The Role of Vitamin D and Ultraviolet Radiation in Inflammatory Bowel Disease

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THESIS DECLARATION

I, Simon Ghaly, certify that:

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The research involving human data reported in this thesis was assessed and approved by the South Metropolitan Area Health Service, Western Australia (Ref: 07/589), QIMR Berghofer Medical Research Institute (trial 2010/0423) and Human Research office fo the University of Western Australia (RA/4/1/6796)

Written patient consent has been received and archived for the research involving patient data reported in this thesis.

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DEDICATION

This thesis is dedicated to my wife, Yostina who has been my rock and support. She has patiently endured my absence during the years of this PhD degree. I am eternally grateful to her and our three daughters, Elyssa, Emilia and Eve.

ABSTRACT

Vitamin D deficiency has been associated with the onset and complications of the inflammatory bowel diseases (IBD), Crohn's disease (CD) and Ulcerative colitis (UC). Oral vitamin D supplementation has been proposed as an adjunctive treatment of these conditions, however, a causal relationship between vitamin D deficiency and IBD has not been proven, and the possibility of reverse causality remains. This thesis examined the relationship between vitamin D and the course of IBD in a retrospective cohort of patients with prospectively collected data, a mouse model of chemical colitis examining the effects of 3 different vitamin D-containing diets and ultraviolet (UV) radiation, and finally a separate prospective observational study of vitamin D metabolites in patients with IBD.

Baseline total and bioavailable serum $25(OH)D_3$ levels did not predict subsequent disease relapse in patients with CD remission. High doses of vitamin D supplementation did not protect mice against chemically-induced colitis but increased their susceptibility to colitis as measured by colonoscopy and greater weight loss. High dose vitamin D shifted the faecal microbiome to a more "inflammatory" profile, which may explain the predisposition to worse colitis. A diet sufficient in vitamin D protected against colitis-induced weight loss compared to vitamin D deficient diets, although endoscopic severity of colitis was no different. Importantly, serum $25(OH)D_3$ levels fell by up to 63% among mice on the highest vitamin D-containing diet after induction of colitis suggesting vitamin D deficiency occurs in response to intestinal inflammation.

Suberythemal UV irradiation did not provide any additional protection against chemical colitis, though distinct changes in the faecal microbiome of UV-irradiated mice were detected independently of vitamin D, including enrichment of members of the phylum *Firmicutes.*

Differences in vitamin D metabolism were observed between mice and humans. A greater proportion of $25(OH)D_3$ was present in the less active epimerised form, C3-epi $25(OH)D_3$, in mice compared to

the measured levels in human sera retrieved from two clinical trials. Further, oral vitamin D supplementation was associated with a greater proportion of epimerised metabolites compared to UV irradiation of skin in mice but not humans. These differences in vitamin D metabolites highlight the limitations of extrapolating results of murine studies to humans.

In view of the differences between murine and human vitamin D metabolism, a prospective cohort of patients with CD was recruited to measure vitamin D metabolites over time. Patients with active CD had reduced levels of the downstream metabolite, $24,25(OH)_2D_3$, but not $25(OH)D_3$. After achieving remission with medical therapy there was a spontaneous increase in circulating levels of $25(OH)D_3$ and $24,25(OH)_2D_3$.

Considered together, these data demonstrate that circulating $25(OH)D_3$ falls in response to active intestinal inflammation at least in the mouse and increases with treatment of the underlying inflammation in both humans and mice. Oral vitamin D supplementation and exposure to UV radiation alter the faecal microbiome, however, this is not protective against chemical colitis in the mouse. There are also differences between murine and human vitamin D metabolism and so adequately powered clinical trials of vitamin D supplementation in patients with IBD are still needed.

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ABBREVIATIONS

APC	Antigen-presenting cells
ARE	Au-rich elements
ASA	Aminosalicylate
ATG16L1	autophagy-related 16-like 1
CD	Crohn's disease
CDAI	Crohn's disease activity index
CRF	Cytokine receptor family
CRP	C-reactive protein
DC	Dendritic cells
DN	Dominant negative
DSS	Dextran sodium suphate
GC	Group specific component
GWAS	Genome-wide association study
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IGRM	Immunity-related GTPase family member M
IL	Interleukin
IOM	Institute of Medicine
KO	Knockout
LC/MS/MS	Liquid chromatography tandem mass spectroscopy
LEfSe	Linear discriminant analysis effect size
MEICS	Murine endoscopic index of colitis severity
NOD2	Nucleotide-binding oligomerisation domain-containing protein 2
NSAID	Non-steroidal anti-inflammatory
OTU	Operating taxonomic unit
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PRR	Pattern recognition receptors
PTPN2	Protein tyrosine phosphatase, nonreceptor type 2

PUFA	Polyunsaturated fatty acid
PVC	Polyvinyl chloride
rRNA	ribosomal RNA
Samp	Senescence accelerated mouse P1/Yit
SCFA	Short chain fatty acid
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor
Treg	Regulatory T cells
TSLP	Thymic stromal lympopoietin
UC	Ulcerative colitis
UV	Ultraviolet
UVR	Ultraviolet radiation
VDBP	Vitamin D Binding Protein
VDR	Vitamin D receptor
VDRE	Vitamin D response element
ZO	Zonula occludens

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This thesis contains work that has been published and/or prepared for publication.

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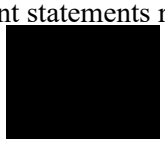
I, Ian C Lawrance certify that the student statements regarding their contribution to each of the works listed above are correct    Coordinating supervisor signature: Date: 17 March 2019

Chapter 1

General introduction

GENERAL INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are chronic lifelong diseases of the bowel that can have a debilitating impact and require long-term treatment. Often the first reaction when a patient is told of the diagnosis is "Why have I got this? Is it something I've eaten?". Unfortunately, the underlying cause of IBD is not well understood but is thought to be due to a dysregulated immune response against antigens within the gastrointestinal tract in a genetically predisposed individual. Vitamin D has been an attractive target for investigation due to its effect on adaptive and innate immune responses as well as epidemiological associations between vitamin D deficiency and IBD onset, complications and the need for surgery.

With the surge in interest in vitamin D for many conditions including IBD, there is increased primary care physician and patient awareness that has translated into a surge in vitamin D testing and supplementation. The true benefit of vitamin D supplementation in gastrointestinal health, however, is unknown. The overarching aim of this research is to better understand the relationship between vitamin D and intestinal inflammation as relevant to IBD.

The specific aims of this research include:

1. To determine if serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) can predict future disease relapse in patients with CD in remission. The hypothesis being that if vitamin D has a protective effect, patients in remission with vitamin D deficiency are likely to have a shorter time to flare.

2. To determine if vitamin D supplementation can modify the susceptibility to chemically-induced colitis in a mouse model and if an effect exists, to then determine whether there is an associated dose-response effect.

3. To investigate the effect of exposure to ultraviolet radiation (UVR) on the susceptibility to colitis in a mouse model. Humans and many animals obtain most of their vitamin D from skin exposure to sunlight. Does exposure to UVR have a different effect on intestinal inflammation compared to oral vitamin D supplementation?

4. To determine the differences in vitamin D metabolism with UVR exposure compared to oral vitamin D supplementation among mice and humans, with emphasis on vitamin D epimer metabolism.

5. To determine the change in vitamin D metabolites in a prospective cohort of patients with IBD with changing inflammatory activity.

Significance and Impact of research

An estimated 80,000 individuals suffer IBD in Australia, and the cost for hospitalisations alone are estimated at $100 million per annum [1]. In 2007 the annual economic cost of IBD in Australia was AUD$2.7 billion which included a financial cost of AUD$500 million and a net cost of lost wellbeing of AUD$2.2 billion. Current therapeutic options are mostly limited to lifelong therapies that are usually immunosuppressive and in the long-term predispose patients to opportunistic infections and malignancies. Environmental factors are thought to account, in part, for the rising incidence. UVR exposure and vitamin D are modifiable environmental factors that need to be carefully evaluated for their roles in disease onset and progression. Narrowband UVB is used therapeutically in other autoimmune disorders such as psoriasis and vitamin D supplementation is widely available at low cost,

thus both of these interventions could be added to the therapeutic armamentarium if proven to be of benefit. Understanding the interaction between UVR, vitamin D and systemic immune responses may reveal new insights into disease pathogenesis. Conversely, if vitamin D and UVR do not significantly impact disease pathogenesis then physicians and patients should be educated about this, to avoid unnecessary vitamin D testing and supplementation.

LITERATURE REVIEW

1.1. Inflammatory Bowel Disease (IBD)

CD and UC are chronic relapsing, incurable inflammatory conditions of the bowel that are increasing in both incidence and prevalence. IBD is more prevalent at increasing latitudes globally and thought to also be associated with urbanisation and western lifestyle [2-4]. Australia has one of the highest incidence rates in the world at 29.6 per 100,000 [5]. The underlying pathogenesis of these diseases is not completely understood but they are thought to be due to dysregulation of the immune response to normal intestinal flora in a genetically susceptible host.

Clinicopathological reports of the disease later to be termed "Crohn's disease" date back to 1761 [6]. In 1932, Crohn, Ginzberg and Oppenheimer reported the disease of "terminal ileitis" which was later revised to regional enteritis [7]. The eponym, CD, has gained general acceptance in recent decades and encompasses the many clinical presentations of this disease that are not restricted to the terminal ileum but rather can involve the entire gastrointestinal tract from mouth to anus. UC was first described by Dr. Samuel Wilks in 1859 as "idiopathic colitis" being a distinct entity from bacillary dysentery. More complete descriptions of the relapsing and remitting nature of the disease and the sigmoidoscopic appearances were published by Hawkins and Hurst [8].

1.1.1 Epidemiology of IBD:

While it is accepted that the incidence and prevalence of the IBDs are increasing, rigorous epidemiological studies are lacking due to the lack of a gold standard for the diagnosis and overlap with infectious disease that can mimic the presentation of IBD. A population-based cohort study from Olmsted County in the United States found that between 1935 and 1975, the age-adjusted incidence of CD rose from 1.9 cases per 100,000 in the period 1935-1954 to 4.0 per 100,000 in 1955-1964, as high

as 6.6 per 100,000 by 1975 [9]. A more recent update of the cohort demonstrated a less steep increase in disease incidence between 1984-1993 of 6.9 cases per 100,000 and 7.9 per 100,000 in 2000 [10]. Similar trends are seen with UC with an increase in risk from 0.6 cases per 100,000 person-years in 1940, to 8.3 cases per 100,000 person-years in 1993, and stabilizing to 8.8 cases per 100,000 person-years by 2000 [10, 11].

A north-south gradient in disease incidence has been described where individuals residing at increasing latitudes from the equator are at increased risk of developing IBD. Even in individual countries, such as France, Italy, Spain, Portugal and North America a north-south gradient has been observed for both CD and UC [12-14]. In a more recent study from France, the incidence of CD was higher in the North, whereas the incidence of UC was evenly distributed throughout the country [15]. The effect of latitude has been postulated to be due to differences in sun exposure and resultant changes in vitamin D status. Two recent studies from France have correlated high residential sun exposure to incident cases of CD, but not UC [16, 17].

The peak age of onset for CD is 20-30 years; for UC, it is 30-40 years. Some studies report a second peak between 60-70 years [18]. UC occurs more frequently in men (60%), whereas CD occurs 20-30% more frequently in women in high incidence areas, the same trends are not seen in areas of low-incidence [3, 19, 20].

Traditionally, Asian countries such as Japan, Singapore and South Korea have had a very low incidence of IBD, but as these countries have become Westernised the incidence of UC increased first, followed by CD [21]. A cohort study from the Asia-Pacific examined incidence and phenotype of IBD in predefined catchment areas from eight countries across Asia and Australia over a 1-year period between 2011 and 2012. A total of 419 new cases of IBD (232 UC and 187 CD) were identified. The overall

incidence was 1.4 per 100,000 in Asia compared to 23.7 per 100,000 in Australia [22]. UC comprised only one-third of new diagnoses of IBD in Australia but over two-thirds of new cases in Asia. The reason for a rise in UC incidence before one occurs in CD remains unclear but may be due to different environmental factors. The overall increase in incidence in Asian countries is likely related to westernisation of lifestyle and industrialisation. Urbanisation of societies is associated with changes in diet, antibiotic use, hygiene status, microbial exposures and pollution.[23]

Migration studies are very useful in examining the influence of risk factors on the development of IBD, because while these individuals maintain the same genetics as their country of origin, over time they adopt the lifestyle, diet and behaviours of their adopted country. Indian migrants to the UK were found to have an incidence of UC similar to native European control populations, despite having a very low incidence of disease in India [24]. Similar findings were described among Bangladeshi immigrants to East London [25]. These findings again suggest a strong influence of environmental risk factors.

1.1.2 Environmental risk factors

Environmental determinants of IBD risk in Western countries include smoking, appendicectomy, diet, medications such as the non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics and vitamin D. Current smokers are twice as likely to develop CD, have more flares after diagnosis and are more likely to require surgery, and experience recurrence after surgery [26, 27]. Intriguingly, smoking has the opposite effect in UC, where never and former smokers are more likely than current smokers to develop UC and are more likely to require an early colectomy [26]. A number of small clinical studies have suggested a potential therapeutic benefit with nicotine therapy in UC, though the mechanism for this remains unclear [28].

Appendicitis and appendicectomy are protective against the development of UC, especially in those who have the surgery under the age of 10 [29]. There is no proven relationship between appendicitis,

appendectomy and CD [30]. There have been mixed reports about the benefit of therapeutic appendectomy in ulcerative proctitis, with one Australian case-series reporting short-term positive outcomes [31].

A number of early-life exposures alter the predisposition to IBD. Exposure to antibiotics in early life predisposes to IBD in Western populations. Of children with IBD, 58% had one or more courses of antibiotics in their first year of life compared with 39% of controls [32]. This association has been confirmed in a meta-analysis investigating antibiotic use as a risk factor for developing IBD, where the greatest risks were seen with metronidazole (OR 5.01, 95% CI 1.65-15.25) and fluoroquinolones (OR 1.79, 95% CI 1.03-3.12) [33]. Breastfeeding has been shown to have a marked impact on infant gut microbiota, and in a meta-analysis of 17 studies there was a strong inverse association between breastfeeding and the incidence of both CD (OR 0.45, 95% CI 0.26-0.79) and UC (OR 0.56, 95% CI 0.38-0.81) [34, 35]. Babies born by caesarean section compared to vaginal delivery are deprived of contact with the maternal gut and vaginal microbiota, and changes in the gut microbiota were shown to persist in 7-year old children [34, 36]. Despite changes in microbiota, epidemiological studies have not shown an association between mode of delivery and IBD risk [37].

Diet also influences gut microbiota and susceptibility to both CD and UC. There is an inverse relationship between fruit and vegetable consumption and CD risk. A prospective cohort of 170,776 women suggested those with the highest quintile of dietary fibre intake were less likely to develop CD (OR 0.59, 95% CI 0.39-0.90). This risk reduction was mainly attributable to fibre from fruits and vegetables, rather than whole grains, cereal or bran [38]. N-3 polyunsaturated fatty acid (PUFA) intake is inversely associated with the risk of UC [39], whereas n-6 PUFA increased the risk of incident UC [40]. These studies were conducted in adults, with very few evaluating diets in early life, which is where diets are likely to have the strongest effect on the development of IBD.

A number of other risk factors such as environmental pollution, NSAIDs and high altitude have been studied and associated with IBD risk. The increased incidence of IBD at increased latitudes from the equator has given rise to interest in studying the effects of Vitamin D and UVR. These two factors will be reviewed in detail in sections 2 and 3.

1.1.3 Pathogenesis of disease

IBD is thought to result from environmental triggers in a genetically susceptible host leading to a dysregulated immune response against luminal antigens resulting in a chronic bowel inflammation.

1.1.3.1 Genetics

Genetic predisposition is evidenced by the observation that family members of affected persons are at significantly increased risk of developing IBD. Among monozygotic twins there is a 58.3% concordance rate for CD but only 6.3% for UC [41]. Siblings of patients with CD are between 15-35 times more likely to develop CD. There is an eightfold increased risk of CD among first-degree relatives and a fourfold increase in UC [42]. The first susceptibility locus for CD was identified in 2001 as the *NOD2* (nucleotide-binding oligomerisation domain 2) gene, also known as *CARD15 [43].* Allelic variants of this gene remain one of the strongest genetic risks for CD. Carriage of disease-associated allelic variants on both chromosomes confers an odds ratio for CD of 17.1 (95% CI, 10.7-27.2), and heterozygous persons have an OR of 2.5 (95% CI, 2.0-2.9) [44]. *NOD2/CARD15* polymorphisms have been associated with younger onset, ileal location of disease and stricture formation [45]. The gene product of *NOD2/CARD15* is a cytosolic protein expressed in monocytes and enterocytes, specifically found in Paneth cells. It functions as an intracellular sensor of bacteria and produces antimicrobial peptides called defensins.

