chronic immune modulated disease state by further induction of regulatory immune response should be considered.

Acting as an inhibitory T cell receptor, CTLA4 is responsible for regulation of immune response. Analysis of the effect of vitamin D on the CTLA4 MFI showed that there is a statistically significant rise in the CTLA4 MFI in SEB stimulated cells is noted with both 1,25(OH)₂D₃ and 25(OH)D₃ supplementation. This regulatory effect of vitamin D correlates with previous findings by Jeffery et al [252] and the findings concur with a previous study which determined that 1,25(OH)₂D₃ was readily produced by DCs upon maturation at a level sufficient to promote an anti-inflammatory T cell phenotype, exemplified by high CTLA4 and decreased expression of IL17, IFN γ [228].

The CTLA4 MFI values are blunted in PPD stimulated cells overall, both in health and latent TB. The reason for this is unclear but may be multifactorial. This may be due to a problem with the concentration of the PPD in producing adequate stimulation, as the blunted response is common in both the healthy control and latent groups. There may have been a more non-specific T cell response to PPD and this may not have therefore been identified in this experiment looking specifically at activated regulatory T cells. Alternatively, the culture may not have been of adequate duration and this may have resulted in a suboptimal response being identified.

The latent group also show a reduced CTLA4 MFI response and the reason is again unclear, but disease specific T reg identification should be considered, particularly at disease sites, rather than relying solely on peripheral measurement. Peripheral blood measures may not be reflective of the immune state at sites of disease for example in the lymph nodes or lavage washings from the lungs.

Overall the findings support the role of vitamin D enhancing the expression of both T reg markers and CTLA4 immune regulatory receptor, confirms findings of previous work and is further suggestive of the immune regulatory role of vitamin D.

Vitamin D - effect of supplementation on cytokine activity

SEB and PPD stimulated T cells were cultured in the presence of both 1,25(OH)₂D₃ and 25(OH)D₃. The findings clearly show a significant reduction in IL2 IFN γ TNF α , and under certain conditions IL17 production with the supplementation of vitamin D (either 25(OH)D₃ and / or 1,25(OH)₂D₃). Figure 4.20 summarises the effect of vitamin D on cytokine production in T cell cultures.

The findings show that vitamin D results in a reduction in cytokine levels in health and this is reflected in the latent TB response. The response to PPD is blunted in all groups and this may indicate failure of PPD to stimulate the sufficiently.

The resultant cytokine response to vitamin D may be multifactorial, including a reduction in the ability of the T cells to stimulate antigen presenting cells, as they require stimulation in order to produce the resultant cytokine response. Another reason may be direct inhibition on T cell expression. VDR binding sites within cytokine promoter / effector genes have been identified, supporting the likelihood of such a mechanism of action of vitamin D. Chang et al identified a post transcriptional mechanism of suppression of T17 cell function (IL17) [253] by VDR as a result of 1,25(OH)₂D₃ treatment as did Selvaraj et al on IFN γ production [254].

The levels of cytokines were comparable between the healthy and latent populations and there does not appear to be a significant difference in the response to vitamin D between health and latent TB indicating that T cells of TB infected cells are unlikely to be defective in their immune response.

4.2.2 The effect of genetic polymorphisms of the vitamin D axis in determining the in vitro / ex vivo response to vitamin D supplementation in patients with mycobacterial infection

Studying the same SNPs mentioned above statistical analysis using Mann Whitney U and Wilcoxon tests showed that there was no significant association between any of the above SNPs and vitamin D response (measured by IL6 suppression) in monocyte supernatants. However, the experiments with pooled plasma containing a range of DBP genotypes from patient plasma sources on cytokine production from monocyte cultures yielded some interesting results. Monocytes are incompletely differentiated but stimulation results in effects which are essentially antimicrobial and make them ideal cells to use in vitro for analysis of the effect of various stimulants / suppressors of immune function.

Chun et al [255] identified the ability of monocytes to take up DBP at low level, without megalin mediated mechanisms in order to direct vitamin D to CYP27a1 for hydroxylation intracellularly. By adding pooled plasma to monocytes of differing genotypes the study by Chun et al was able to determine if cathelicidin production could be affected in this way and that it is likely that genotype will affect the response to vitamin D according to variations in affinity of DBP.

Jefferey et al [256] noted that the cytokine response of T cells could be affected by the availability of vitamin D to dendritic cells. By converting vitamin D to its active metabolite, this could increase the bioavailability to T cells which inherently, due to lack of Cyp27b1 expression are unable to use 25(OH)D₃ to establish a normal cytokine response. The presence of DBP appeared to play a role in the availability of 25(OH)D₃ for immune regulation.

In a similar way, this study aimed to determine if cytokine response could be affected by the presence of varying DBP genotype plasma in the monocyte culture, thereby resulting in varying availability of vitamin D to the cells. Possible problems with this method would include the use of high levels of vitamin D in the culture which may saturate the DBP and thereby lose the effect of its differential affinity for vitamin D. Also the baseline vitamin D levels after storage of the samples, which were then pooled are not known. By pooling, the heterogeneity of the effect is reduced and the likelihood is that the levels were low as the plasma was retrieved from TB patients used in the earlier experiments and thus it was known that the levels of vitamin D in these patients were significantly lower than in the general population. Therefore the additive effect of the vitamin D supplementation to the culture would likely outweigh any variation in the pooled plasma levels between genotypes.

By affecting the bioavailability of vitamin D to monocytes, this may in turn affect the cytokine response.

The four cytokines analysed were GCSF, IL6, TNF α , and IL1 β . These were measured in relation to exposure to pooled plasma from various DBP genotypes. The DBP effects transport of vitamin D intracellularly and thereby modifies the release of inflammatory cytokines as a result. The hypothesis that variation in the DBP genotype may affect susceptibility to tuberculosis with

particularly low vitamin D levels has already been studied [257]. However this in vitro work looks more closely at four of the cytokines listed above and the effect of genotype on inflammatory response, in the presence of vitamin D sufficiency.

GCSF was chosen to be measured due to the fact that previous studies have identified that GCSF modifies pro-inflammatory cytokine release from monocytes such as TNF α , IL1 β and IFN γ [258].

GCSF which is secreted endogenously by monocytes, used in the described experiments has been traditionally used to increase neutrophil counts in patients who had neutropenic sepsis secondary to chemotherapy. It can be released from a variety of sources if it is stimulated by an appropriate cytokine e.g. TNF α / LPS etc. and it increases phagocytic function and antibody mediated killing by its effect on neutrophils and a reduction in neutrophil apoptosis [259]. Therefore it is an interesting cytokine to study in relation to TB and whether its activity is modulated by vitamin D or genotypes of plasma which may in turn effect differing responses to infection and degree of phagocytic killing.

Previous studies have noted that GM-CSF concentrations were significantly higher in vitamin D severely deficient individuals as compared to vitamin D sufficient individuals [260]. Murine studies have also shown that VDR-/VDR- mice are unable to respond to GCSF in mobilisation of hematopoietic stem/progenitor cells which indicates the pluripotent effect of the vitamin D axis on the immune response [261].

The results in this study show that GCSF levels decline with the addition of pooled plasma in the Luminex assays carried out on monocyte cultures. They did not however significantly decrease with the addition of vitamin D (small number of samples with wide variation in GCSF levels on analysis). A possible reason for the significant decline on adding plasma within the culture medium was that the levels of GCSF in plasma are normally low, rising with infection [262] and declining

with recovery. Although the elevated production of GCSF with LPS stimulation was evident this may have been counterbalanced in some way by the plasma. This may suggest binding, inactivation or dilution of the GCSF by an element in the plasma, though clearly it is still detectable by the Luminex assay as shown in the figures even after the addition of plasma.

Interestingly as shown in Figure 4.4, the expected suppression of LPS with 25D in this group did not occur in the GCSF group. This effect may not have been apparent due to small numbers or the wide variation in GCSF titres obtained. Further analysis of larger study cohort would be helpful.

IL6 expression in the presence and absence of vitamin D in vitro has been discussed in the previous results section, noting significant reductions in IL6 production in responders to vitamin D supplementation. Indeed figure 4.4 shows a reduction in IL6 with vitamin D supplementation in the presence of LPS stimulation of monocytes. Although the graph indicates no significant difference in IL6 production between LPS and LPS+25(OH)D₃, it can be seen that one of the six results is an outlier, and once this is removed from the calculation, there is a statistically significant reduction in IL6 with vitamin D supplementation ($p=0.043$) which correlates with the findings in the previous monocyte experiments.

The presence of DBP containing plasma results in a significant reduction in IL6 despite the continued presence of LPS in the culture media. This theme is reproduced in all but the IL1 β graphs and suggests perhaps that the addition of plasma at the concentration / dilution chosen may have an effect on suppressing cytokine expression. However it is notable that the inflammatory response varies with genotype.

IL1 β is an inflammatory cytokine responsible for the activation of blood monocytes. The trend for IL1 β remains the same with the addition of plasma but the initial IL1 β response to LPS induction is blunted compared to the other cytokines and this is the case for all of the healthy control monocytes with no outliers in the group.

IL1 β is produced by a mechanism of TLR activation (known to be a key part of the inflammatory response in tuberculosis) and involves intracellular production of pro-IL1 β and this in turn, cleaved by precursors, producing a mature form which is found extracellularly. The fact that only when it is extracellular, we may obtain a measurement of it in the cell culture medium may not be reflective of the entire effect of vitamin D on the pathway. Further studies may include a larger cohort with a view to assessing some of the precursors of the mature IL1 β which may reflect its activity more accurately.

TNF α followed a similar pattern to GCSF and IL6 response with both the addition of LPS and pooled plasma with some genotypes exerting a more potent effect on reduction in cytokine response than others. This is discussed further in the section *monocyte genetics*.

In all the cytokine responses there is a reduction in levels when pooled plasma is added to the LPS stimulated cultures. This suggests possible formation of LPS-plasma complex [263] [264] which may then affect cytokine response. Another possibility is the presence of something in the plasma of TB patients that may be affecting the cytokine response to LPS, rather than purely the DBP or other globulin. To further analyse this, it would be necessary to perform experiments with both TB

plasma of varying DBP genotypes as well as healthy control plasma of varying genotypes to determine any difference between the groups.