Multiple genes in the autophagy pathway have also been associated with the risk of CD. Autophagy is the process by which segments of cytoplasm are isolated within a membrane and delivered to lysosomes to clear proteins that are long-lived, misfolded, or aggregated, and to clear apoptotic bodies which might otherwise trigger inflammation [46]. Genome-wide association studies (GWAS) have identified two autophagy genes associated with CD, the autophagy-related 16-like 1 *(ATG16L1)* gene and the immunity-related GTPase family member M (IGRM) [47, 48]. *NOD2/CARD15, ATG16L1 and IGRM* are not associated with UC, but the interleukin (IL)-23 gene has been associated with both CD and UC [49].

Protein tyrosine phosphatase, nonreceptor type 2 (PTPN2) is located in chromosome 18p11.2 and is expressed in intestinal epithelial cells and helps to maintain barrier function [50]. The coded protein (T cell protein tyrosine phosphatase) is involved in regulation of the immune system. In intestinal cells, PTPN2 regulates epithelial permeability and is a key negative regulator of the immune mediators, STAT1 and STAT3, as well as p38 and ERK1/2 phosphorylation, by modulating cytokine secretion and epithelial barrier defects [51]. Consequently, it attenuates both IFN-gamma and TNF-alpha-induced signalling and release of proinflammatory cytokines. A meta-analysis of 17 studies with 18,308 patients and 20,406 controls confirmed an association between PTPN2 polymorphisms and a susceptibility to CD and UC [52].

GWAS have identified 235 susceptibility loci for IBD [53, 54]. These studies have highlighted the important interaction between host immune responses and the handling of intestinal microbiome in IBD pathogenesis, specifically identifying genes associated with T-cell activation, the IL-23/T helper 17 pathway, autophagy and microbial recognition [55]. Using GWAS data and determining genetic risk scores, it has been suggested that there may be three genetically distinct subtypes of IBD: ileal/ileo-

colonic CD, colonic CD and UC [56]. Despite advances in our understanding of IBD genetics, significant gaps remain and the currently described susceptibility loci do not explain the heritability seen from twin studies. The IBDs are a spectrum of genetically complex disorders, and cohorts are phenotypically heterogenous limiting the ability to identify all relevant genetic variants.

1.1.3.2 Microbiome

Many of the identified host genetic susceptibility loci relate to the handling of microbiota. The gut microbiota comprise a large collection of microbes and the largest within the body, reaching 10^{12} cells/g of luminal contents in the colon [55]. These contain more than 150 times the genetic material of the entire host. Commensal bacteria are essential for the normal development of the immune system [57]. Germ-free mice have marked reductions in the size of all lymphoid organs, low serum immunoglobulin levels, fewer mature T cells and reduced immune responses, especially of the Th1 and Th17 types. In the intestine of germ-free mice, Peyer's patches do not develop normally, isolated lymphoid follicles are absent, and there are severely reduced numbers of T lymphocytes in both the lamina propria and the epithelium. Furthermore, the intestinal microbiota are known to be important in maintaining health and mediating disease [58]. Dysbiosis, a change in the normal microbial ecology of the gut, is seen in both animal models of colitis and in humans with IBD. Gut inflammation in IBD is characterised by reduced diversity of the microbiota [59, 60]. To date, however, it is not known if the dysbiosis is a cause or simply the result of intestinal inflammation in IBD.

A number of observations suggest a primary role for intestinal microbiota in the pathogenesis of IBD. First, IBD is commonest where the number of bacteria is greatest, i.e the colon and terminal ileum. Second, antibiotic therapy can lead to improvement in symptoms of IBD, albeit a transient effect. Third, diversion of the faecal stream is an established therapy in patients with CD. Finally, therapy with faecal microbiota transfer from a healthy donor has been shown in two randomised-controlled trials to induce

remission compared to placebo in UC at 8 weeks [61, 62]. Longer-term studies of the utility of faecal microbiota transfer, however, have yet to be undertaken.

Among IBD patients, lower proportions of Firmicutes are consistently reported alongside increases in Proteobacteria and Bacteroidetes phylum members [63-65]. In CD, *Faecalibacterium prausnitzii* levels have been consistently lower in ileal adult CD patients, though increased in paediatric colonic CD [66, 67]. There may be differing functional capabilities between strains [68]. Increases in *Escherichia coli,* including pathogenic variants, are also reported in ileal CD [69]. Importantly, the structural changes in the microbiota have functional significance. Broadly speaking, the functional changes in IBD include enrichment in host metabolite uptake, oxidative stress tolerance, immune evasion and decreases in microbial metabolism, including short chain fatty acid (SCFA) biosynthesis [55].

1.1.3.3 Immune Response

1.1.3.3.1 Innate Barriers

The surface of the intestinal mucosa is lined by a layer of mucus, produced by goblet cells, which acts as a protective barrier from microbial antigens. In the small intestine there is a single layer of mucous, whereas in the colon there are two layers. The inner layer is firmly adherent to the epithelium, and is rich in anti-microbial peptides and mucins, and has a low bacterial density. The outer layer, closest to the lumen, is also comprised of mucin, diluted antimicrobial peptides and some bacteria. The *Muc2* gene encodes a major intestinal secretory mucin and polymorphism in this confer susceptibility to UC in humans and *Muc19* has been associated with CD [70, 71].

The epithelium is comprised of absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. The epithelial layer forms a mucosal barrier with tight junctions between enterocytes that can

exclude entry of most substances. Defects in the epithelial barrier increase intestinal permeability and allow free passage of pathogens across the epithelial layer triggering an immune response [72]. Apart from a physical barrier, intestinal epithelial cells (IEC) express pattern recognition receptors (PRRs, discussed below) that recognise and respond to intestinal microbes through secretion of cytokines and antimicrobial peptides [73].

1.1.3.3.2 Pattern Recognition Receptors (PRRs)

Microorganisms have highly conserved structures called *pathogen-associated molecular patterns* (PAMPS), which are recognised by PRRs that in turn activate cytokine and chemokine signalling pathways, antimicrobial killing and initiate adaptive T- and B-cell responses. Host PRRs include the family of Toll-like receptors (TLRs), C-type lectins, nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) and retinoic acid-inducible gene I-like receptors [74]. The distinct PRRs differ in the repertoire of microbial and host ligands they recognise that, in turn, activate different signalling pathways, though there is overlap with common pathways such as in activation of the transcription factor nuclear factor-κB (NF-κB). It remains unclear how the intestinal immune system discriminates between resident commensal micro-organisms and pathogens, to which it must respond. Maintaining this tightly-regulated balance is crucial to maintain the integrity of the intestinal mucosa.

1.1.3.3.3 Adaptive immune responses

Innate immune responses, particularly those mediated through dendritic cells, initiate adaptive immune responses. The adaptive immune system is a highly specialised system and provides long-lasting immunity (memory). T cells play a pivotal role in the adaptive immune response. Activated naïve T cells (Th0) can differentiate into Th1, Th2, Th17 or Th22 cells. The adaptive immune response contributes significantly to IBD pathogenesis. Traditionally Th1 responses were thought to be the main drivers of CD, and Th2 responses the drivers of UC, however, more recently a significant overlap

between the two pathways has been recognised, along with the contributory role of Th9, Th17 and Th 22 cells, and T regulatory (Treg) cells [75-77]. Th1 cells are thought to be activated predominantly by presentation of microbial antigens that induce IFN-γ and IL-12 release, and activation of signal transducer and activator of transcription-1 (STAT-1) [78]. Th2 cell activation is induced by secretion of IL-4 from innate effector cells, which leads to activation of STAT6, and further activation of the transcription factor GATA-3 [79]. The characteristic cytokines released by activation of Th2 cells are IL-4, IL-5, IL-13, IL-21 and IL-25.

IBD is thought to be due to the initial activation of the innate immune system causing a non-specific response, followed by the upregulation and maintenance of this inflammation by activation of the adaptive immune system, involving various feedback loops generating and sustaining chronic inflammation [80].

1.2 Vitamin D

1.2.1 Vitamin D in IBD

The impact of vitamin D deficiency on bone and muscle health is well recognized but over the last decade *in vivo* studies suggest a role for vitamin D as a regulator of both the innate and adaptive immune responses [81]. This observation has prompted investigators to explore the potential links between vitamin D and a variety of autoimmune disorders, cardiovascular disease, hypertension and common cancers. To date, vitamin D deficiency has been associated with numerous aspects of IBD including, its onset [82], activity [83-85], the need for hospitalisation and surgery [86], as well as the risk of malignant transformation [87]. Vitamin D supplementation has also been suggested as a potential adjunctive therapy in CD [88-90].

The Institute of Medicine (IOM) in 2010 defined vitamin D sufficiency as 25-hydroxy vitamin D (25(OH)D) level ≥50 nmol/L, insufficiency 25-50 nmol/L and deficiency <25 nmol/L [91]. Similarly a recent expert consensus group agreed vitamin D deficiency as 25(OH)D <50nmol/L and severe deficiency as 25(OH)D <30nmol/L [92]. Unfortunately, these are in contrast to those published by the Endocrine society where sufficiency is 25(OH)D ≥75 nmol/L, insufficiency 50-75 nmol/L and deficiency <50 nmol/L [93]. These higher values reflect more recent studies which demonstrate that the classical physiological targets for vitamin D, circulating levels of parathyroid hormone, and intestinal calcium uptake show a correlation with serum $25(OH)D_3$ at levels as high as 75 nmol/L [94, 95]. The largest study on vitamin D status in IBD patients originates from an electronic medical record-based cohort in the Greater Boston area that included 3217 UC and CD patients [86]. Of these patients 32% had a $25(OH)D_3$ level of <50 nmol/L and was similar in both CD and UC patients. However, this level of 25(OH)D3 may be no more common than is observed in other chronic diseases, or indeed, healthy controls living in the same area [96-98].

The cause of vitamin D deficiency in IBD is multifactorial, however, inadequate exposure to sunlight, specifically ultraviolet B (UVB) radiation, is potentially the most important [99]. The amount of dermal exposure to UVB is determined by the time spent outdoors, the number of daylight hours, the UV index, sunscreen use, clothing, skin pigmentation, latitude and season. The time spent outdoors by IBD patients is often limited by disability. In addition, many patients cover skin that would otherwise be sun-exposed with clothing and sunscreen as they are on immunomodulating thiopurine medications that have a peak absorption in the middle of the UVA waveband and have been associated with an increased risk of skin malignancies [100, 101]. In the setting of limited UVR exposure, the dietary intake of vitamin D becomes increasingly important. The IOM's recommended daily allowance of vitamin D is 600 IU between the ages of 1 and 70 years, with the aim to maintain a $25(OH)D_3$ level of at least 50 nmol/L. The guidelines, however, recognise that in the presence of poor nutrition, living in northerly latitudes, institutionalisation, or for those people with dark skin pigmentation higher doses may be

required [91]. Limited oral intake due to abdominal pain has been thought to cause reduced intake of vitamin D in the IBD population, however studies specifically examining this have not found a significant difference in vitamin D intake among IBD patients compared to non-IBD controls [102, 103]. It was previously suggested that disruption of the enterohepatic circulation of bile acids due to disease, or small bowel resection, may cause vitamin D deficiency due to reduced re-circulation of $25(OH)D_3$. Studies from the 1980's however, have shown that the enterohepatic circulation plays very little role in the reabsorption of 25(OH)D [104]. Clinical studies, likewise have yielded mixed results about the true impact of terminal ileal resection and active disease on vitamin D absorption [84, 105].

In addition to UVB exposure and the absorption of dietary vitamin D, genetic factors may regulate vitamin D transport proteins, which are important determinants of circulating $25(OH)D_3$ concentrations. These, however, have yet to be examined within the IBD population [106, 107]. Smoking is also associated with vitamin D deficiency and is also a recognised risk factor for CD development, progression to stricturing and penetrating phenotypes and progression to surgery [108, 109]. Longer CD disease duration, prolonged corticosteroid use and body mass index >30 have also been associated with vitamin D deficiency [110, 111].

1.2.2 Vitamin D biology

The major source of vitamin D in man is via the photolysis of 7-dehydrocholesterol (provitamin D_3) into pre-vitamin D_3 in the skin by UVB at wavelengths of 290-315 nm [112]. Pre-vitamin D_3, however, is thermodynamically unstable and rapidly transforms into vitamin D_3 (cholecalciferol), which is then able to enter the extracellular fluid space and bind to the vitamin D binding protein (VDBP). Vitamin D_3 bound to VDBP then enters the circulation via the dermal capillary bed [113, 114].

Vitamin D_2 (ergocalciferol) is a second form of vitamin D and is produced by UVB irradiation of ergosterol in plants and fungi [115]. Due to structural differences from vitamin D_3, it has a lower affinity

to the VDBP resulting in faster clearance from the circulation and more limited conversion to 25(OH)D [116]. Both vitamin D_2 and D_3 are available as supplements and if taken daily, they are equally effective at maintaining circulating concentrations of 25(OH)D [117]. Dietary sources of vitamin D_3 include oily fish and egg yolk, and vitamin D_2 is predominantly available from plant sources.[118]

Activation of vitamin D_3, whether endogenous, or exogenous, requires 25-hydroxylation in the liver by the cytochrome P450 family of enzymes CYP2R1 (microsomal) and to a lesser degree CYP27A1 (mitochondrial), producing $25(OH)D_3$. $25(OH)D_3$ subsequently undergoes 1α-hydroxylation in the proximal convoluted tubules of the kidney by CYP27B1 to produce the metabolically active hormone $1\alpha,25\text{-}(OH)_2D_3$ [119]. Extra-renal production of $1\alpha,25\text{-}(OH)_2D_3$ was first demonstrated in the placenta [120-122] and from macrophages in granulomatous diseases such as sarcoidosis, where circulating $1,25(OH)_2D_3$ was also elevated. Later, cloning of the CYP27B1 gene enabled the identification of widespread expression of this gene in the skin, lymph nodes, colon, pancreas, adrenal medulla and brain [123]. While there is local production of $1,25(OH)_2D_3$ at these extrarenal sites, there is still debate if this is able to "spill over" into the circulation and cause a physiologically significant change to circulating levels of the hormone in a non-disease setting [124]. $1,25(OH)_2D_3$ and $25(OH)D_3$ also induce the expression of CYP24A1 which is the main catabolic enzyme, thus completing a classic endocrine negative feedback loop.

$1,25(OH)_2D_3$ exerts its effect by binding to, and activating, the nuclear vitamin D receptor (VDR) to form a heterodimer with the retinoid-X receptor that interacts with specific DNA sequences on gene promoters called vitamin D response elements (VDREs) [125]. Binding of the heterodimer to VDREs leads to recruitment of cofactors that can induce, or repress, gene expression. $1\alpha,25\text{-}(OH)_2D$ is thus able to regulate gene expression to fulfil its pleiotropic functions in calcium and phosphate regulation as well as its proposed local effects on immune cell proliferation, differentiation and function.

1.2.3 Gastrointestinal immunity and Vitamin D

The role of vitamin D in the pathogenesis of IBD is complex and not well defined, but it may play a role in the development of gastrointestinal inflammation.

1.2.3.1 Barrier function

Tight junctions seal the space between adjacent epithelial cells, and the claudin family membrane proteins together with occludin and members of the 'zonula occludens' (ZO) protein family are key components of the structure and function of tight junctions [126]. Pretreatment with $1,25(OH)_2D_3$ protects intestinal cells from increased permeability in the murine dextran sodium sulphate (DSS) model of colitis [126, 127]. Further, VDR deletion in intestinal epithelial cells leads to decreased claudin 2 and 12 and $1,25(OH)_2D_3$ downregulates cadherin-17 and upregulates claudin 2 and 12 in the intestine [127, 128]. DSS treatment in VDR knock-out (KO) mice compared to $VDR^{+/+}$ mice reduced the immunostaining of occludin and ZO-1 proteins on colonic epithelial cells, and correlated with reduced transepithelial resistance, a measure of increased permeability. Together these studies suggest an important role for vitamin D in maintaining intestinal epithelial barrier function.

1.2.3.2 Innate antibacterial immunity

Almost 30 years ago it was shown that $1,25(OH)_2D_3$ suppresses the proliferation of *Mycobacterium tuberculosis (M. tb)* in human monocytes [129]. It was then observed that *M. tb* activation of macrophage TLR2/1, a pathogen recognition receptor, led to increased expression of CYP27B1 and VDR resulting in intracellular production of $1,25\text{-}(OH)_2D_3$ [130]. $1,25(OH)_2D_3$ then can induce the expression of the cathelicidin gene (LL-37) which promotes intracellular killing of bacteria [131]. Importantly, increasing levels of precursor $25(OH)D_3$ can also lead to enhanced bacterial killing [130]. Interestingly *Mycobacterium avium paratuberculosis* (MAP) is a bacterium that causes inflammation

of the intestine in livestock, Johne's disease, and it is believed that these bacteria may play a role in the pathogenesis of Crohn's disease in a subset of patients [132, 133].