Further work which may add additional information to this area may be to fluorescently tag the DBP and using flow cytometry, determine the degree of cellular internalisation of DBP according to the genotype and the assessment of cathelicidin and 24-hydroxylase by mRNA measurement.

CHAPTER 5

5.1 Limitations of the Study and Future Work

The study has been useful in confirming some findings from previous work in the field and stimulating consideration for future studies and experiments in mycobacterial infection.

The size of the study was a limitation due to the difficulty in obtaining enough participants to adequately power the study. This meant that firm conclusions regarding clinical effects of vitamin D supplementation e.g. sputum conversion / radiological changes etc. was not possible. However, further in vitro work was added to the original trial protocol through substantial amendments, with the aim that both in vivo and in vitro work would provide further data on which to base additional conclusions.

In the main study, treatment with vitamin D resulted in failure to achieve sufficiency in a percentage of the patients and this has been a problem in previous studies to date. Supplementation with higher initial doses or IM dosing may help to achieve sufficiency in greater numbers of participants would be useful in ensuring sufficient dosing in all individuals thereby reducing variability in the study.

The study, as an observational trial, only allowed analysis of those who were treated with vitamin D and given the general health benefits of vitamin D it would need to be debated whether withholding treatment once deficiency has been identified, would be in the patient's best interest.

The likely interaction of the vitamin D axis on TB outcomes / response to supplementation has been highlighted in this study, however it is likely that larger randomised controlled trials would be useful in identifying whether the immune response in individuals are only due to vitamin D supplementation or genotype response, rather than the sole or additional effect of standard antimycobacterial therapy. Studies have reached varying conclusions as discussed in the introduction regarding whether the genotype influences diseases susceptibility, although a recent study by Junaid et al notes that although vitamin D correlates with susceptibility to TB, polymorphisms in DBP, VDR and CYP2R1 do not [265]. Larger analyses are required.

The in vitro work had planned to include active TB patients particularly for the study of T cell activity and response. However due to time limitations this was not possible and this would be a further research opportunity in future work both to expand the original population numbers of control and latent patients and add to this with active samples.

The flow cytometry was subjective to a degree when carrying out the gating and frequency analysis. To minimise this the initial samples, collected as part of an associated BMedSci project were reanalysed to ensure that both the results were similar and to ensure that all samples were calculated for final analysis in the same manner to reduce heterogeneity of results.

Despite this some interesting findings have been noted for potential further study.

It is clear that there is a sizeable group of patients who suffer with vitamin D deficiency, particularly those with tuberculosis and that treatment of these patients may be of benefit. Not all individuals will necessarily benefit from identical treatment regimens and the possibility of individualised regimens is highlighted. This may be of further interest in aiming to personalise treatment depending on genotype and severity of vitamin D deficiency in order to achieve optimal therapeutic

effect. Considerations would have to be taken regarding whether this is a practical approach or whether broad dosing regimens are more suitable with the by-product of potentially over-treating some individuals whilst achieving sufficiency in all.

Additionally, consideration for future work should be considered using different dosing regimens. The benefits and disadvantages of high dose intermittent vitamin D supplementation versus daily dosing is debated and there have been suggestions that doses in the region of 100,000 units of cholecalciferol will only improve short term levels and fail to suppress PTH, whereas higher dosing must be used with caution due to side effects [266].

There is also the potential for future work to consider the possibility that vitamin D assays may be affected by the possibility of C3 epimer in the assay, which exists in an identical structure with the exception of a stereochemical difference at one site. Higher levels of this epimer have been found at greater 25(OH)D doses [267], such that dosing regimens which avoid large rises and falls of the levels may be beneficial in avoiding the presence of substances which may interfere with the accuracy of the current assays.

Increasing numbers of new entrants to the UK means that the pool of population suitable for latent TB treatment is expanding and in this population it would be interesting to investigate any benefit that high dose vitamin D supplementation may have in the likelihood of progression to active disease. This could include a supplementation arm of those who are treated and those who remain untreated for latent disease.

On this theme, the cytokine responses in latent TB would be interesting to investigate, identifying those who are likely to have been recently infected and determining if there is any difference in their in vivo cytokine production prior to and during treatment. This may enable the identification of one

or more biomarkers which may help to help identify this population and the likelihood of progression to active disease.

Genetic associations appear to be an important dimension in the consideration of which individuals may respond to or benefit most from vitamin D treatment and the study highlights the complexity of the vitamin D axis in mycobacterial infection.

Further work of interest would be to confirm differences in active disease states in the proliferation of T regulatory cells and the effect of vitamin D on this activity in disease states. Studies such as that by He et al [251] suggest that *M. tuberculosis* infection induces circulating CD4⁺CD25⁺FoxP3⁺. Whether vitamin D in this context is beneficial may be debatable and the possibility of inducing a chronic immune modulated disease state by further induction of regulatory immune response should be considered.

Study of the anti-mycobacterial response in the form of LL37 has yielded results which stimulate further consideration regarding the mechanisms and importance of this substances production in TB. Further work in this area would be to measure hCAP gene protein as part of the analysis in order to see if this correlates with the LL37 picture. Analysis of the LL37 levels at weeks 2, 4 and 6 may have been useful in order to determine whether the levels at week 8 are purely spurious results or whether there is indeed a gradual decline in the initial stages of treatment.