To investigate the role of 25(OH)D_3 in bacterial killing, transformed human colonic epithelial cell line Caco-2, and the Caco-2 clone BBE were used and these cells did not produce cathelicidin or the β-defensin, Defensin Beta 4A (DEFB4), in response to TLR-induced CYP27B1. This may mean that alternative pathways are involved in the production of cathelicidin and β-defensins in the human colonic cells. One such alternative pathway may involve the nucleotide-binding oligomerisation domain-containing protein 2 (NOD2), which is an intracellular PRR that activates NF-kB and potentiates vitamin D-mediated transcription of both cathelicidin and DEFB4 [134]. Aberrant innate immune handling of the microbiota has been implicated as an initiator of tissue inflammation. NOD2 is also a major CD susceptibility locus [135]. Thus, one can postulate that the effect of vitamin D in protecting against CD involves NOD2 modulation and the subsequent induction of innate antibacterial responses against enteric bacteria.

Another antimicrobial protein, angiogenin-4, may also be important in IBD. Angiogenin-4 is typically implicated in tumour-associated angiogenesis, but it is also known to promote bactericidal innate immune activity against intestinal microbes. In a study of vitamin D-deficient mice, DNA microarray analysis of colonic tissue identified 27 genes consistently up-regulated or down-regulated in vitamin D-deficient vs vitamin D-sufficient mice [136]. This included angiogenin-4, which was significantly decreased in vitamin D-deficient mice even in the absence of colitis. Interestingly, this was associated with elevated levels (50-fold) of bacteria in colonic tissue. Thus, vitamin D leads to the upregulation of anti-microbial peptides against enteric bacteria, and disruption of this balance may lead to the dysbiosis thought to be important in CD pathogenesis.

1.2.3.3 Antigen presentation and innate immune response

An adequate immune response against microbes requires cooperation with the adaptive immune system. Specialised cells need to process and present microbial antigens to stimulate T-cell differentiation and appropriate cytokine responses. Dendritic cells (DC) are effective antigen-presenting cells (APC) and express the VDR and CYP27B1 [137]. However, exposure to $25(OH)D_3$ and $1,25(OH)_2D_3$ attenuates the capacity of DCs for antigen presentation and these cells become less responsive to antigens and less effective at activating the adaptive immune response. This is known as tolerogenesis [138, 139]. The mechanism by which $1,25(OH)_2D_3$ influences DC phenotype, antigen presentation and T-cell function, is not yet known. Despite this, the following three observations have been made; **1**) $1,25(OH)_2D_3$ preferentially inhibits the maturation of myeloid DCs, which are more active than plasmacytoid DCs at priming naïve T-cell responses and thus, the key effect of vitamin D may be to suppress the activation of naïve T-cells [140]. **2)** Immunostimulatory IL-12 activity is inhibited by $1,25(OH)_2D_3$ in DCs and other APCs [141]. IL-12 shifts T-cell cytokine production towards a Th1 phenotype (IFNγ, TNFα and IL-2) rather than a Th2 (IL-4, 5, 6, 10 & 13) phenotype. **3)** DCs treated with $1,25(OH)_2D_3$ enhance IL-10 expression which is both anti-inflammatory and immunosuppressive and can oppose the pro-inflammatory effects of IL-12 [142].

$1,25(OH)_2D$ is also able to inhibit the IL-23 receptor pathway. Group 3 innate lymphoid cells (ILC3s) are tissue resident lymphocytes that functionally resemble Th17/22 cells in the adaptive system. When ILC3s are stimulated by IL-23 plus IL-1β they are rendered responsive to $1,25(OH)_2D_3$ by upregulating the VDR, which subsequently causes downregulation of proinflammatory IL-23R pathways [143].

1.2.3.4 Vitamin D and adaptive immunity

T lymphocytes and B lymphocytes express the VDR and thus $1,25(OH)_2D_3$ can suppress their proliferation and phenotype [144, 145]. This effect is independent of DC regulation. *In vivo* studies

suggest that $1,25(OH)_2D_3$ can directly enhance Th2 and suppress Th1 cytokine expression [146]. VDR KO mice, however, have reduced Th1 cytokines, thus the effect on T-cells *in vitro* is more complex than a simple Th1 to Th2 shift [147]. This is particularly relevant in IBD, as the early stages of CD are characterised by a Th1 and Th17 response, whereas in UC a Th2-like response predominates [148]. Thus, the disparate findings from *in vivo* and *in vitro* studies of Th1 vs Th2 responses may simply reflect the limitations of animal models in replicating the different IBDs.

$1,25(OH)_2D_3$ also influences the development and function of Th17 cells, regulatory T cells (Treg) and CD8 T cells [149]. Naïve T cells can differentiate into Th1, Th2 or Th17 cells. Th17 cells differentiate in response to TGF-β in the presence of IL-6 or IL-21. IL-23, is thought to be important for the pathogenicity of Th17 cells, and can amplify Th17 differentiation in the presence or absence of TGF-β [150]. CD4+ T cells from VDR KO mice more readily develop into Th17 cells [151]. IL-17 is a proinflammatory cytokine produced by Th17 cells and has been implicated in several autoimmune conditions including IBD. *In vivo* mouse models of IBD demonstrate that treatment with $1,25(OH)_2D_3$ down-regulates the expression of IL-17 [152]. Tregs suppress the activation and effector functions of mature self-reactive lymphocytes by releasing mediators such as TGF-β and IL-10 and thus maintain self-tolerance in peripheral tissues. Reduced Treg numbers occur in autoimmune diseases including IBD [153]. $1,25(OH)_2D_3$ enhances the suppressive capacity of Tregs, which then inhibit the proliferation of other CD4+ T cells [154]. $1,25(OH)_2D_3$ also stimulates Treg development directly, and through the induction of immature DC phenotypes, to promote Treg activity [155, 156].

$CD8^+$ T cells, also known as cytotoxic T cells, have an essential role in the elimination of virally infected cells and increased proportions of circulating $CD8^+$ cells occur in CD and UC [157]. Transfer of VDR KO $CD8^+$ T cells, but not wild type (WT) $CD8^+$ T cells, induced colitis in Rag KO mice, where more severe colitis was associated with rapidly proliferating naïve VDR KO $CD8^+$ T cells. It thus appears that VDR is required to prevent replication of quiescent $CD8^+$ T cells [158]. VDR is also important for homing of $CD8^+$ T cells to the small intestine as demonstrated in murine experiments where IL-10/VDR

double KO mice were reconstituted with WT CD4 T cells or VDR KO CD4 T cells. The WT CD4 T cells compared to reconstitution with VDR KO CD4 T cells inhibited the development of colitis and were associated with twice as many intraepithelial CD8αα cells (a subset of $CD8^+$ T cells) in the small intestine [159].

1.2.4 Genetic aspects of Vitamin D

With advances in molecular biology techniques, an understanding of the VDR and the DBP gene structure has evolved. This has opened new avenues to investigate how vitamin D biology may be linked to IBD.

1.2.4.1 Vitamin D Receptor (VDR)

The VDR is a nuclear receptor that binds $1,25(OH)_2D_3$ and modulates many biological activities via gene transcription. The $1,25(OH)_2D_3$-VDR-RXR complex binds to VDREs on gene promoters and replaces transcription repressors with transcription activators within the target genes [125]. Over 100 genes with VDREs in the promoter regions have been identified by microarray analyses, these are all potential targets for the $1,25(OH)_2D_3$-VDR complex. In addition to the well-known targets for calcium homeostasis, the VDR is also expressed by most immune cells. Recent chromatin immunoprecipitation parallel DNA sequencing (ChIP-seq) studies have identified widespread VDR binding throughout the human genome, in all 2776 genomic positions are occupied by the VDR [160]. Due to its widespread targets and pleiotropic effects, there has been interest to investigate the genetic variants of the VDR and its relationship to disease states.

The VDR gene maps to chromosome 12q13.11. Alternative gene splicing results in multiple transcript polymorphisms that encode the VDR protein resulting in different protein lengths [161]. For example, two VDR isoforms identified in the human population arise from a frequent single base pair transition

(ATG-ACG) [162, 163]. This mutation results in the production of a shortened VDR with 424 amino acids instead of 427, and this polymorphism can be detected through the presence, or absence, of a *FokI* restriction site [163]. This occurs in the homozygous form in approximately 37% of people and in the heterozygous form in approximately 48% of the population [164]. The functional significance of these different VDR isoforms are not known.

VDR genetic variants are associated with a variety of different diseases/phenotypes including various cancers, asthma, height, longevity/mortality, bone mineral density and obesity [125]. The six common VDR genetic variants include *Cdx2, GATA, Fok1, Bsm*I*, Apa*I and *Taq*I.

VDR variants are associated with IBD. In one of the earliest studies Simmons and colleagues identified that *Taq*I polymorphism homozygotism (genotype "tt") was more common in CD compared to UC Caucasian patients (P=0.017) [165]. This finding was reproduced in two other Caucasian populations where an association was detected between male CD, UC and IBD patients and the *Taq*I polymorphisms [166, 167]. A meta-analysis of all studies has further suggested that the *Taq*I tt genotype increases the risk of CD in Europeans, while males with the genotype have a moderately increased risk of developing either UC or CD [168]. The same study found that in Asians the ff genotype of *Fok*I was associated with an increased risk of developing UC and the minor allele carrier status of *Apa*I appeared to be protective against CD. The mechanism by which the tt genotype of *Taq*I influences the susceptibility to CD in Caucasians, however, remains unclear, but this genotype is associated with lower VDR mRNA levels and may be associated with less efficient VDR-induced inhibition by $1,25(OH)_2D_3$ of the production of IL-12, which is involved in the immune activation seen in CD [169-171].

1.2.4.2 IBD susceptibility genetic loci regulated by vitamin D

1,25(OH)D regulates the expression of several CD-specific gene loci, such as *NOD2, ATG16L1* and *PTPN2.* Transcription of the *NOD2* gene is directly stimulated by $1,25(OH)_2D_3$-bound-VDR in human monocytic and epithelial cells pre-treated with $1,25(OH)D_3$ [134]. Activated NOD2 recruits ATG16L1

to the cell membrane at the entry site of bacterial products such as lipopolysaccharide. Mutations in NOD2 or ATG16L1, however, lead to functional impairment of autophagy and pathogen clearance resulting in an exaggerated inflammatory response [172]. Thus, through its regulation of *NOD2,* vitamin D could indirectly regulate autophagy and inflammation levels. CHiP-seq has defined a genome-wide map of VDR binding and identified *PTPN2* as a notable target gene for VDR binding [160]. Thus 1,25(OH)D-VDR complex-induced PTPN2 expression may inhibit epithelial barrier pore formation and altered intestinal permeability [173].

1.2.4.3 Vitamin D binding protein (VDBP)

VDBP is a multifunction glycosylated protein that is structurally related to albumin and α-fetoprotein. The VDBP gene is encoded on chromosome 4q11-q13 [174]. The primary function of VDBP is to transport vitamin D_3 metabolites and fatty acids to target tissues, and to sequester globular actin with a possible role in modulating inflammation and the immune system. Interestingly, VDBP-null mice are viable and do not display an obvious phenotype when fed a normal diet [175]. No humans, however, have been detected with a total absence of VDBP, suggesting that one, or more, functions of VDBP may be essential to human viability [176].

The *VDBP* gene, however, does exist in humans as three common variants, and over 120 rare variants [176]. Three major isoforms are referred to as Gc1F (wild-type), Gc1S, and Gc2. Compared to the wild-type Gc1F protein, the Gc1s contains the Asp416Glu polymorphism, and the Gc2 contains the Thr420Lys polymorphism. A GWAS of 25(OH)D_3 concentrations in 33,996 Caucasians of European descent observed that three loci reached genome-wide significance in predicting a risk of vitamin D insufficiency [106]. One of these involved the GC gene encoding VDBP at 4p12 (overall $p=1{\cdot}9\times10^{-109}$ for rs2282679, in GC). The other loci related to cholesterol synthesis and the CYP2RI gene controlling 1α-hydroxylation. The link between *VDBP* variants and 25(OH)D_3 concentrations has been previously

reported; these studies also found that *GC* variants associated with low $25(OH)D_3$ concentrations were strongly related to reduced concentrations of VDBP [177, 178]. Several mechanisms for this have been postulated. Concentrations of the binding protein may affect delivery of $25(OH)D_3$ and $1,25(OH)_2D_3$ to target organs, as well as clearance of vitamin D metabolites from the circulation. Alternatively, the difference in quantity or function of the binding protein could be accompanied by changes in the relative proportion of free and bound 25(OH)D, with the free portion being the potential rate-limiting factor for 1,25(OH)D production. Arnaud et al showed there was a higher affinity for binding $25(OH)D_3$ to Gc1, compared with Gc2 supporting this concept [179].

Few groups have examined the VDBP genetic variants in IBD specific populations. One group however, examined two SNPs in exon 11 of the *VDBP* gene looking for the Asp426Glu polymorphism associated with GC-1s isoform and the Thr420Lys associated with the Gc2 isoform, in an IBD group compared to controls [174]. It was found that the Thr420Lys containing phenotypes were less frequent in IBD (both CD and UC) with the difference most notable when comparing those with IBD and homozygous for the Thr420Lys polymorphism with the controls. The authors concluded that the Thr420Lys associated with *GS-2* could have a protective role against IBD. This seems paradoxical as this isoform is associated with lower plasma concentrations of $25(OH)D_3$ and lower affinity of VDBP to vitamin D metabolites, however, this could result in higher free $25(OH)D_3$ to be available to target tissues such as the intestine.

A small study incorporating proteomics technology to determine predictors of primary non-response, response and remission to anti-TNFα therapy in IBD, also found that VDBP concentrations were significantly increased in the serum of patients achieving remission compared to baseline samples [180]. There are no studies, however, examining the impact *VDBP* polymorphisms to patient response to anti-TNFα therapy.

1.2.5 Clinical aspects of Vitamin D in IBD

1.2.5.1 Vitamin D and incidence of IBD

Few studies have correlated vitamin D deficiency with the incidence of IBD, however, those developing IBD from the Nurses' Health Study (72719 women) were identified and compared to non-IBD controls [82]. Participants completed diet and lifestyle assessments from which a $25(OH)D_3$ prediction score was developed. It was noted that those in the highest quartile of $25(OH)D_3$ levels had a significantly lower risk of developing CD (adjusted HR 0.54, 95% CI 0.30-0.99, p=0.02). There was an insignificant association with UC, but the small number of incident cases of CD (122) and the older age of the cohort may have underestimated the associations.

Seasonal effects on the incidence of IBD have also been examined in numerous studies, as low $25(OH)D_3$ levels can be expected during months of low sunlight exposure. There are no consistent findings to suggest the season of birth increases the risk of later developing either CD or UC, and similarly there are no consistent seasonal effects with the onset of CD or UC [181].

1.2.5.2 IBD Activity

An association between CD activity and vitamin D status was first reported by Harries and colleagues in 1985 when they published on 40 undernourished and 40 well-nourished patients with CD, and found that the undernourished group were more likely to have active CD and low plasma $25(OH)D_3$ [85]. Since then a number of cross-sectional and retrospective cohort studies have correlated CD activity with plasma $25(OH)D_3$ levels [83, 84, 99]. A cross-sectional Australian study found a negative correlation between faecal calprotectin levels (but not C-reactive protein) and total, free and bioavailable $25(OH)D_3$ even after controlling for sunlight exposure, vitamin D intake and obesity, further supporting a potential role for vitamin D in controlling intestinal inflammation [182]. In smaller cross-sectional studies, however, including one study from an Australian paediatric population, an inverse association between active CD and $25(OH)D_3$ was not found [183]. The results from UC studies have also been mixed [84, 184].

In a large multi-institution IBD cohort, serum $25(OH)D_3$ <50 nmol/L was associated with an increased risk of surgery (OR 1.76; 95% CI 1.24-2.51) and hospitalisation (OR 2.07; 95%CI 1.59-2.68) compared with those with sufficient levels [86]. Similar estimates were seen in UC. Taking this a step further, the same study found that patients with CD who had initial levels $25(OH)D_3$ <75 nmol/L, but subsequently increased their $25(OH)D_3$ level, had a reduced likelihood of surgery (OR, 0.56; 95% CI, 0.32-0.98) compared with those with persisting $25(OH)D_3$ <75nmol/L suggesting that increasing vitamin D levels may alter the CD course. Another prospective cohort study involving 965 IBD patients followed for up to 5-years found those with mean $25(OH)D_3$ <75nmol/L required significantly more steroids, biologics, narcotics, computed tomography scans, emergency department visits and hospital admissions. Those who received vitamin D supplements with an increase in 25(OH)D levels had significant reduction in their health-care utilisation [185]. While the authors imply a causal link between improved vitamin D levels and health outcomes, the observation could be equally explained by those with milder disease burden at baseline being more likely to have improvement in 25(OH)D levels and associated reduced health-care utilisation.

The effect of seasonal variation on disease flare has been examined in a number of studies, as low sunlight exposure and subsequently low $25(OH)D_3$ levels might be expected to result in disease flare. Almost all the data comes from the northern hemisphere, and these have reported peaks in Crohn's disease activity in spring, winter and summer, but a number of studies did not report a seasonal effect [181]. Ulcerative colitis has more consistently not been shown to have a seasonal effect [181].

1.2.5.3 Vitamin D as treatment

There is evidence from animal models of colitis that vitamin D may be effective in ameliorating the inflammation, with DSS-induced colitis being more severe in vitamin D deficient, VDR KO and CYP27B1KO mice, than in controls [136, 186, 187]. The oral administration of 1,25(OH)D to IL-

10/vitamin D double-deficient mice also reduced the severity of spontaneous colitis [188]. Similarly the vitamin D analog 22-3n3-25-oxa-vitamin D was noted to reduce the severity of colitis in the 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced mouse model [189].

Despite the observed effects in animals, there are relatively few human studies that demonstrate a similar therapeutic benefit from vitamin D. A placebo-controlled trial has evaluated the effect of vitamin D in CD, and there are no placebo-controlled trials examining its effect in UC [90]. In this randomised double-blind placebo-controlled trial, 94 CD patients in remission were randomised to receive either 1200 IU vitamin D or placebo once daily for 1 year [90]. Treatment reduced the rate of clinical relapses from 29% to 13% (P=0.06) but this study was noted to be underpowered and that may be why the impact of vitamin D did not reach statistical significance. The study also did not exclude patients who already had relatively high 25(OH)D_3 levels, which could affect the findings. In addition, the study dose and the serum levels of 25(OH)D_3 achieved may have been too low to induce a clinical effect. Selecting patients with vitamin D insufficiency, or deficiency, and supplementing in order to achieve higher serum levels and including greater patient numbers could improve a similar study's statistical power and may define an effect for vitamin D.

Another pilot randomised double-blind controlled study compared 10,000 IU to 1,000 IU daily vitamin D3 for 12 months in 34 patients with CD in remission and found that high dose is more effective at raising serum 25(OH)D levels, however, on intention-to-treat analysis the rate of relapse was not significantly different between the two groups, and borderline significant in per-protocol analysis (p=0.049) [190]. Again, most of the subjects in this study were vitamin D sufficient at baseline with a mean 25(OH)D of 71.3 and 73.5 nmol/L, respectively, in the two groups. A pilot study in UC supplemented vitamin D in patients with active and inactive UC and in healthy controls. The study demonstrated reduced faecal calprotectin, increased albumin levels as well as an increase in faecal Enterobacteriaceae abundance but no change in clinical activity [191].

1.3. Ultraviolet radiation-induced systemic immunosuppression

UVB is the major source of vitamin D and as the incidence of CD is more prevalent at higher latitudes, vitamin D may explain this observation [15]. To date, however, studies examining a therapeutic benefit of vitamin D replacement in IBD have been negative, but there could be vitamin D-independent pathways that are involved in the mechanisms of UVR-induced systemic modulation of immunity. In female, but not male, vitamin D deficient mice, acute erythemal UVR increased serum $25(OH)D_3$ levels despite similar systemic immune suppression responses demonstrated in both sexes, suggesting that $25(OH)D_3$ may not be essential for the mediation of the immunosuppressive effects of erythemal UVR [192]. Two studies have examined the effect of phototherapy in an animal model of IBD using oral DSS which causes a chemical injury to the gastrointestinal tract that is then repaired by innate immune mechanisms [193]. These studies reported a reduction in disease severity with light therapy, but the findings are limited by the subjective measures of colitis severity, the small numbers of mice used and most importantly, the lack of definition and consistency of the light sources used.

The vitamin-D independent pathways by which UVR may suppress immunity have yet to be fully elucidated, however, a number of mechanisms have been proposed and reviewed in detail elsewhere [112, 194]. In brief, UVR exposure of skin cells can damage their DNA and it has been proposed that enhanced repair of UVR-induced DNA lesions limits UVR-induced immunosuppression. It has recently been shown that the addition of $1,25(OH)_2D_3$ enhances the repair of UVR-induced DNA damage [195]. Further, UVR exposure of keratinocytes can alter double-stranded domains of noncoding RNAs, which stimulate the production of inflammatory mediators (such as ATP, cytokines, chemokines, and biolipids), surface markers (including TLRs and RANK-Ligand), cyclooxygenase-2, and antimicrobial peptides [196]. If these innate responses are of sufficient concentration, they can lead to infiltration of neutrophils, monocytes, macrophages and Th-17 cells into the irradiated skin, and receipt of

inflammatory signals by the bone marrow to regulate stem cell and progenitor cells and their daughter myeloid cells, thus influencing systemic innate immune responses [197].

A second pathway leads to reduced antigen-specific responses. After UV irradiation of skin and application of antigen to the irradiated site or to nonirradiated skin, there are fewer antigen-specific effector and memory T cells generated. These cells have reduced capacity to migrate to distant tissues, thus enabling a dampened immune response on subsequent antigen exposure [198].

In another pathway, nitric oxide (NO) synthase-mediated production of NO is stimulated by UV irradiation of skin. The NO can induce suppressive Tregs [199]. Further, UVR-induced molecules of interest include prostaglandin E_2 (PGE_2) which has been implicated in systemic immunosuppression [200]. Interestingly, in murine IBD models, the effect of PGE_2 has been conflicting, with one study showing that PGE_2 shifts the IL-12/IL-23 balance in DCs in favour of IL-23 and exacerbates the inflammatory process, while work from our group has shown that PGE_2 reduced the severity of TNBS colitis [201, 202] . Thus, PGE_2 appears to be a regulator of intestinal inflammation but the mechanisms for this remain unclear. Other UVR-induced molecules of interest include *cis*-urocanic acid (UCA), which when added to cultures of human peripheral blood mononuclear cells stimulates a higher percentage of $FoxP3^+$ Tregs, increased IL-10 and decreased IFN-γ [203]. Similarly, UVR activation of the aryl hydrocarbon receptor induces human $FoxP3^+$ Tregs.

1.4. Animal Models of IBD

Animal models of IBD are valuable for studying pathophysiological mechanisms of intestinal inflammation and the effect of emerging therapies. While none of the available animal models represent the complexity of human disease and do not replace studies with patient material, they do provide the

opportunity to examine the effect of treatments in a controlled and reproducible manner, avoiding the heterogeneity inherent with clinical studies. Broadly speaking, animal models of IBD are based either on chemical induction, immune cell transfer or gene targeting, and only in some models, disease occurs without any exogenous manipulation.

1.4.1 Disruption of epithelial integrity

A number of models induce intestinal inflammation by disrupting the integrity of the intestinal epithelial layer, allowing translocation of intestinal bacteria into the laminal propria and initiating an overwhelming inflammatory response [204]. In otherwise asymptomatic CD patients, increased intestinal epithelial permeability preceding clinical relapse has been observed suggesting that a barrier defect may be an early event in disease recurrence [205].

1.4.1.1 DSS Colitis

Feeding mice for several days with DSS polymers in the drinking water induces a very reproducible acute colitis characterised by bloody diarrhoea, ulceration and infiltration with acute inflammatory cells [206]. It is believed that DSS is directly toxic to gut epithelial cells. This model is particularly useful for studying innate immune mechanisms of colitis and epithelial repair mechanisms. T- and B-cell-deficient C.B-17^{scid} or Rag1$^{-/-}$ mice also develop severe colitis, indicating the adaptive immune system does not play a major part in this model [207].

1.4.1.2 TNBS/Oxazolone Colitis

Colitis can be induced by intrarectal instillation of the haptenated substances, TNBS/DNBS or oxazolone, dissolved in ethanol [208, 209]. Ethanol breaks the mucosal barrier, whereas TNBS/Oxazolone haptenises colonic autologous, or microbiota proteins, rendering them immunogenic

to the host immune system. $CD4^+$ T cells play a central role in chronic TNBS colitis, thus this model is useful to study T helper cell-dependent mucosal immune responses [210]. Inflammation is most frequent in the distal colon, and inflammation predominated by Th2 responses, making this a good model for UC.

1.4.1.3 Dominant negative (DN) N-cadherin transgenic mice/keratin $8^{-/-}$ mice

Transgenic/chimeric mice expressing a DN mutant of the cell adhesion molecule N-cadherin in intestinal epithelial cells along the crypt villus axis, develop areas of leaky epithelium and chronic IBD and at later times intestinal neoplasia [211]. Inflammation only occurs in the vicinity of porous epithelium.

1.4.1.4 Senescence accelerated mouse P1/Yit (Samp) mice

In this model, inflammation occurs spontaneously in the terminal ileum, making it an excellent model to study mechanisms of CD [212]. Similar to CD, inflammatory lesions are characterised by transmural inflammation, granulomas and change in epithelial morphology. Increased epithelial permeability precedes the onset of inflammation.

1.4.2 IBD development related to dysregulated immune cell function

1.4.2.1 STAT3 deficiency in myeloid cells

Mice with specific disruption of the STAT3 gene in macrophages and neutrophils develop a spontaneous enterocolitis [213]. STAT3 in macrophages and neutrophils is a critical factor within the signal transduction pathway of IL-10. This suggests that the absence of an IL-10-mediated

counterregulatory effect on colonic macrophages, continuously subjected to stimulation by luminal bacterial or food antigens, is sufficient for the development of chronic intestinal inflammation.

1.4.2.2 TNF$^{\Delta ARE}$ MICE

Gene targeting of AU-rich elements (ARE) in the untranslated region of the TNF-α mRNA in mice is associated with increased constitutive and inducible levels of TNF-α due to dysregulated processing of TNF-α mRNA [214]. Overproduction of TNF-α leads to polyarthritis and chronic intestinal inflammation with infiltrating inflammatory cells.

1.4.2.3 IL-10/orphan receptor cytokine receptor family 2 (CRF2-4) deficient mice

IL-10 is a well-known suppressor of Th1 cells and macrophage effector functions. Non-germ-free mice with targeted deletion of the IL-10 gene spontaneously develop chronic enterocolitis with massive infiltration of lymphocytes, activated macrophages and neutrophils [215]. Further studies identified that T-cell specific IL-10 deficiency is most relevant for induction of intestinal inflammation. IL-10 expression from other cell types seems to be more important in the context of acute septic shock. IBD like disease is also present in mice deficient in CRF2-4 [216] and the IL-10 receptor beta chain, and in mice with myeloid cell specific STAT3 deficiency that reveals a defect in IL-10 signalling.

1.4.3. Choice of model

Most models cause inflammation of the colon rather than the small bowel, with only few causing ileitis. Unfortunately, the onset and severity of disease in most of the spontaneous gene targeted or transgenic models are highly variable and depend on environmental factors such as the enteric flora of the animal

facility [193]. Thus, for evaluating novel therapeutic agents, experimentally induced acute colitis with DSS- or TNBS- are widely used. These models are cost effective and highly reproducible.

1.5. Linking vitamin D to IBD pathogenesis and gaps in current understanding

Epidemiological data link reduced UVR-exposure and vitamin D deficiency to the development of IBD. The role of vitamin D as a regulator of innate and adaptive immune responses directly, and through modulating the expression of genes such as NOD-2 and ATG16L, makes vitamin D a candidate regulator of the gut mucosal immune system. To date, a number of critical questions remain which form the basis of this thesis;

1. Whether vitamin D deficiency is the cause, or result, of intestinal inflammation.
2. Whether the effect on gut mucosal immunity is due to vitamin D or if vitamin D is a surrogate for UV-irradiation, which has vitamin D independent immunomodulatory properties.
3. Whether vitamin D alters gut mucosal immunity. If so, is there a dose response where a maximal effect is achieved?

1.6. Organisation of thesis

To address these questions this thesis has been organised into a series of papers.

1.6.1 Paper 1 – High Vitamin D-Binding Protein Concentration, Low Albumin, and Mode of Remission Predict Relapse in Crohn's Disease [217]

To address the issue of reverse causality of vitamin D deficiency and IBD, I examined a cohort of CD patients in remission with stored serum and DNA. Vitamin D metabolites were analysed to determine if $25(OH)D_3$ levels predicted time to relapse. In this study, the VDBP concentration, and not circulating $25(OH)D_3$, was found to be associated with disease relapse.

1.6.2 Paper 2 – High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis [218]

While serum $25(OH)D_3$ levels did not predict CD relapse, this did not exclude a benefit of vitamin D supplementation in reducing active intestinal inflammation. To examine this, we utilized a mouse model of colitis. The advantage of murine models is the ability to control for background genetics, diets, UV exposure and disease phenotype, which is not possible in clinical trials. Mice were maintained on diets containing A. no vitamin D, B. sufficient vitamin D or C. high doses of vitamin D before treatment with DSS to induce colitis. Mice on the highest dose of vitamin D developed the most severe colitis. Serum 25(OH)D levels also dropped significantly after the induction of the DSS colitis, consistent with $25(OH)D_3$ being a negative acute phase reactant.

1.6.3 Paper 3 – Ultraviolet Irradiation of Skin Alters the Faecal Microbiome Independently of Vitamin D in Mice [219].

As oral vitamin D supplementation was not protective, we sought to determine if UV-irradiation independently impacts faecal microbiome and protects against colitis. To do this, UV phototherapy was added to the three groups of mice on the different vitamin D diets. It was determined that UV-irradiation impacts faecal microbial composition independently of vitamin D, but this did not impact the inflammation level associated with DSS.

1.6.4 Paper 4 – Vitamin D C3-epimer levels are proportionally higher with oral vitamin D supplementation compared to ultraviolet irradiation of skin in mice but not humans [220].

Lower serum vitamin D C3-epimer levels in the initial cohort of CD patients were noted in comparison to those measured in mice. This led me to examine in more detail the differences between murine and human vitamin D metabolism. Serum samples from experimental mice and participants in two clinical trials were retrieved to determine if this was the case using the same 25(OH)D assay, and whether oral supplementation versus UV-irradiation had different effects on epimer production in humans.

1.6.5 Paper 5 – $24,25(OH)_2D_3$ is lower with active Crohn's disease and spontaneously recovers with remission *(unpublished, presented European Crohn's and Colitis Organisation Congress, Barcelona 2017)*

In view of the differences between murine and human vitamin D metabolism, I led a team to examine a cohort of CD patients to determine if $25(OH)D_3$ is indeed a "negative acute phase reactant" after carefully accounting for confounders such as corticosteroid use, vitamin D supplementation, dietary intake and sunshine exposure. Participants with active CD disease, or CD in remission, were enrolled and circulating $25(OH)D_3$, $1,25(OH)_2D_3$ and the downstream breakdown metabolite $24,25(OH)_2D_3$ were measured at baseline and at 6 months. At baseline, levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ were not different between those with active disease and those in remission. $24,25(OH)_2D_3$ levels were reduced

in active disease. It is likely, that in the setting of active disease there is relative vitamin D deficiency with a homeostatic downregulation of CYP24A1 activity to maintain 25(OH)D and 1,25$(OH)_2$D concentrations, and as a consequence reduced 24,25$(OH)_2D_3$ levels. Importantly, when remission was achieved without vitamin D supplementation and independently of changes in sunlight exposure and dietary intake, there was a spontaneous increment in 25$(OH)D_3$ and 24,25$(OH)_2D_3$ levels. This supports the previous findings that 25$(OH)D_3$ is a negative acute phase reactant that falls in the setting of inflammation and spontaneously increases with treatment of inflammation.

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Chapter 2

Materials and methods

2.1 MATERIALS

All chemical reagents, commercial kits and equipment used for experimentation in the murine and human studies are shown below. These were all of analytical or biochemical grade. Following this there is an outline of methodology used for experiments performed for each of publications included in this thesis.