The finding that the vitamin D has an inhibitory effect on pro-inflammatory cytokine production, whilst promoting the expression of CTLA4, may be of interest specifically in the mycobacterial field where modulation of T cells could provide the potential for managing difficult or severe disease (for example cerebral tuberculomas with mass effect) or in the field of the paradoxical immune response which sometimes occurs on commencing TB therapy and may result in a significant worsening of

the inflammatory component of the disease. Further work should include similar T cell studies in both active and latent disease to compare and contrast the effects between these populations.

Collaboration to consider the effects of vitamin D in an immunomodulatory role in other diseases would be helpful and work has already shown that in asthma, vitamin D supplementation appears to reduce the severity of exacerbation requiring corticosteroids and number of hospital admissions [268], suggesting an immune modulatory role, although the mechanism is unclear and only mild to moderate severity patients were included.. Additionally a recent study by Chambers et al has shown that in severe, steroid resistant asthma phenotypes, a subgroup with increased IL17A production in sputum and BAL, has a steroid independent response to calcitriol in reducing this inflammatory cytokine [269]. Work has also recently been carried out in a multicentre trial on the effects of 120,000 units of vitamin D supplementation in COPD. The findings have shown that there was no effect of supplementation on infection or exacerbation rates, although subgroup analysis did suggest that vitamin D supplementation in those with lower baseline 25(OH)D levels (<50nmol/l) conferred a protective effect on moderate or severe exacerbations [270].

5.2 Conclusions

This study has enabled both in vivo and in vitro analysis of the immune regulatory effects of vitamin D in mycobacterial infection. It has confirmed previous findings regarding vitamin D deficiency in tuberculosis and the relevance of this in latent and active disease.

Optimisation experiments carried out prior to and during the study have enabled in vitro measurements of immune cells, along with examination of the cytokine response to differing stimulation.

In addition the in vitro work shows and confirms previous work that there is a significant immune modulatory effect of vitamin D, particularly around the effect on the CTLA4 antigen expression.

It is possible that vitamin D may be useful in particular subsets of patients, for example, those with profound vitamin D deficiency or those with particular genotypes of DBP or VDR which may confer an improved response to treatment when combined with vitamin D. It is likely that not all individuals will benefit from 'standard' vitamin D supplementation and the dosing may need to be adjusted according to requirements / genotype.

Consideration around the safety of 'blanket' supplementation with vitamin D should be considered particularly with regard to the possible immune regulatory role which has been confirmed in this study, which may theoretically enhance persistence or dissemination of disease following pulmonary infection.

Currently however, it would seem reasonable to check patients' vitamin D levels and supplement deficiency, in order to provide patients with the multifactorial benefits (particularly musculoskeletal) which are already proven.

The work enhances the already large array of information available and prompts consideration of future research directions in this field of infection and immunity.

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ABBREVIATIONS

ARDS	Adult respiratory distress syndrome
ATP	Adenosine triphosphate
BCG	Bacille-Calmette Guerin vaccine
BMI	Body mass index
BSA	Bovine serum albumin
CCL3L1	Chemokine (C-C motif) ligand 3-like 1
CCR5	C-C chemokine receptor type 5
CD	Cluster of differentiation
CFP-10	10 kDa culture filtrate antigen
CHRNA5	Cholinergic receptor nicotinic alpha 5 subunit
CISH	Cytokine-inducible SH2-containing protein
CNV	Copy number variation
COPD	Chronic obstructive pulmonary disease
CRP	C reactive protein
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CXR	Chest X-ray
CYP27b1	Cytochrome P450 family 27 subfamily B member 1
CYP2R1	Cytochrome P450 2R1 / vitamin D 25-hydroxylase
DBP	Vitamin D binding protein
dbSNP	Single nucleotide polymorphism database
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEF4B	Defensin B4
DHCR7	7-Dehydrocholesterol Reductase
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPTB	Extrapulmonary tuberculosis
ESAT-6	Early secretory antigenic target
FACS	Fluorescence activated cell sorting
FCS	Fetal Calf Serum
FoxP3	Forkhead box P3
GATA3	GATA transcription factor 3
GC	Group-specific component
G-CSF	Granulocyte colony stimulating factor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GWAS	Genome wide association study
HapMap	Haplotype map
HBC	High burden country
HC	Healthy control
h-CAP	Human cathelicidin antimicrobial protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish Peroxidase
Ig	Immunoglobulin
IGRA	Interferon gamma release assay
IL	Interleukin 23 Receptor
IL1B	Interleukin 1 beta
IL23R	Interleukin 23 Receptor
IQR	Interquartile range
ISC	Indian subcontinent
LD	Linkage disequilibrium
LL-37	Cathelicidin derived antimicrobial peptide
LPS	Lipopolysaccharide
LTB	Latent TB
M.tb	Mycobacterium tuberculosis
MAF	Macrophage activating factor
MAP-c	Mycolic acid arabinogalactan peptidoglycan complex
MBL	Mannose binding lectin
MDG	Millennium development goals
MDR-TB	Multidrug resistant tuberculosis
MFI	Median fluorescence intensity
MHRA	Medicines and healthcare products regulatory agency
MRI	Magnetic resonance imaging
mRNA	Microsomal RNA
MUAC	Mid upper arm circumference
NAAT	Nucleic acid amplification test
NADSYN1	Nicotinamide adenine dinucleotide synthetase 1
NF-kB	Nuclear factor kappa B
NICE	National institute for health and care excellence
NOS2A	Nitric oxide synthase
NRAMP1	Natural resistance-associated macrophage protein 1
OMIM	Online Mendelian Inheritance in Man
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
PMA	Phorbol 12-myristate 13-acetate