2.1.1 General Reagents and Materials

Anaesthetic (murine) - ketamine, xylazine	Troy Laboratories, NSW, AUS
Agarose	Sigma Aldrich, St. Louis, MO, USA
Chloroform	Sigma Aldrich
Dextran sodium sulphate (DSS)	MP Biomedicals, LLC, OH, USA
EDTA	Merck Pty Ltd, NSW, AUS
Ethanol (100%)	Sigma Aldrich
Ethidium bromide	Merck Pty Ltd
Hydrogen chloride (HCl)	Sigma Aldric
Magnesium chloride ($MgCl_2$)	Merck Pty Ltd
PBS tablet	Amersham Biosciences, Buckinghamshire, UK
Potassium chloride (KCl)	Merck Pty Ltd
Sodium chloride (NaCl)	Merck Pty Ltd
TAE Buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA)	Thermo Fisher Scientific, Waltham, MA, USA
Tris (Hydroxymethyl Methane)	BDH Biochemicals, UK
Tween-20	Sigma Aldrich
Water, “Ultrapure”, DNAse/RNase-free	Gibco® Invitrogen, Waltham, MA, USA

2.1.2 DNA and RNA studies and reverse transcription reagents

2X TaqMan universal PCR mix	Life Technologies, Carlsbad, CA, USA

Deoxyribonucleotide triphosphate (dNTP)	Invitrogen, Carlsbad, CA, USA
PCR reaction buffer, 10X	Invitrogen
Magnesium chloride, 25mM	Invitrogen
DNA AWAY™Surface Decontaminant	Thermo Fisher Scientific
Platinum Taq DNA polymerase	Invitrogen
TRIzol™ Reagent	Invitrogen
RT^2 SYBR Green qPCR mastermix	Qiagen, Hilden, Germany
GoTaq® DNA Polymerase	Promega, Madison, WI, USA

2.1.3 Commercial Kits

$1,25(OH)_2D_3$ IDS EIA ELISA	Immunodiagnostic Systems, Fountain Hills, AZ, USA
Bio-Plex Pro ™Mouse Cytokine 23-plex assay	Bio-Rad, Hercules, CA, USA
MicroAmp® 96-well Optical reaction plates	Life Technologies
PowerSoil DNA Isolation Kit	MoBio, Carlsbad, CA, USA
QuantiTect® Reverse Transcription kit	Qiagen
Qubit dsDNA broad range (BR) Assay Kit, 100	Invitrogen
RNeasy® Plus Universal Mini Kit	Qiagen
Vitamin D binding protein Quantikine ELISA	R&D Systems, Minneapolis, MN, USA

2.1.4 Equipment and Software

3730XL DNA Analyzer	Applied Biosystems, Foster City, CA, USA
24 well microtitre plates	BD Biosciences, San Jose, CA, USA
96 well microtitre plates	BD Biosciences

0.6, 1.5 & 2mL MAXYMum recovery™microfuge tubes	Axygen Scientific, Union city, CA, USA
15 & 50 ml Falcon tubes	BD Biosciences
19 G needles	Terumo Medical Corp, NSW, AUS
23 G needle	Terumo Medical Corp
10, 20, 50ml syringes	BD Biosciences
Agilent 1290 UPLC binary pumps	Agilent technologies, Santa Clara, CA, USA
Agilent 6460 triple quadrupole tandem mass spectrometer	Agilent technologies
Applied Biosystems 7900HT Fast Real-Time PCR System	Applied Biosystems
Architect c1600 Analyzer	Abbott Diagnostics, Chicago, IL, USA
BioPlex 200 system	Bio-Rad
Benchtop centrifuge 5414D (refrigerated)	Eppendorf, Hamburg, Germany
FastPrep-24 homogenizer	MP Biomedicals
Gel Doc™XR System	Bio-Rad
Heating Block 1.5mL	
Heating Block 2mL	
Microtome: Microm HM 325	GMI Inc, Ramsey, MN, USA
Nanodrop 1000 spectrophotometer	Thermo Fisher Scientific
Qubit 2.0 Fluorometer	Invitrogen
Rotor-Gene 3000	Qiagen
Rotor-Gene 6 software	Corbett Research, NSW, AUS
Superfrost® plus slides	Menzel-Glaser, Germany

Wallac 1420 multilabel plate reader	PerkinElmer Life and Analytical Sciences, Waltham, MA, USA

2.2 Methods for retrospective analysis of Vitamin D metabolites in a Crohn's disease cohort

These methods are relevant to the published manuscript, ***"High Vitamin D-Binding Protein Concentration, Low Albumin, and Mode of Remission Predict Relapse in Crohn's Disease"***, presented as Chapter 3 of this thesis.

2.2.1 Patients and design

Prospectively maintained databases at three Inflammatory Bowel Disease (IBD) units in Australia were interrogated to identify patients with CD in remission at the time of serum and DNA banking. The three IBD centres included were: 1) The Centre for IBD, Fremantle Hospital, Fremantle, W.A; 2) Flinders Medical Centre, Adelaide, South Australia; and 3) The IBD Unit, Royal Brisbane and Women's Hospital, Brisbane, Queensland. The study was approved by the human research ethics committee of the South Metropolitan Area Health Service, Western Australia (Ref: 07/589), with site-specific approvals at the secondary sites.

Data were collected at the time of first clinical visit and included disease activity, number, dates and types of CD-related surgeries, smoking, family history and medication use. These data were updated for each patient at each subsequent visit to determine changes in disease activity and medication use. Blood was collected at the first visit so that DNA and serum could be cryopreserved at -20 ^{0}C.

Clinical remission was defined as disease controlled without any change in CD medications and without corticosteroids for at least 4 weeks before inclusion. Patients were not on vitamin D supplementation at inclusion or during the follow-up period. In addition, both physician and patient assessment identified no CD symptoms or evidence of active disease requiring escalation of therapy. Patients had not been hospitalised in the previous 12 weeks. Disease relapse was defined as worsening in CD symptoms where the physician deemed a change in CD medications to be necessary.

2.2.2 Biochemical measurements

25(OH)D and C3-epimer-25(OH)D were measured using liquid chromatography tandem mass spectroscopy (LC/MS/MS) at the University of Western Australia, Centre for Metabolomics, using two Agilent 1290 UPLC binary pumps coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). This assay is certified by the Centre for Disease Control (NIH, USA) for both precision and accuracy of $25(OH)D_3$ measurement with a correlation of R^2 0.9979, and also for 3-epi-$25(OH)D_3$ R^2 0.9961.[1]

VDBP was measured by immunonephelometry (Dade Behring, Liederbach, Germany) (inter-assay coefficient of variation, CV, <6%), and albumin was measured by dye binding reaction to bromocresol green (inter-assay CV<5%). Free and bioavailable (free plus albumin-bound) concentrations of 25(OH)D were calculated using VDBP and albumin concentrations, according to modified "Vermeulen" equations [2] as shown below in Figure 2.1.1.

$[D_{total}]$ = serum 25(OH)D concentration
[Alb] = serum albumin concentration
[DBP] = serum vitamin D binding protein concentration
$[D_{Alb}]$ = albumin-bound 25(OH)D concentration
$[D_{DBP}]$ = DBP-bound 25(OH)D concentration
$[D_{free}]$ = free 25(OH)D concentration
$[D_{bioavailable}]$ = bioavailable 25(OH)D concentration = $[D_{free}] + [D_{Alb}]$
K_{alb} = affinity constant between vitamin D and albumin = $6 \times 10^5\ M^{-1}$ for 25-OH D
K_{DBP} = affinity constant between vitamin D and DBP = $7 \times 10^8\ M^{-1}$ for 25-OH D

Equation			
1	$[D_{DBP}]$	=	$[D_{total}] - [D_{Alb}] - [D_{free}]$
2	$[D_{Alb}]$	=	$K_{alb} \cdot [Alb] \cdot [D_{free}]$
3	$[D_{free}]$	=	$[D_{DBP}] \div K_{DBP} \div [DBP_{free}]$
4	$[DBP_{free}]$	=	$[DBP_{total}] - [D_{DBP}]$
From equations 3 and 4			
	$[D_{free}]$	=	$[D_{DBP}] \div K_{DBP} \div ([DBP] - [D_{DBP}])$
From equations 1 and 2			
	$[D_{DBP}]$	=	$[D_{total}] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{free}]$
	$[D_{free}]$	=	$\frac{[D_{total}] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{free}]}{K_{DBP}\ ([DBP] - ([D_{total}] - (K_{alb} \cdot [Alb] + 1) \cdot [D_{free}]))}$
Simplified to fit a second-degree polynomial ($ax^2 + bx + c = 0$) where $x=[D_{free}]$:			
$a =$	$K_{DBP} \cdot K_{alb} \cdot [Alb] + K_{DBP}$		
$b =$	$K_{DBP} \cdot [DBP] - K_{DBP} \cdot [D_{total}] + K_{alb} \cdot [Alb] + 1$		
$c =$	$-[D_{total}]$		
	$[D_{free}]$	=	$[-b + \sqrt{b^2 - 4ac}] \div 2a$
	$[D_{bioavailable}]$	=	$[D_{free}] + [D_{Alb}]$
		=	$(K_{alb} \cdot [Alb] + 1) \cdot [D_{free}]$

Figure 2.2.1 – Vermeulen equation to calculate free and bioavailable 25(OH)D

Seasonal variation in circulating 25(OH)D concentrations can be large relative to mean values. As single measurements may misclassify annual exposure, a sinusoidal model incorporating month of blood collection was fitted to the actual vitamin D concentration for each subject to calculate "de-seasonalised vitamin D" as previously described [3].

2.2.3 Vitamin D Binding Protein - Single nucleotide polymorphism (SNP) Genotyping

In a DNA-free room, primers were diluted by adding 188 µl of 1x TE buffer to 188 µl of thawed TaqMan primer to make 20x primer mix from 40x. Dilute primers (375 µl) were then aliquoted into 4 x 0.6 ml PCR clean Eppendorf tubes and stored away from light at -20 ^{o}C. The mastermix was prepared by adding 0.5 µl of TaqMan SNP primer (Life Technologies, USA), 5 µl TaqMan Genotyping Mastermix (Life Technologies) and 3.5 µl RNA/DNase-free H_2O

2.2.3.1 Dilution of DNA

DNA concentration was checked with the Nanodrop 1000 spectrophotometer, and subsequently diluted to 50 ng/µl using 1X TE buffer.

2.2.3.2 PCR

9 µl of pre-prepared mastermix was reverse pipetted into 0.1 mL strip PCR clean tubes. DNA samples (0.5 µl, i.e 25 ng DNA from 50 ng/µl) were added to each PCR clean tube. Amplification conditions on the thermocycler were as follows: 1 cycle of 95^{o}C for 10 min, followed by 50 cycles of 92^{o}C for 15 sec, and 60 ^{o}C for 1 min.

2.2.3.3 Allele Discrimination

Using the Rotor-gene 6 thermocylcer software, allelic discrimination was performed to determine wild type, mutant and heterozygosity for each of the two SNPs of interest. An example readout is shown below.

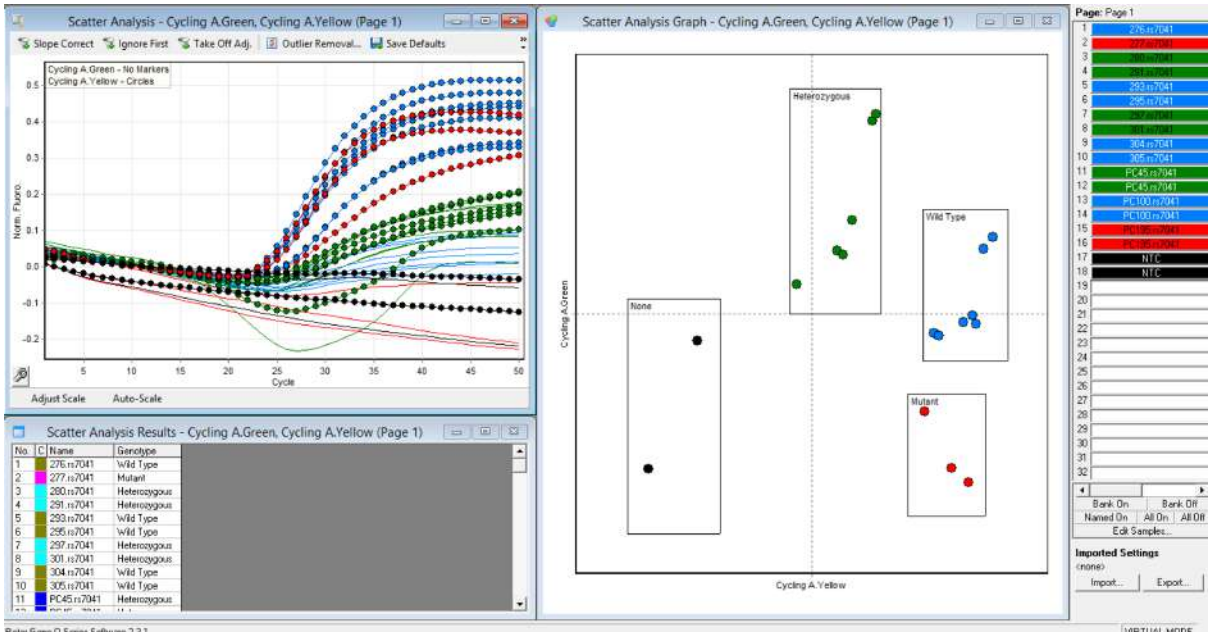


Figure 2.2.2 – Scatter analysis graphs generated by Rotor-gene 6 software for allelic discrimination

2.2.4 Sanger Sequencing

To confirm the genotype, a subset of samples underwent Sanger sequencing

2.2.4.1 Primer resuspension, determining yield and dilution

The forward primer 5' agccaagttacaataacaccaggaa 3' (C_A15629; Life Technologies) and the reverse primer 5'ttccaattcagcagcgatttgt3' (C_A15630; Life Technologies) were used. Primers were diluted to give a 5 µM working stock.

In the DNA-free room 2.25 µl of 5 µM primer was combined with Rnase-free water, dNTPs (10 mM), $MgCl_2$ (50mM), 10X reaction buffer and Platinum® *Taq* DNA polymerase (5 U/ µl) to a total volume of 42 µl. The preparation (24 µl) was aliquoted into each tube with 1 µl of template or TE buffer and placed on the Rotor-Gene thermocycler to determine the yield of each primer.

2.2.4.2 Preparation in-house Taq PCR master mix

In the DNA-free room and using a cooling block, 5 µl of 10X PCR buffer, 1 µl 10 mM dNTP mixture, 1.5 µl 50 mM $MgCl_2$, 0.2 µl Platinum® *Taq* DNA polymerase (5 U/ µl) and 39.3 µl RNase- and DNase-free water were added to 1 µl of primer mix (5 µM).

2.2.4.3 PCR

48 µl of the Taq mastermix were aliquoted into 0.2 µl thin wall tubes and 2 µl DNA (from 50 ng/ µg stock) were added. Tubes were placed on the thermocycler and the PCR program started with the following steps:

Step 1: 94 °C for 3 min – Incubation,

Step 2: 94°C for 30s – Denature,

Step 3: (annealing temperature specific for primer)for 30s – Anneal,

Step 4: 72°C for 30s – Extend,

Step 5: 35 cycles for steps 2 to 4,

Step 6: 4°C Hold

2.2.4.4 Checking PCR product on 1% Agarose Gel

A 10-comb agarose gel made with 1g agarose powder, 100mL 1X TAE buffer and 1.6 µl ethidium bromide were used. The ladder (0.5 µl of 1kB, Invitrogen) and 1.6 µl of 6X loading dye (Invitrogen) were added to each well with 7.9 µl RNase and DNase-free water. Sample wells were made of 5 µl PCR product, 1.6 µl loading dye and 3.4 µl water. The agarose was run at 90V for 30-45min and images were captured on the Gel Doc XR System (Bio-Rad).

2.2.4.5 Sequencing

Sanger sequencing was performed at the Institute of Immunology and Infectious Disease, Murdoch, WA, on the 3730XL sequencer (Applied Biosystems) and the sequences obtained were aligned using the Assign software (Conexio Genomics, Australia).

2.2.5 Statistical analysis

Summary statistics, including counts and percentages for categorical variables, and means and standard deviation for continuous variables, have been presented overall and broken down by whether the patient flared. The time to flare was investigated using Cox Proportional Hazards models to describe the risk of flaring by the various predictors. Results from univariate (one-predictor) have been provided for all potential predictor variables (age, gender, disease location, smoking status, mode of remission induction, season of blood draw, VDBP mg/L, total, bioavailable and free 25(OH)D, C-3-epimer-25(OH)D and percentage epimer of total 25(OH)D and albumin), along with results from model selecting from this group of variables to determine one final multiple predictor model. Hazard ratios and 95% confidence intervals are presented along with the Kaplan Meier charts for the variables identified as statistically significant in this final model and for other non-significant comparisons of interest. Model selection was carried out using a 0.05 significance level.

Genetic analysis was carried out in two ways. Firstly, analysis of variance models were used to determine whether there were differences in the levels of the two responses of VDBP and $25(OH)D_3$ in three different ways, one for each factor, VDBP genotypes rs4588 (CC, CA, AA), rs7041 (GG, GT, TT) and Diplotype (*GC* 1f-1f, 1f-1s, 1s-1s, 1-2 and 2-2). Pairwise comparisons were then undertaken between the levels to establish where the differences occurred. Secondly, Cox Proportional Hazards models were fitted, as previously described, using the genotypes as predictors of risk of flaring. All analyses were carried out using the R language and Environment for Statistical Computing.[4]

2.3 Methods for animal model experiments

Methods described here are relevant to the published manuscripts:

1. ***"High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis"*** presented in chapter 4; and
2. ***"Ultraviolet Irradiation of Skin Alters the Faecal Microbiome Independently of Vitamin D in Mice"*** presented in chapter 5.

2.3.1 Animal care and housing

Female 6-week-old C57Bl/6 mice were purchased from the Animal Resources Centre, Western Australia. They were fed semi-pure diets supplemented with higher than usual doses of vitamin D (SF14-069, Specialty Feeds, Perth, Western Australia, 10,000 IU/kg vitamin D_3, 0.5% calcium), standard doses of vitamin D to achieve vitamin D sufficiency similar to standard chow (SF05-34, Specialty Feeds, 2,280 IU/kg vitamin D_3, 1% calcium) or no added vitamin D to induce vitamin D deficiency (SF05-033, Specialty Feeds, 0 IU/kg vitamin D_3, 2% calcium). Previous work from our group has used the vitamin D deficient and standard vitamin D diet to generate vitamin D deficient ($25(OH)D_3$ <20 nmol/L) and replete ($25(OH)D_3$ >50 nmol/L) mice that reflect similar vitamin D status to humans [5]. A study of allergic asthma used the high vitamin D diets and generated mice with mean $25(OH)D_3$ levels of 167.5 nmol/L [6]. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia with the approval from the Telethon Kids Institute Animal Ethics Committee (AEC #276). Animals were maintained under specific pathogen free conditions. Mice were allowed food and water *ad libitum*.