PPD	Purified protein derivative
PTB	Pulmonary tuberculosis
ROR γ t	RAR-related orphan receptor gamma
RXR	Retinoid X receptor
SAC	South African Coloured population
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SP110	SP110 nuclear body protein
T reg	Regulatory T cells
Tbet	T-box expressed in T cells
TGF	Transforming growth factor
TH1/2	T helper 1/2 lymphocytes
THP-1	Human leukaemia monocytic cell line
TIRAP	Toll-interleukin 1 receptor (TIR) domain-containing adapter protein
TMB	Tetramethylbenzidine
TNF α	Tumour necrosis factor alpha
US	Unstimulated
UT	Untreated
UVB	Ultraviolet B
VDDR1A	Vitamin D dependent rickets type 1A
VDR	Vitamin D receptor
VDREs	Vitamin D response elements
WCC	White cell count
WHO	World health organisation
WT	Wild Type
WT1	Wilm's tumour 1
XDR-TB	Extensive drug resistant tuberculosis

APPENDICES

APPENDIX 1 Patient Information Leaflets

Patient information leaflet and Consent for Main Study Version 1.

Study code: 2010-023189-27

Title: The pharmacogenetics of vitamin D response in tuberculosis

You have been asked to participate in a clinical study for research purposes. Before you decide to take part it is important that you understand why the study is being performed, what it involves, and the possible risks and benefits for you. Take your time reading the following information and discuss it with others if you wish.

Introduction to the research & invitation to take part

This study is being carried out at the University of Birmingham and participants are being recruited from the Heart of England NHS Trust and University Hospital Birmingham. You have been chosen because your hospital doctor has identified you as possibly having tuberculosis and believes that you are suitable to take part in the study.

What is the research about?

The main purpose of the study is to look for Vitamin D deficiency amongst patients with tuberculosis. We will treat the Vitamin D deficiency if identified and also look at whether the protein that binds vitamin D varies from person to person. We may then be able to establish if some patients respond better than others to treatment. We also hope to determine if Vitamin D supplements have a positive effect in those with tuberculosis. We will do this by looking at changes in X-rays, blood tests and symptoms.

What will I have to do?

Please read this section carefully as it details the procedures that are specific to research and not part of usual clinical care

If you agree to take part you will be seen by a member of the research team at the time of your usual out-patient clinic appointment. They will explain more about the study and what it would mean for you. The study will take place over 24 weeks (6 months) which is the minimum amount of time you should expect to be followed up if treated for tuberculosis. It will involve seeing a member of the research team after one of your out-patient appointments after 2 months, 4 months and 6 months. There will be no extra visits to the hospital other than those for your usual out-patient appointments.

A blood sample will be collected from you. This can be done at the same time as any other blood tests your hospital doctor has asked for. We will collect blood at each of your outpatient appointments.

We will process the blood and sputum samples so that they can be stored safely in secure freezers at the University of Birmingham. For this study they will be used to examine markers relevant to vitamin D and tuberculosis. They will not be used for other studies without prior ethical approval.

We will also extract your DNA from your blood, which will be stored in the same way. Your DNA will be used to look for genes that may influence the response of individuals to vitamin D supplements. Your DNA will not be used for other studies.

As well as collecting these samples we will also collect information about you by talking to you and by looking at your medical notes. We will also ask some questions about symptoms that people with tuberculosis often experience, and ask you to fill in a questionnaire about the way your illness affects you. We will also look in your medical notes to see what medicines

you take, and scans you have had (not all patients will have needed scans). All of this information will be recorded in written form only.

All of the above procedures should take no more than half an hour on the first occasion, and be a little shorter on later visits. This will be in addition to seeing your usual hospital doctor.

What are the benefits?

You will benefit by being identified and treated if you have vitamin D deficiency. Your participation and donation of samples may benefit patients with lung disease in the future by helping us to understand the role of vitamin D in the treatment of infectious lung diseases better.

What are the risks?

We do not expect any harm to come to you as a result of providing samples or talking to the researchers. Sometimes blood tests can be uncomfortable, or leave bruising, but this will be temporary. There is a theoretical risk of elevated calcium levels in the blood with vitamin D supplementation but this will be monitored.

What if I do not want to take part?

The study is entirely voluntary and if you do not wish to participate it will not affect your future care.

What happens to the information?

If you decide to take part you will need to allow access to your medical records. They may be looked at by the doctors carrying out the research, by the hospital research and development department and by regulatory authorities who check that the study is being carried out properly. By signing this form you are giving permission for this to be done.