Mice were housed under perspex-filtered fluorescent lighting, which emitted no detectable UVB radiation as measured using a UV radiometer (UVX Digital Radiometer, Ultraviolet Products Inc., Upland, CA, USA). Serum $25(OH)D_3$, serum calcium and full blood count were measured in a subset of mice from each

group in preliminary experiments to ensure safety of diets (Centre of Metabolomics, UWA and Vetpath Ascot, Western Australia).

2.3.2 Ultraviolet radiation

A bank of six 40 W lamps (Philips TL UV-B, Eindhoven, The Netherlands) emitting broadband UVR, 250-360 nm, with 65% of the output in the UVB range (280-315 nm), was used to irradiate mice and to deliver 1 kJ/m^2 of UVR onto clean-shaven 8 cm^2 dorsal skin. The lamps used have a greater proportion of UVB radiation compared to sunlight, with only 5% of the solar UV radiation reaching the surface of the earth being in the UVB range. The dose of UVR used is approximately 50% of the minimal erythemal dose for C57Bl/6 mice, i.e. 50% of the lowest amount of UVR causing just perceptible erythema after 24 h. UV lamps were turned on one hour before irradiation, the UVB output (flux) of the lamps was measured using a hand-held UVX radiometer and the time required to deliver the required amount of UVB calculated. To deliver 1 kJ/m^2, this was approximately 5 minutes on each occasion. UV-irradiation was performed consistently between the hours of 8 am and 11 am. The dorsal skin of mice not treated with UVR was also shaved and the mice were handled in an identical fashion to UV-irradiated mice including being placed in the UV irradiation room for the same duration.

A new sheet of PVC plastic film (0.22 mm) was taped to the top of each perspex cage immediately before irradiation to screen wavelengths <290 nm. Sunlamps were held 20 cm above the cages.

Figure 2.3.1 – Setup of mice prior to UV irradiation

After 4 weeks on the respective diets, half of the mice within each group were irradiated with 1 kJ/m^2 for each day of 4 days, followed by twice weekly dosing for the remainder of the experiment (Figure 2.3.2).

2.3.3 Dextran Sodium Sulphate Colitis

After 4 weeks and 4 days of being fed the respective diets and the UVR groups receiving 4 doses of treatment, colitis was induced by the addition of 3% DSS to the drinking water for 6 days. Control mice received water without DSS. As the efficacy of DSS varies between batches, the experiments were conducted using the same batch [7]. In preliminary experiments, 3% DSS induced adequate colitis with peak weight loss ranging between 0.3% to 10.3% at day 7. Following induction of colitis, mice recovered over a period of 0-4 weeks without ongoing DSS treatment (Figure 2.3.2). Mouse body weight was assessed daily during DSS treatment and weekly during recovery. The experiment was repeated, with a total of 35 mice per group. Mice were sacrificed at day 7, 14 and 35. For control non-DSS mice the experiment was repeated, with total of 21 mice per group (Figure 2.3.3).

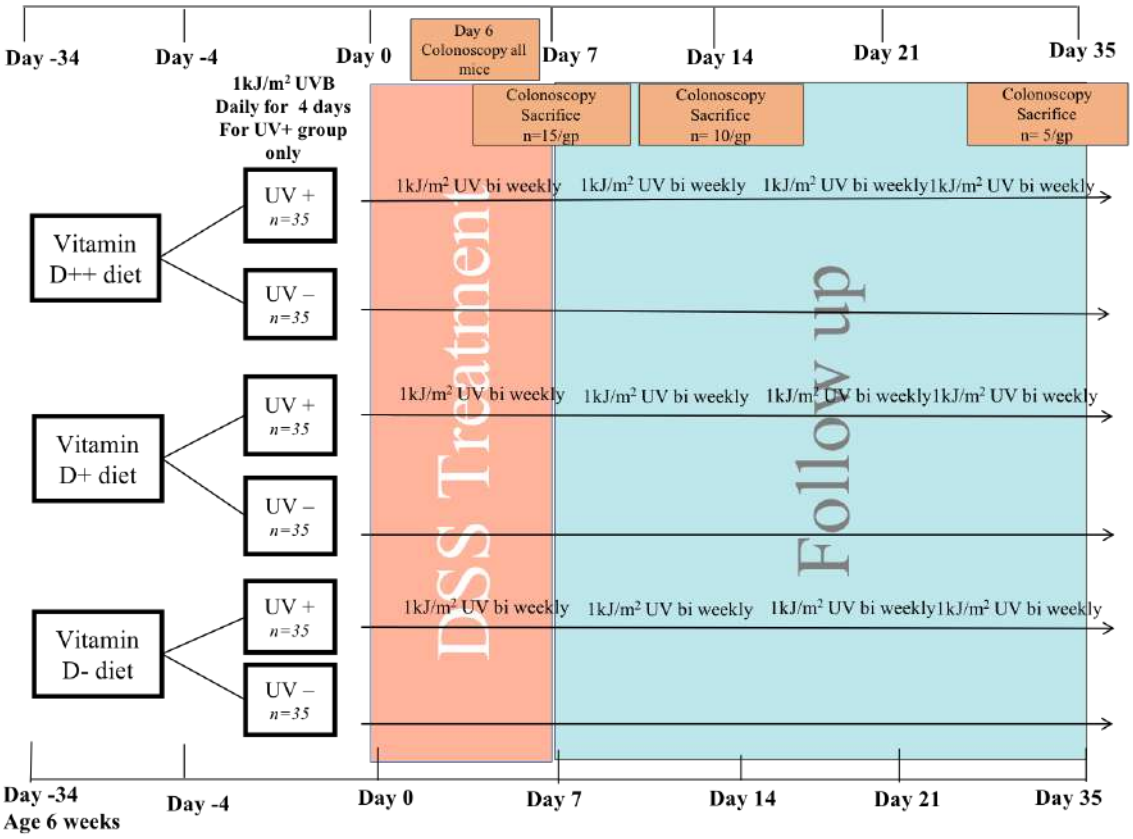


Figure 2.3.2. Study flow chart for DSS groups

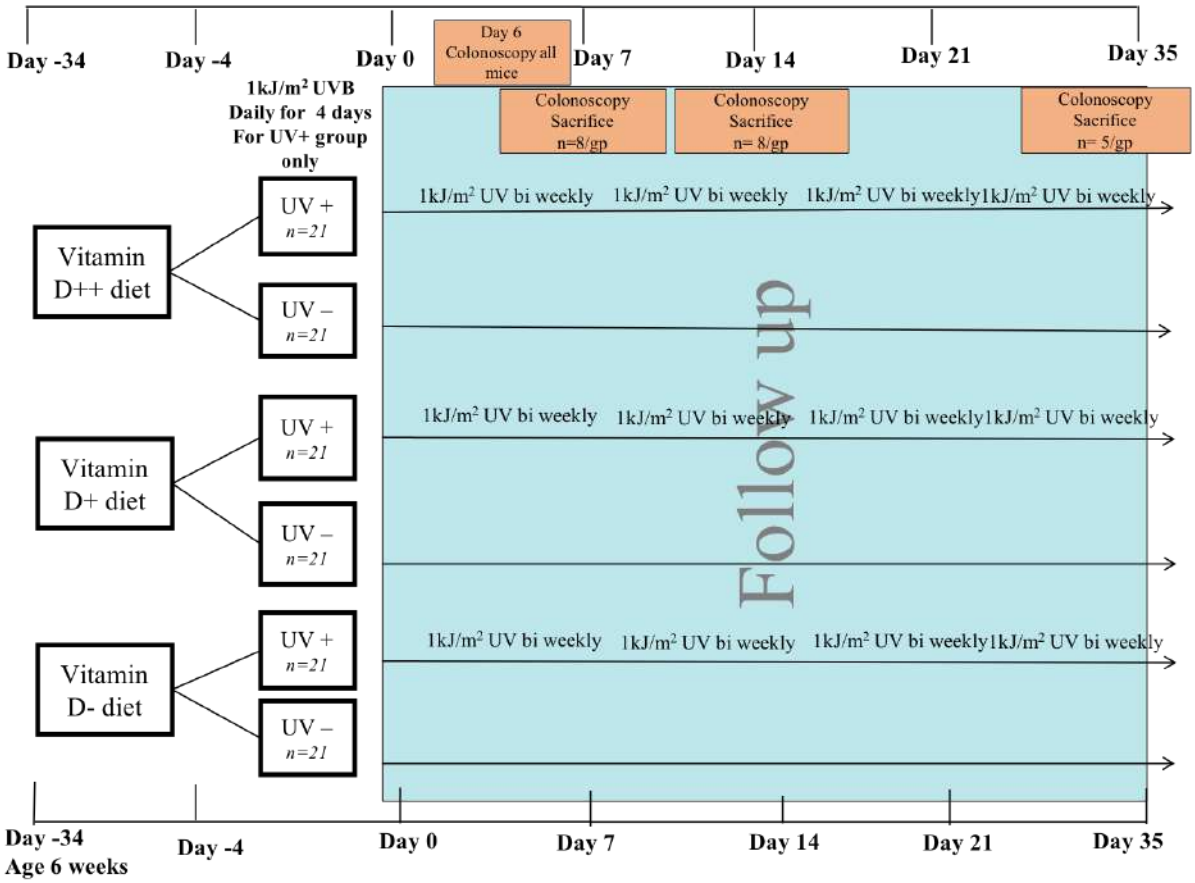


Figure 2.3.3 – Study flow chart for control non-DSS groups.

2.3.4 Murine colonoscopy

A high-resolution mouse video endoscopic system was used to assess the level of colitis. All mice were colonoscoped on day 6 after commencing DSS treatment and then at the time of sacrifice. Mice were anaesthetised using isofluorane unless the colonoscopy was being performed at the end-point when ketamine 20 mg/ml and xylazine 2 mg/ml by intraperitoneal injection was used. All procedures were digitally recorded then scored in a blinded fashion. The experimental endoscopy setup consisted of a miniature endoscope (1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Karl Storz, Germany) to achieve regulated inflation of the mouse colon (Figure 2.3.4).

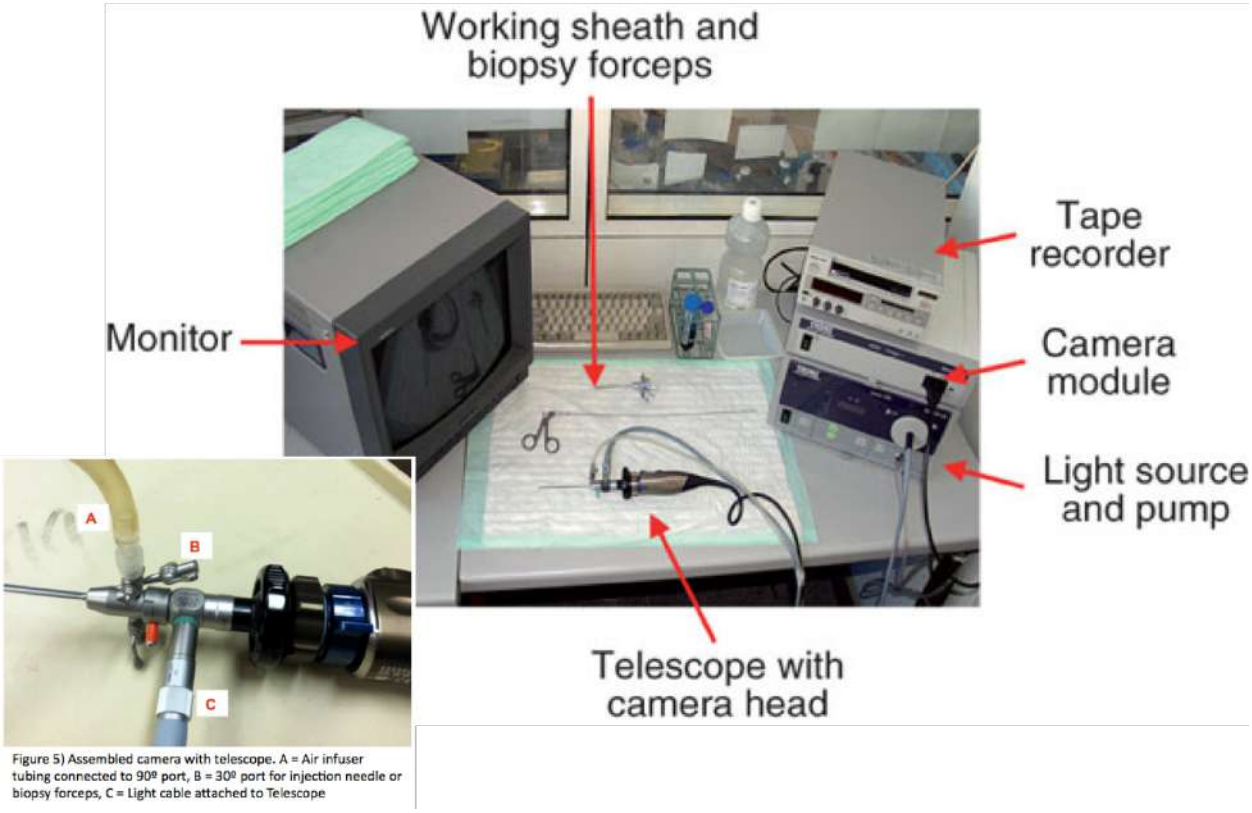
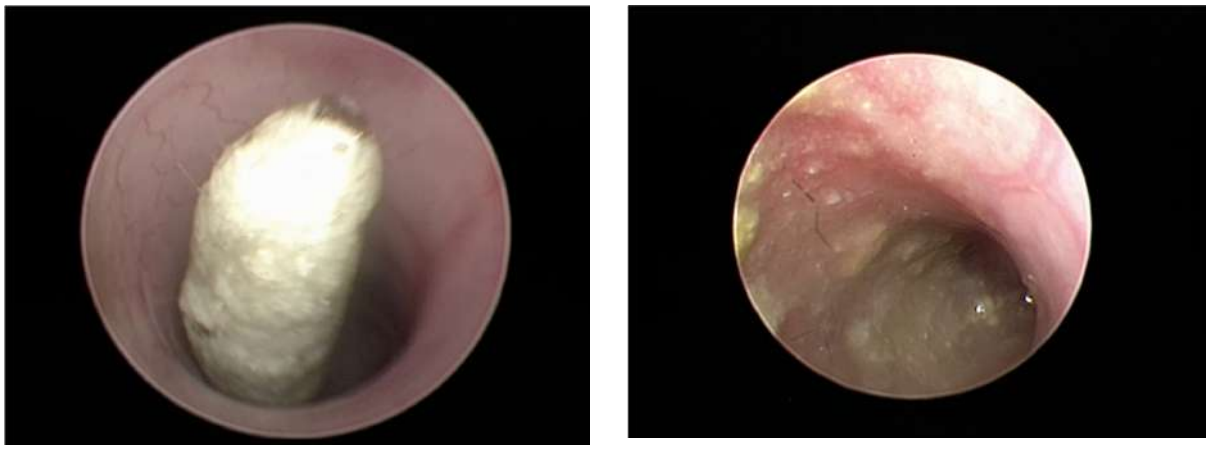


Figure 2.3.4 – Murine Endoscopy equipment

The severity of colitis was determined using the modified '**m**urine **e**ndoscopic **i**ndex of **c**olitis **s**everity' (MEICS)[7, 8]. The MEICS system consists of 5 parameters: thickening of the colon wall, changes of the normal vascular pattern, presence of fibrin, mucosal granularity and stool consistency. These are graded as shown in Table 2.3.1 below. Healthy mice had a score of 0-3. Examples of the endoscopic appearances of a healthy colon (Figure 2.3.5A) compared to a colon with colitis (Figure 2.3.5B) are shown below.

Endoscopic Colitis Grading					Total
Thickening of the colon	0 transparent	1 moderate	2 marked	3 Non-transparent	0-3
Changes of the vascular pattern	0 transparent	1 moderate	2 marked	3 bleeding	0-3
Fibrin visible	0 transparent	1 moderate	2 marked	3 extreme	0-3
Granularity of the mucosal surface	0 transparent	1 moderate	2 marked	3 extreme	0-3
Stool consistency	0 normal+solid	1 still shaped	2 unshaped	3 spread	0-3
				Overall	0-15

Table 2.3.1 – Murine Endoscopic Index of Colitis Severity (MEICS)



A. Healthy Colon

B. Colitis

Figure 2.3.5. Endoscopic Images

2.3.5 Colon Dissection

All mice were sacrificed by cervical dislocation while anaesthetised post colonoscopy procedure. The colon was removed and the colon length measured. Approximately 1mm from the anus was discarded as the rectum has a different fibro-structure to the colon. The next 5mm were removed for paraffin embedding

(labelled A in Figure2.3.6), the following section (B) from the distal colon was removed for RNA analysis as was section C from the proximal colon, just distal to the caecum.