The information collected will be stored on a secure computer, but your name will not. This is known as linked anonymised data, meaning that only the lead researching doctor will have

access to your details. They will have sole access to a written record of your information, stored in a secure facility at University Hospitals Birmingham. All the data collected, samples you provide, and their results, including any information about your genes, will be coded with a number. The results of tests on your samples and about your genes will not be available to anyone outside of the research team and our collaborators. The link to your name will be destroyed after 15 years. Once the data is collected it will be the property of the research department.

The results of the study may be published in a medical journal, but your identity will not be revealed. The results may be used in statistical tests, research and development of new treatments, diagnostic tests and medical aids.

Who else is taking part?

About 200 other patients with tuberculosis will be asked to take part.

What if something goes wrong?

Since the study involves only simple tests and treatments that could form part of your routine care, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future care. If you are harmed by taking part in this research project there are no special compensation arrangements.

What happens at the end of the study?

Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to monitor you.

What happens if I have more questions?

If you do not understand something in this leaflet, or have further questions you may ask the researcher now, or your hospital doctor.

What happens now if I decide to take part?

If you decide to take part you will be asked to read, sign and date the Written Consent Form attached to this sheet. By signing it you acknowledge that you have understood the aims of the research, and what you are being asked to do.

In summary

The research aims to find a relationship between the response to vitamin D supplementation and the genetic type of that individuals protein that binds the vitamin. It will also look at whether there is an improved response to tuberculosis treatment with vitamin D supplementation.

If you take part you will be asked to provide blood and sputum samples, and answer questions about yourself and your symptoms at four out-patient appointments.

All information will be stored in such a way that you cannot be identified by anyone without the lead researcher's permission

Will my General Practitioner (GP) be informed?

If you give your permission, your GP will be told about your participation in the study.

What happens if I change my mind during the study?

You are free to withdraw your participation at any time, and it will not affect your future care. If you withdraw your consent after your samples have been analysed it will be the responsibility of your hospital doctor to ensure that the samples are destroyed if you so wish.

Who can I contact about the study?

In the first instance any concerns or questions should be addressed to either your GP or hospital doctor. If you have further concerns you can contact

Dr Alice Wood 

Dr Gemma Hawthorne

Thank-you for reading this information leaflet

Study code: 2010-023189-27

Title: The pharmacogenetics of vitamin D response in tuberculosis

I..... (Name in BLOCK CAPITALS)

Initials

Have read the attached information concerning my participation in this study and have had the opportunity to discuss it and ask questions. All my questions have been answered in a satisfactory way.

I voluntarily consent to take part in this study.

I know that at any time, and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected.

I understand that I will have a copy of this Patient Information Leaflet and Written Consent to keep.

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals 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.

I understand that samples taken as part of this study will not be used in other studies without prior ethical approval.

I hereby give permission for my GP and hospital consultant to be informed about my

participation in this research study.

.....
.....

Patient's signature

Date

.....

Name in BLOCK CAPITALS

Responsible investigator

I have explained the nature and purpose of this study for the person named above

.....
.....

Responsible investigator/representative signature

Date

.....

Name in BLOCK CAPITALS

For patients who do not wish for their records to be used in research tick here

ADDITIONAL INFORMATION AND CONSENT FORM FOR LATENT TUBERCULOSIS PATIENTS VERSION 1A

Introduction to the research & invitation to take part

This study is being carried out at the University of Birmingham and participants are being recruited from the Heart of England NHS Trust and University Hospital Birmingham. You have been chosen because your hospital doctor has identified you as possibly having latent tuberculosis and believes that you are suitable to take part in the study.

What is the research about?

The purpose of this study is to look at the numbers and types of immune cells in the blood amongst patients with active and latent tuberculosis. We may also evaluate the response of these cells to vitamin D in the laboratory. We are also carrying out a larger clinical trial researching vitamin D deficiency but this will not affect you.

What will I have to do?

Please read this section carefully as it details the procedures that are specific to research and not part of usual clinical care

If you agree to take part you will be seen by a member of the research team at the time of your usual out-patient clinic appointment. They will explain more about the study and what it would mean for you. The study will involve seeing a member of the research team at your initial outpatient. There will be no extra visits to the hospital other than those for your usual out-patient appointments.

A blood sample will be collected from you. This can be done at the same time as any other blood tests your hospital doctor has asked for.

We will process the blood samples for analysis and some may be stored safely in secure freezers at the University of Birmingham. They will not be used for other studies without prior ethical approval.

All of the above procedures should take no more than fifteen minutes. This will be in addition to seeing your usual hospital doctor.

What are the benefits?

Your participation and donation of samples may benefit patients with tuberculosis in the future by helping us to understand the role of immune cells in the activity of tuberculosis.

What are the risks?

We do not expect any harm to come to you as a result of providing samples or talking to the researchers. Sometimes blood tests can be uncomfortable, or leave bruising, but this will be temporary.

What if I do not want to take part?

The study is entirely voluntary and if you do not wish to participate it will not affect your future care.

What happens to the information?

If you decide to take part you will need to allow access to your medical records. They may be looked at by the doctors carrying out the research, by the hospital research and development department and by regulatory authorities who check that the study is being carried out properly. By signing this form you are giving permission for this to be done.

The information collected will be stored on a secure computer, but your name will not. This is known as linked anonymised data, meaning that only the lead researching doctor will have access to your details. They will have sole access to a written record of your information, stored in a secure facility at University Hospitals Birmingham. All the data collected, samples you provide, and their results, will be coded with a number. The results of tests on your samples and about your genes will not be available to anyone outside of the research team and our collaborators. The link to your name will be destroyed after 15 years. Once the data is collected it will be the property of the research department.

The results of the study may be published in a medical journal, but your identity will not be revealed. The results may be used in statistical tests, research and development of new treatments, diagnostic tests and medical aids.

Who else is taking part?

About 30 other patients with active and latent tuberculosis will be asked to take part.

What if something goes wrong?

Since the study involves only simple tests that could form part of your routine care, we do not expect any harm to come to you. Whatever part of the study you choose or decide not to take part in will not affect your future care. If you are harmed by taking part in this research project there are no special compensation arrangements.

What happens at the end of the study?

Throughout the study, and when it ends, your hospital doctor and general practitioner will continue to monitor you.

What happens if I have more questions?

If you do not understand something in this leaflet, or have further questions you may ask the researcher now, or your hospital doctor.

What happens now if I decide to take part?

If you decide to take part you will be asked to read, sign and date the Written Consent Form attached to this sheet. By signing it you acknowledge that you have understood the aims of the research, and what you are being asked to do.

Will my General Practitioner (GP) be informed?

If you give your permission, your GP will be told about your participation in the study.

What happens if I change my mind during the study?

You are free to withdraw your participation at any time, and it will not affect your future care. If you withdraw your consent after your samples have been analysed it will be the responsibility of your hospital doctor to ensure that the samples are destroyed if you so wish.

Who can I contact about the study?

In the first instance any concerns or questions should be addressed to either your GP or hospital doctor. If you have further concerns you can contact

Dr Alice Wood

Dr Gemma Hawthorne [REDACTED]

Study code: EUDRACT No: 2010-023189-27 Enrolment No:

Title: The pharmacogenetics of vitamin D response in tuberculosis.

Sub- study looking at T cell proportions in latent tuberculosis

Sub- study looking at response of immune cells in latent tuberculosis

I..... (Name in BLOCK CAPITALS)

Initials

Have read the attached information concerning my participation in this study and have had the opportunity to discuss it and ask questions. All my questions have been answered in a satisfactory way.

I voluntarily consent to take part in this study.

I know that at any time, and without giving a reason, withdraw my participation in the study and that my future care and management will not be affected.

I understand that I will have a copy of this Patient Information Leaflet and Written Consent to keep.

I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals 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.

I understand that samples taken as part of this study will not be used in other studies without prior ethical approval.

I hereby give permission for my GP and hospital consultant to be informed about my participation in this research study.

.....
.....

Patient's signature

Date

.....

Name in BLOCK CAPITALS

Responsible investigator

I have explained the nature and purpose of this study for the person named above

.....
.....

Responsible investigator/representative signature

Date

.....

Name in BLOCK CAPITALS

For patients who do not wish for their records to be used in research tick here

APPENDIX 2 Data Collection Proforma

PROFORMA FOR STUDY OF PHARMACOGENETICS OF VITAMIN D RESPONSE IN TUBERCULOSIS

DEMOGRAPHICS

NAME	
HOSPITAL NUMBER	
GENDER (m/f)	
DATE OF BIRTH (dd/mm/yyyy)	
GP (name and address)	
Ethnic Origin	
Year of Entry to UK	

MICROBIOLOGICAL CONFIRMATION OF TUBERCULOSIS

Smear positive (circle one)

+

++

+++

Smear negative

Results of subsequent sputum smear

HIV Status (circle one)

Positive

Negative

Unknown

CULTURE

Positive

Sputum

BAL

Lymph node

Other – specify

Negative

VITAMIN D LEVELS

	Baseline	8 weeks	16 weeks	24 weeks
1,25 (OH) ₂ D ₃				
25 (OH) D ₃				

VDBP GENOTYPE (circle one)

GC1S

GC1F

GC2

CALCIUM LEVEL

<2.65

INFLAMMATORY MARKERS

Full Blood Count

Hb	
WCC (total)	
Neutrophils	
Monocytes	
Eosinophils	
MCV	
Haematocrit	
Platelets	
CRP	

U+Es (if checked)

Na	
K	
Urea	
Creatinine	

LFTS (if checked)

Protein	
Albumin	
Alkaline phosphatase	

ALT	
Bilirubin	

RADIOLOGY

	Lobar	Multilobar	Unilateral	Bilateral
Baseline				
8 weeks				
16 weeks				
24 weeks				

If no chest X-ray changes please tick box

TB SCORE

Score 1 point for each positive response. Positive responses to all questions will give maximum score of 13.