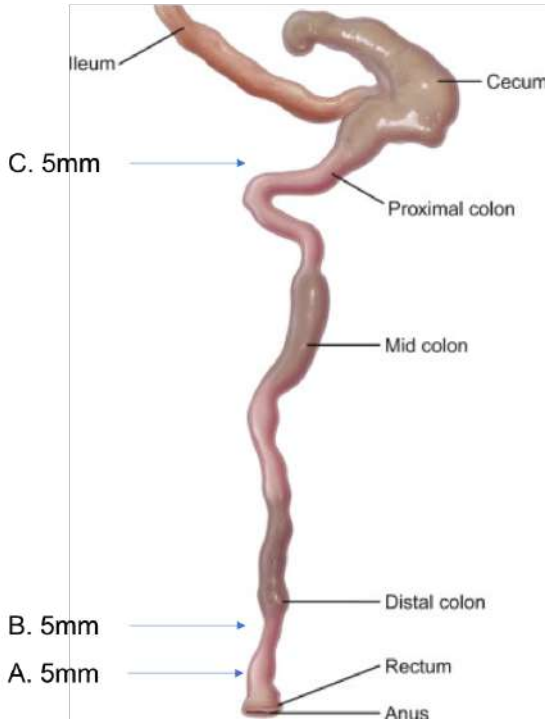


Figure 2.3.6 Dissection of Colon illustrating where sections were harvested from. Section A) paraffin section, B) RNA analysis and C) RNA analysis.

2.3.6 Serum collection

Blood was drawn by cardiac puncture immediately prior to sacrifice and collected into plain microtubes and allowed to clot at room temperature for 1-2 hours. The tube was then centrifuged at maximum speed on a benchtop centrifuge for 15 min at room temperature (21ºC). Serum was carefully pipetted into fresh microtubes and allowed to cool at 4ºC for 2 hours before freezing at -20ºC.

2.3.7 Faeces collection

Prior to colonoscopy mice were separated into individual sterile cages. The first two faecal pellets that were spontaneously passed were retrieved using a 24 gauge sterile needle and placed into a sterile microtube. These were then frozen at -20ºC immediately.

2.3.8 Preparation of paraffin sections

Colonic tissue was cleaned and then fixed by immersion in neutral-buffered formalin and processed in the Sakura Tissue Tek VIP-E150 tissue processor (GMI Inc, USA) at Pathwest Histopathology Department at Princess Margaret Hospital, WA. It was then embedded in paraffin wax and stored at RT. Sections, 4.5 μm thick, were cut using a Microm HM 325 Microtome (GMI Inc, USA), floated in a 45ºC water bath prior to mounting on Superfrost ™slides. Paraffin sections were dried at 55ºC overnight and stored at RT until H&E staining.

2.3.9 Haematoxylin and eosin (H&E) staining

Paraffin section were stained with H&E using the Shandon Linistain™GLX Linear Stainer (Thermo Scientific, AUS) at Pathwest, Princess Margaret Hospital WA.

All H&E stained paraffin sections were assessed blindly by Dr. Cynthia Forrest (Gastrointestinal histopathologist, Department of Anatomical Pathology, Pathwest, Fiona Stanley Hospital, Murdoch, W.A). The scoring system by Dieleman *et al* was used [9]. In this scoring system, the severity and depth of inflammation as well as the level of crypt damage and regeneration are scored. As shown in Table 2.3.2, the grading includes severity and depth of inflammation, level of crypt damage and regeneration. These changes are quantified as a percentage of the total colonic circumference involved by the disease process. A score of 0-56 is calculated by multiplying the score of each feature by the percentage of the tissue involved and adding the totals together.

Table 2.3.2. Histological grading of colitis

Feature	Grade	Description
Inflammation	0	None
	1	Slight
	2	Moderate
	3	Severe
Depth	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Precent involvement	1	1-25%
	2	26-50%
	3	51-75%
	4	76-100%

2.3.10 Measurement of serum metabolites

2.3.10.1 $25(OH)D_3$

Serum (100µL) was sent to Dr. Michael Clarke at the Centre for Metabolomics, UWA to carry out $25(OH)D_3$ analysis using the liquid chromatography tandem mass spectroscopy (LC/MS/MS) method as described in section 2.1.2.

2.3.10.2 $1,25(OH)_2D_3$

Levels of $1,25(OH)_2D_3$ were measured using IDS EIA ELISA kits (Immunodiagnostic Systems, USA) as described by the manufacturer. Due to the very small quantities of $1,25(OH)_2D_3$ (pmol) in serum, the

protocol required a delipidation step prior to immunoextraction. Immunoextraction required evaporation using a nitrogen evaporation unit with a heating block, the resulting immunopurified samples were then incubated overnight at 2-8ºC after the addition of the antibody solution. All samples were analysed in duplicate. The assay ELISA plate was read on the Wallac 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Finland)

2.3.10.3 Vitamin D binding protein (VDBP)

Serum VDBP concentration was measured in duplicate using the Quantikine ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. This immunoassay uses a monoclonal antibody specific for mouse VDBP that has been precoated onto a microplate. Standards, control, and samples are added into the wells and mouse VDBP is bound by the immobilised antibody. After washing away unbound substances, an enzyme-linked monoclonal antibody specific for mouse VDBP is added to the wells. After further washing step, a substrate solution is added to the wells and the enzyme reaction yields a blue product that turns yellow when stop solution is added. The intensity of the colour measured is in proportion to the amount of mouse VDBP bound in the initial step. The assay ELISA plate was read on the Wallac 1420 multilabel counter (PerkinElmer Life and Analytical Sciences, Finland), with concentrations of VDBP measured in μg/mL.

The R&D VDBP ELISA has been criticised in clinical studies because of different antibody affinities for the different VDBP genotypes.[10] This was not an issue in our murine studies due to the same genetic background of all mice and therefore single VDBP genotype, however due to the theoretical concern with this kit we collaborated with Professor Roger Bouillon, Katholieke Universiteit Leuven who is a world expert on VDBP analysis. Serum samples were sent to his laboratory where repeat analysis was carried out using a radial immunodiffusion method that has been previously validated.[11]

2.3.11 Cytokine bead assay

The cytokine bead assay, uses fluorescently dyed microspheres (beads), where each microsphere has a distinct spectral address to permit discrimination within a multiplex suspension. This allows simultaneous detection of multiple cytokines within a single well of a microplate. A specialised flow cytometer with two lasers measures the different molecules bound to the surface of the beads and a high-speed digital signal processor manages the fluorescence data.

The Bio-Plex Pro™ mouse cytokine 23-plex assay (Bio-Rad, USA) was used to detect the following cytokines and chemokines simultaneously in serum samples from individual mice: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17A, KC, MCP-1(MCAF), MIP-1α, MIP-1β, RANTES, TNF-α. Serum samples were diluted 1:4 in assay diluent. The assay was performed according to the manufacturer's instructions. A typical standard was obtained by serial dilution of the recombinant protein. Samples were analysed on the Bio-Plex 200 system (Bio-Rad, USA).

2.3.12 RNA analysis

All buffers, distilled water, microfuge tubes, micropipette tips, and any implements that were used in RNA work were RNase free.

2.3.12.1 Tissue homogenisation for RNA isolation from liver, kidney and colon tissue

Liver, kidney and colon tissue were harvested and snap frozen in liquid nitrogen then stored at -80ºC. The tissue pieces were transferred to an ice-cold surface and dissected into tiny pieces before being transferred to microtubes. TRIzol reagent (500 μL) was added before vortexing. Tissue homogenisation was performed using a mechanical sonicator (Thermo Fischer Scientific) with a 1 mm probe on maximum amplification. The supernatants were transferred to fresh microtubes and snap-frozen.

2.3.12.2 mRNA extraction

Supernatants from homogenised tissue were thawed rapidly. Chloroform (200 µL) was added to each sample and vigorously shaken for 15 sec then incubated at room temperature for 3 min. After centrifugation at 14,000 g 4°C for 5 min, 300 µL of the aqueous phase were transferred to new microtubes on ice. The lysates were incubated at 37°C for 2 min and then 300 µL of lysates were transferred to the gDNA eliminator columns in 2 mL collection tubes included in the RNeasy®Plus Universal mini kits (Qiagen, USA). The tubes were centrifuged at 9,300 g for 30 sec at RT, then the columns discarded. Ethanol (300µL 50% v/v) was added to samples. The solution was then transferred to a RNeasy spin column in 2 mL collection tubes and then centrifuged at 9,300g for 30 sec at RT. The flow through was discarded. The proprietary RW1 buffer (700 µL) was added to the RNeasy column then centrifuged again (9,300g, 30 sec, RT) and the flow through discarded. The kits proprietary RPE buffer solution (500 µL) was added to the RNeasy column and incubated at RT for 5 min and then centrifuged (9,300g, 30 sec, RT). The flow through was discarded, and 500 µL of 80% ethanol added to the RNeasy column and centrifuged (9,300g, 2 minutes, RT). The 2 mL collection tube was discarded and the RNeasy column placed in a new 2 mL centrifuge tube and centrifuged at maximum speed (16,100g for 1 min). The RNeasy column was then placed in a pre-labelled 1.5 mL collection tube. Pre-heated RNase-free water (40 µL) was added to each column and centrifuged (9,300g, 1 minute, RT). The flow through was taken and placed back in the RNeasy column and centrifuged at (10,000 rpm, 1 min, RT). RNA was kept on ice, and the RNA quality checked using a spectrophotometer (NanoDrop™)

2.3.12.3 Quantification of RNA

The concentration of RNA was determined undiluted using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). The A_{260}/A_{280} ratio of the RNA is an indication of its purity. The RNA samples were then snap frozen in liquid nitrogen and stored at -80°C.

2.3.12.4 Reverse transcription of RNA

Reverse transcription of RNA was performed using the QuantiTect® Reverse Transcription kit (Qiagen, USA) according to the manufacturer's instruction. In brief, RNA, the QuantiTect kit buffers and the transcriptase were kept on ice. The genomic DNA elimination required 2 µL of gDNA wipeout buffer to be added to microtubes, followed by 6 µL of template RNA and 6 µL of water. Non-reverse transcription (NRT) controls were used. The gDNA elimination reaction was incubated for 2 min at 42°C then the tubes were placed directly on ice. The reverse-transcription master mix was prepared and 6 µL added to each sample and 3 µL to NRT controls then incubated at 42°C for 15 min, followed by further incubation at 95°C for 3 min to inactivate the reverse transcriptase. PCR water (80 µL) was added to each sample and 40 µL to each NRT control. The cDNA samples were then stored at -20°C.

2.3.12.5 Real-time Quantitative Polymerase Chain Reaction

Real-time quantitative PCR was performed using continuous monitoring of the amplification process with SYBR green, a high-affinity double-stranded DNA-binding fluorescent dye. The RT^2 SYBR Green ROX qPCR mastermix (Qiagen) was used. Each PCR reaction consisted of 4 µL cDNA, 4.8 µL Rnase-free water, 10 µL 2Xmastermix, 0.6 µL of 10µM forward primer, 0.6 µL of 10µM reverse primer to give a final volume of 20 µL per reaction. MicroAmp® 96-well Optical reaction plates with barcodes (Life Technologies, USA) were used for the reactions.

PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) and analysed using the 7900 SDSv2.4.2 software (Applied Biosystems). The SYBR green dye fluoresces when bound to double-stranded DNA. When the DNA is denatured the SYBR green dye is released and the fluorescence is drastically reduced. During extension, primers anneal and PCR product is generated (polymerisation). When polymerisation is completed, SYBR Green dye binds to the double-

stranded product resulting in a net increase in fluorescence detected by the instrument. The level of fluorescence is measured after the 60ºC extension step of each cycle.

The PCR cycling profiles included initial PCR activation for 15 min at 95ºC, then 40 cycles consisting of denaturation at 94ºC for 15 sec, annealing at 60ºC for 20 sec and primer extension of 60 ºC for 60 sec. Data were acquired after each cycle. After the 40 cycles, the reaction was held for 15 sec at 72 ºC followed by a melting curve program (72-99 ºC with a rising heating rate by 1 ºC on each step and a continuous fluorescence measurement) and finally a cooling step to 40 ºC for 3 min. At the completion of the PCR, the melt curve was generated.

All genes were normalised to a housekeeping gene (elongation factor 1α (*EeF1α1)* or Tata-box-binding protein (*Tbp)*) and the fold change expressed relative to controls using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are shown in Table 2.3.3.

Table 2.3.3 – Primer sequences

Gene Name	Primer sequences (5'-3')	Annealing temp (ºC)
CYP27b1	**Fwd:** AGTGTTGAGATTGTACCCTG **Rev:** CGTATCTTGGGGAATTACATAG	57
CYP24A1	**Fwd:** CCCAAAGGAACAGTCTTAAC **Rev:** GGTCTAAACTTGTCAGCATC	57
CYP2R1	**Fwd:** TCAGTTACAGCATCCAAAAG **Rev:** TGTGCACTTTTATTGGTCTC	58
EeF1α1	**Fwd:** GGGGGAGAACGGTATATAAG **Rev:** GATGTGAGTCTTTTCCTTTCC	59

Tbp	**Fwd:** GTTCTTAGACTTCAAGATCCAG **Rev:** TTCTGGGTTTGATCATTCTG	60
VDR	**Fwd:** TCAGTTACAGCATCCAAAAG **Rev:** CAACATGATCACCTCAATGG	58
CAMP	**Fwd:** AGTGAAGGAGACTGTATGTG **Rev:** ATTTTCTTGAACCGAAAGGG	56
IL-1β	**Fwd:** GGATGATGATGATAACCTGC **Rev:** CATGGAGAATATCACTTGTTGG	60

2.3.13 Bacterial Genomic DNA Purification Procedure

To extract bacterial DNA from faeces, the frozen faecal samples were allowed to thaw for 15 min. Sterile 1X TE buffer (1.4 mL) was aliquoted into 2 sterile screw-capped microtubes and warmed on the 80ºC heating block. The PowerSoil DNA Isolation Kit (MoBio, USA) was used and the procedure was carried out according to the manufacturer's instructions. In brief, one faecal pellet was placed into the respective labelled PowerBead tube. Extraction controls were also used but no sample was added to these. The PowerBead tube was gently vortexed. After adding the appropriate solutions to each tube and warming to 70ºC for 10 min, the cells were disrupted on the FastPrep-24 homogenizer (MPBio, USA) at 6.5 meter/second for 30 sec. The microtubes were then centrifuged at 13,000g for 1 minute and then the supernatant transferred to a clean collection tube. Solution C2 (250 µL) was added, vortexed then incubated in at 4ºC for 5 min, followed by a centrifugation at 13,000g for 1 minute. The supernatant (600 µL) was then removed, and the procedure repeated twice after adding solution C3, then solution C4. The supernatant (650 µL) was then transferred onto the Spin Filter and centrifuged at 13,000 g for 30 sec. The flow through was discarded and repeated until all the supernatant was loaded onto the Spin Filter. Solution C5 (500 µL) was added followed by centrifugation at 13,000 g for 30 sec. The flow through was discarded, followed by repeat centrifugation. Finally, the Spin Filter was placed in a clean 2 mL collection tube and 50 µL of pre-

warmed 1X TE, pH 8.0, added directly over the filter in column and allowed to stand at RT for 2 min. The Spin Filter was then removed and discarded.

2.3.14 Bacterial DNA quantification

Quantification of bacterial DNA was performed using the Qubit dsDNA BR Assay kits on the Qubit 2.0 Fluorometer (Invitrogen, USA) according to the manufacturer's instructions. The Qubit working solution was made up by diluting the Qubit dsDNA BR reagent in the Qubit dsDNA BR buffer. Working solution (190 μL) was added to each of the tubes used for standards and then 10 μL of each Qubit standard added to the appropriate tube and mixed. Following this, Qubit working solution was added to individual assay tubes so the final volume after adding sample was 200 μL. The Qubit 2.0 fluorometer was used to quantify the bacterial DNA. For quantification, a new calibration was run using the manufacturer supplied standards and following this each of the test samples tubes were read. DNA was then sub-aliquoted into a new microtube with TE buffer to make a final concentration of 0.3ng/μL and a total volume of 200 μL.

2.3.15 16S rRNA PCR Amplification

Most intestinal microbes are anaerobic and therefore difficult to culture. Portions of the gene encoding the small subunit 16S ribosomal RNA (rRNA)(referred to as 16S) are highly conserved among bacteria [12]. Other internal regions of the gene are highly variable, possessing almost entirely unique sequences in most bacterial clades. PCR amplification, with universal primers, of a pool of DNA from a community of microbes, followed by cloning and sequencing, provides marker genes that can be used to quantify bacterial taxa present within a sample.

The primers used for PCR amplification of the 16S region are shown in the Table 2.3.4. This hypervariable region of the 16S gene was chosen because of previous studies demonstrating it matched approximately

98% of Bacteria and 95% of Archaea ribosomal RNA gene sequences in the Ribosomal Database Project database [13].