		Score (1 point for each positive response)
Symptoms	Cough	
	Dyspnoea (shortness of breath)	
	Night Sweats	
	Haemoptysis	
	Chest pain	
Signs	Anaemia (pale conjunctivae)	
	Tachycardia	
	Positive auscultatory findings (rhonchi, absence of breath sounds, crackles)	
	Temperature >37 degrees Celcius	

	BMI <18	
	BMI <16	
	Mid upper arm circumference (MUAC) <220	
	Mid upper arm circumference (MUAC) <200	
		Total Score = /13

Comorbidities

History of alcohol or drug misuse.

(If yes – explain)

Medication List (free hand)

Allergy information

Vegetarian diet / vegan diet (please specify)

Smoking status

Smoker

Non-smoker

Exclusion Criteria Checklist (please tick appropriate column).

If any of the answers are 'yes', the patient is excluded from the study.

	Yes	No
Managed with drug regime other than standard 6 months		
Known HIV positive		
Known drug resistant TB		
Known intolerance of Vitamin D		
Sarcoidosis		
Vitamin D supplementation in last 8 weeks		
Baseline serum corrected Calcium >2.65		
Current haemodialysis		
Pregnant or breastfeeding		
Use of benzothiadizine, phenytoin, phenobarbitol, cardiac glycoside, carbamazepine, primidone, long term immunosuppressive therapy		

Additional measures

VDBP level

LL37 level

Confirmation of Administration of Dekristol 100000 IU (initial)

Initial box to confirm administration

	0 weeks	8 weeks	16 weeks
Administered			

Name of person completing form _____

Date _____

APPENDIX 3 Standard Operating Procedures for Trial

SOP for the Pharmacogenetics of Vitamin D response in Tuberculosis

SOP for acquisition of serum from whole blood

Materials

Blood collection apparatus (vacutainer set and butterfly needle)

1 red top tube.

Centrifuge

Pasteur pipette

500 microlitre pipette and tips

Sterile eppendorfs

Ice blocks and cool box for transfer or -20 freezer if prolonged wait prior to transfer back to lab.

Labelling

All tubes should be labelled with the patient number e.g. Patient number 1, Visit 1 will be labelled 1.1; patient number 2 visit 3 will be labelled 2.3. In addition labelling should include the date.

All eppendorfs will also be labelled with this patient / visit number.

In addition the eppendorfs should be labelled with the date and serum or plasma marker (S or P).

Processing

Place the red top tube in the centrifuge at 500 rcf for 10 minutes.

Using a Pasteur pipette transfer the supernatant into a sterile tube.

Pipette off 500 microlitre aliquots from this into each sterile eppendorf.

Put on ice / ice blocks and transfer to freezer minus 80 C.

SOP for acquisition of plasma from whole blood

Materials

Blood collection apparatus (vacutainer set and butterfly needle)

1 EDTA tube.

Centrifuge (NOTE: always make sure the rotor is balanced)

Pasteur pipette

500 microlitre pipette and tips

Sterile eppendorfs

Ice blocks and cool box for transfer or -20 freezer if prolonged wait prior to transfer back to lab.

Labelling

All tubes should be labelled with the patient number e.g. Patient number 1, Visit 1 will be labelled 1.1; patient number 2 visit 3 will be labelled 2.3. In addition labelling should include the date.

All eppendorfs will also be labelled with this patient / visit number.

In addition the eppendorfs should be labelled with the date and serum or plasma marker (S or P).

Processing

Place the EDTA tube in the centrifuge at 500 rcf for 10 minutes.

Using a Pasteur pipette transfer the supernatant into a sterile tube.

Pipette off 500 microlitre aliquots from this into each sterile eppendorf.

Put on ice / ice blocks and transfer to freezer minus 80 C.

SOP for obtaining whole blood for DNA extraction.

Materials

Blood collection apparatus (vacutainer set and butterfly needle)

2 EDTA tubes.

Labelling

All tubes should be labelled with the patient number e.g. Patient number 1, Visit 1 will be labelled 1.1; patient number 2 visit 3 will be labelled 2.3. In addition labelling should include the date.

Processing

Freeze the 2 EDTA tubes at minus 80 for DNA genotyping at a later date.

APPENDIX 5 Pharmacy Documentation

PHARMACY

Clinical Trial Prescription

The Pharmacogenetics of Vitamin D Response in Tuberculosis

EUDRACT No: 2010-023189-27

Protocol: RG-10-179

Patient Initials	
Patient ID	
Date of Birth	

Week No (0-8-16)	
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Please supply:

1x5 Vitamin D 20,000 IU capsules (Dekristol)

Open label, for clinical trial use only

Date	
Prescribing Signature	
Printed Name	
Contact details (please supply telephone/bleep No)	

Pharmacy Use Only				
Date Issued:	Sign		Print	
Checked by	Sign		Print	

Collection				
Collected by	Sign		Print	
	Date		Time	

Request Form

The Pharmacogenetics of Vitamin D Response in Tuberculosis

Eudract No: 2010-023189-27

Protocol: RG-10-79

Principal Investigator: Dr Gemma Hawthorne

Chief Investigator: Dr Alice Wood

Please Supply : of bottles of Vitamin D 20000 IU capsules (Dekristol) Supplied in quantities of 5 capsules per bottle equalling a total dose of 100000 IU per bottle

Investigator:

Date:.....

Print Name /Signature

Pharmacy Use Only

Supplied by: _____ Date: _____

Checked by: _____