Table 2.3.4 – 16S Primer sequence

Target Name	Forward Sequence	Reverse Sequence
16S: 341F-806R (V3-V4)	CCTAYGGGRBGCASCAG	GGACTACNNGGGTATCTAAT

In the DNA-free room, the PCR mastermix was made by aliquoting 12.5 µL/reaction of GoTaq® DNA polymerase (Promega, USA), 2 µL for forward primer, 2 µL of reverse primer and 6.5 µL of RNase,DNase -free sterile water. In the post-PCR room, 24 µL of mastermix was aliquoted into each of the 0.2 µL PCR microtubes containing 2 µL of DNA template diluted in TE buffer.

PCR was performed using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) and analysed using the 7900 SDSv2.4.2 software (Applied Biosystems). The PCR Cycling protocol was as shown in the Table below.

Table 2.3.5 – 16S PCR conditions

Target	Cycle	Initial	Disassociate	Anneal	Extension	Finish
16S	29	95 *C for 7 min	94*C for 45sec	50*C for 60sec	72*C for 60sec	72*C for 7 min

The quality of the PCR product was confirmed by running a subset of sample on a 1% Agarose gel at 90V for 30-45 min.

2.3.16 16S high-throughput sequencing

PCR products were sent to the Australian Genome Research Facility, Brisbane, Australia, for sequencing on the Illumina MiSeq (USA) using 2X300bp paired-end chemistry. Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) [14]. Primers were trimmed using Seqtk (version 1.0)[15]. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8)[16] USEARCH (version 7.1.1090)[17, 18] and UPARSE[18] software. Using QIIME, taxonomy was assigned using Greengenes database (Version 13_8, Aug 2013) [19].

The biom file, the OTU table, the taxonomic assignments and associated sample data were imported into R to create a phyloseq object. For all beta diversity analyses, OTUs for which the variance across all samples were very low, were filtered out. For testing a single categorical experimental condition, exact tests for differences in the means between two groups of negative-binomially distributed counts were computed. Data were normalised using the RLE scaling factor method and dispersions estimated. The counts were extracted and ranked by p-value, applying a false discovery rate cut-off of less than 0.001.

2.3.17 Statistical analysis

Statistical significance was calculated using IBM® SPSS® Statistics Version 22 (IBM Corp. Armonk, NY). All graphs and comparison of differences between groups were assessed using Student's unpaired t-test or ANOVA with post hoc LSD analysis for multiple group analysis. Non-parametric data using Mann-Whitney U and Kruskall-Wallis testing. Microbiome statistical analysis was undertaken using the programming language R, specifically the *phyloseq* and *edgeR* packages available through Bioconductor, a project providing tools for the analysis and comprehension of high-throughput genomic DNA. LEfSe (Linear discriminant analysis effect size) was used to identify differentially abundant microbial taxa[20].

2.4 Methods for Vitamin D Epimers in mouse and humans

The methods described here are relevant to the published manuscript **"Vitamin D C3-epimer levels are proportionally higher with oral vitamin D supplementation compared to ultraviolet irradiation of skin in mice but not humans"** – presented in chapter 6

2.4.1 Experimental design

The aim of this study was to compare the levels of vitamin D epimers among mice and humans, and examine the comparative effects of oral vitamin D supplementation versus UV-irradiation on circulating epimer levels.

2.4.1.1 Animal model

Stored samples from the animal study detailed above in Section 2.2 were used. Specifically, control mice on vitamin D sufficient diets without UVR treatment and mice on the vitamin D deficient diet treated with or without UVR, were examined (Figure 2.3.1). As previously described, 6-week-old C57Bl/6 female mice were fed diets sufficient (D+, 2,280 IU/kg vitamin D_3, 1% calcium) or deficient in vitamin D (D-, 0 IU/kg vitamin D_3, 2% calcium) for 4 weeks. After 4 weeks on their respective diets, half of the mice from the D- group received 1 kJ/m^2 UVR for four consecutive days. After this they were irradiated bi-weekly with 1kJ/m^2 UVR for 4 weeks. Mice were sacrificed at weeks 6, 7 and 9.

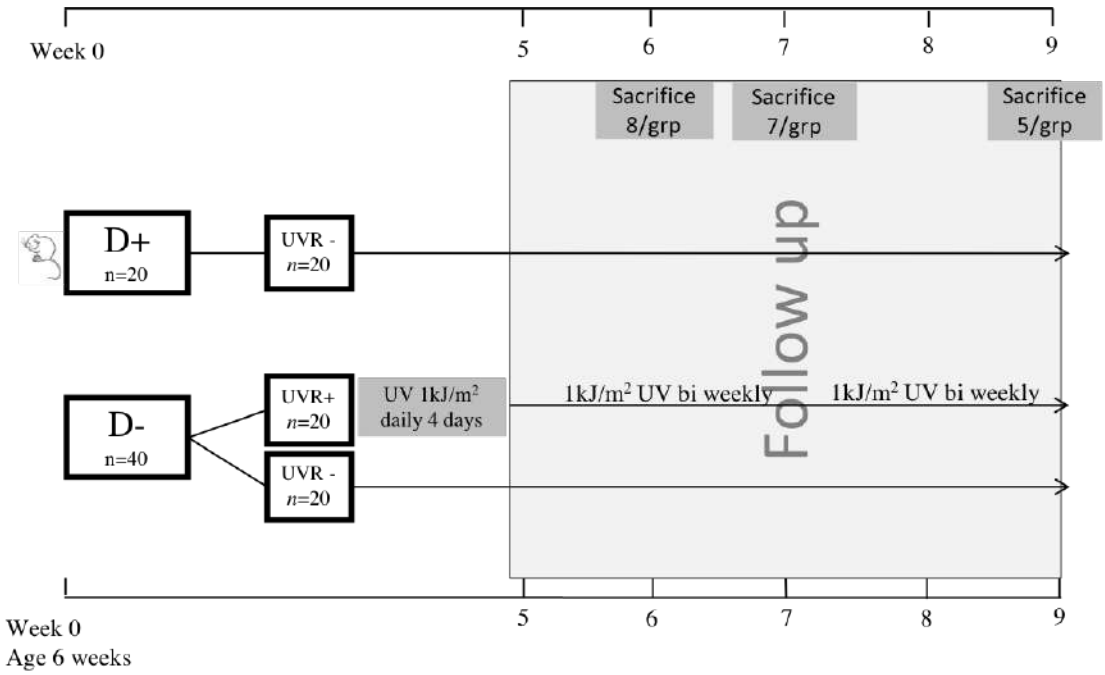


Figure 2.4.1. The experimental approach. 6-week-old C57Bl/6 female mice were fed diets with sufficient (D+) or no (D-) vitamin D_3 for 4 weeks. Half of the mice on the vitamin D deficient (D-) diet were UV-irradiated with 1 kJ/m^2 daily for 4 days, followed by biweekly UVR exposures for the remainder of the study. Mice continued on their respective diets until 9 weeks, and were sacrificed at weeks 6, 7 and 9. The experiment was repeated twice. Total n=20/group.

2.4.1.2 The pilot D-Health and PhoCIS trials

Serum samples were obtained from participants in two clinical trials, the pilot D-Health [21, 22] and PhoCIS trials [23], to analyse 25(OH)D_3 and C3-epi 25(OH)D_3 metabolites. An amendment to the original D-Health ethics application was approved by the human research ethics committee at the QIMR Berghofer Medical Research Institute (trial 2010/0423). The PhoCIS trial (Phototherapy for Clinically Isolated Syndrome) was approved by the Belberry Human Research Ethics Committee (2014-02-083) and endorsed by the Human Research Ethics Office of the University of Western Australia (RA/4/1/6796). Briefly, the D-Health trial was a population-based, randomized, placebo-controlled, double-blind chemoprevention trial of vitamin D_3 in older adults. In total, 644 individuals aged 60 to 84 in the eastern states of Australia were

recruited and randomly assigned to monthly doses of placebo, 30,000 IU, or 60,000 IU vitamin D_3 for 12 months [24]. Blood samples were collected at baseline and within 2 weeks after the last dose of vitamin D. The initial analysis of 25(OH)D_3 for the study was performed using the Diasorin Liaison platform immunoassay which does not distinguish the C3-epi 25(OH)D_3 when reporting total 25(OH)D_3 levels. In this sub-study, serum samples were reanalysed from 11 subjects who were treated with 60,000 IU Vitamin D_3 / month and had demonstrated at least a 30% rise in 25(OH)D_3 according to the Diasorin assay, as these were most likely to demonstrate the differences in epimer metabolites.

The PhoCIS trial examined 20 people with clinically isolated syndrome, which refers to a first episode of neurologic symptoms lasting at least 24 hours and caused by inflammation or demyelination in the central nervous system. This may be part of the multiple sclerosis disease course. Participants were randomised in a non-blinded 1:1 fashion to receive, or not receive, narrow band UVB phototherapy (24 sessions total, 3 exposures/week for 8 weeks) [25]. Blood samples were collected at months 1, 2, 3, 6 and 12. As published, three subjects in the phototherapy group and 5 subjects in the control group (no phototherapy) had a baseline 25(OH)D_3 level <80 nmol/L requiring supplementation [25]. If baseline 25(OH)D_3 levels were <80 nmol/L, then participants in both groups were supplemented with low dose oral vitamin D3 (800 IU/day) to achieve serum 25(OH)D_3 levels >80 nmol/L [23].

2.4.2 25(OH)D_3 and C3-epi 25(OH)D_3 measurement

As described in section 2.1.2, all serum samples were analysed at the University of Western Australia, Centre for Metabolomics. This 2-dimensional ultra-performance liquid chromatography (UPLC) separation coupled tandem mass spectrometry (MS/MS) detection allows quantification of 25(OH)D_3 and C3-epi 25(OH)D_3 with only 50 μL of serum and allows analysis of the small volume serum samples obtained from mice.

Chromatography is a separation technique to separate the individual compound from a mixture using a stationary and mobile phase. Liquid-Chromatography-Mass Spectrometry (LC-MS) is an analytical technique that couples high resolution chromatographic separation with sensitive and specific mass spectrum detection. Mass spectrometers operate by converting the analyte molecules to a charged (ionised) state, with subsequent analysis of the ions and any fragment ions that are produced during the ionisation process [26]. Chromatograms are produced showing typical separate peaks for 25(OH)D_3 and C3-epi 25(OH)D_3 (example shown in Figure 2.3.2)[1].

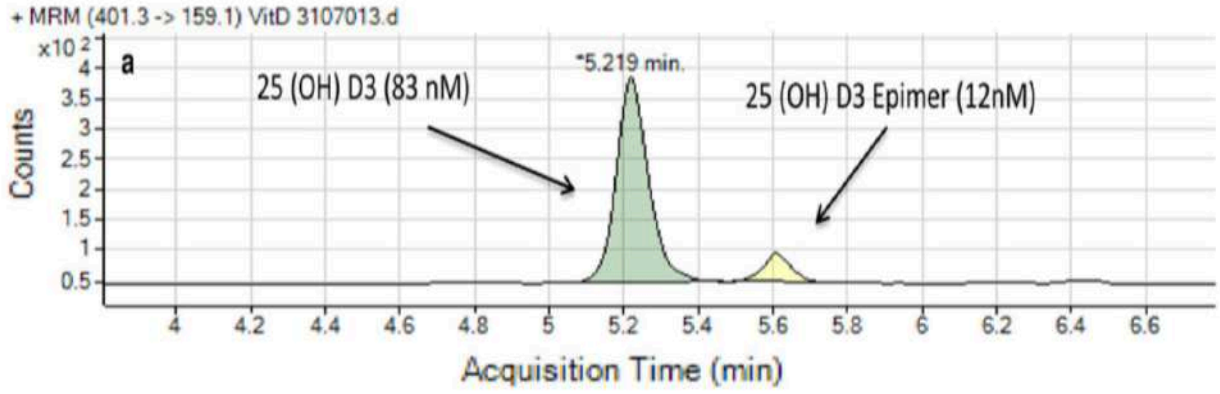


Figure 2.4.2. Typical chromatogram of human sera samples showing separation of 25(OH)D metabolites.

2.4.3 Real time PCR

Messenger RNA expression of key enzymes in the synthesis and metabolism of vitamin D were examined to determine if any differences with UV-irradiation compared to oral vitamin D supplementation exist. mRNA was extracted from snap-frozen kidney and liver tissue with cDNA synthesised and real-time assays performed as previously described in 2.2.12.5 including *CYP27B1*, *CYP24A1*, *CYP2R1* and eEF1α as the housekeeping gene. An additional gene was examined from liver tissue, the *GC (VDBP)* cat#QT00267799 (Qiagen, Germany).

2.4.4 Statistical Analysis

IBM® SPSS® Statistics Version 22 (IBM Corp. Armonk, NY) was used for statistical analysis of samples from experimental animals. Homogeneity of variances was tested with Levene's test. Means were compared using student's unpaired t-tests for murine data, and paired t-test analysis for paired human data. Pearson correlations were performed to examine the relationship between $25(OH)D_3$ and its metabolites.

The change in absolute C3-epi $25(OH)D_3$ and %C3-epi $25(OH)D_3$ was calculated between baseline and subsequent visits (month-2, -3, and -12) for all participants in the Phototherapy group (PhoCIS trial) and between baseline and month-12 for participants in D-Health trial. The changes in both absolute and %C3-epi $25(OH)D_3$ between baseline and subsequent visits were compared using generalized linear models, under the assumption that participants in the D-Health study reached their month-12 level of epimer and %C3-epi $25(OH)D_3$ by month-2. Clinical data were analysed using SAS version 9.4. P-values <0.05 were considered statistically significant.

2.5 Methods for vitamin D metabolites in active and inactive CD.

The methods presented here are relevant to the unpublished manuscript **"$24,25(OH)_2D_3$ is lower with active Crohn's disease and spontaneously recovers with remission"** presented in chapter 7.

2.5.1 Patients and Design

The study was approved by the St. Vincent's Hospital human research ethics committee (LNR/17/SVH/26). Patients with CD were prospectively recruited from the IBD clinic at St. Vincent's Hospital, Sydney, Australia between March and June 2017. All patients had confirmed CD and were phenotyped using the "Montreal Classification". CD was diagnosed according to standard clinical, endoscopic, and radiological criteria [27]. Patients between 16 and 60 years of age with either colonic or ileo-colonic CD in remission or with moderate-severe activity were included. Moderate-severe disease was defined by a CD activity

index (CDAI) of ≥ 220 and an objective marker of active inflammation (C-reactive protein ≥10 mg/L, faecal calprotectin >250µg/g or active ulceration seen at ileo-colonoscopy within 3 months). Remission was defined by CDAI <150 and CRP<10, faecal calprotectin <150 or no ulceration at ileo-colonoscopy within 3 months. Patients were excluded if using vitamin D supplementation or corticosteroids within 4 weeks, if they were pregnant, had short bowel syndrome, isolated small bowel CD or remission that was induced by intestinal resection.

At enrolment, baseline data including demographics, disease and medication history were collected. Participants completed diet and sunlight questionnaires at baseline and at 6 months. Blood was collected and serum stored from all participants at enrolment and 6 months. Vitamin D metabolite testing took place after the study period, therefore treating physicians were blinded to the results. The use of vitamin D supplements or corticosteroids during the study period was left to the discretion of the treating physician. Participants were followed for 6 months unless disease relapse was experienced beforehand. Disease relapse was defined as >100 point rise in CDAI to at least 150 with associated objective marker of relapse (CRP ≥10 mg/L, faecal calprotectin >250µg/g or active ulceration seen at ileo-colonoscopy).

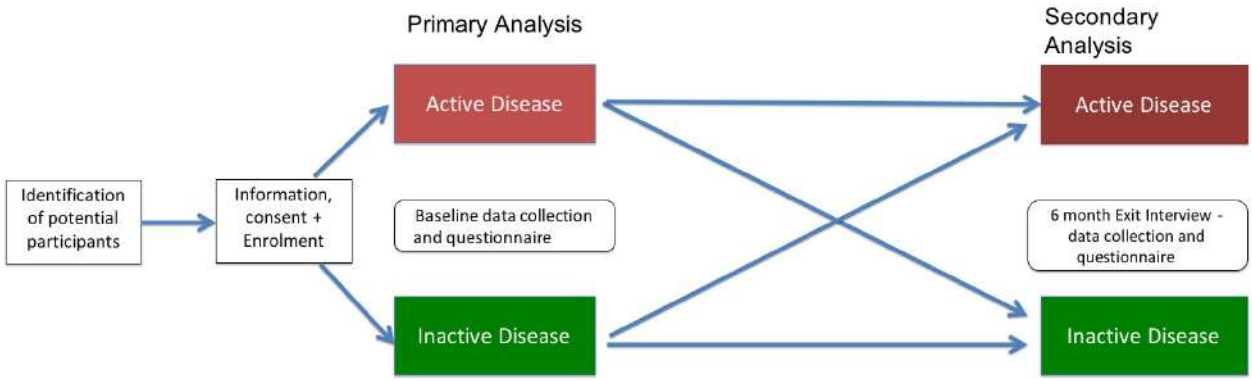


Figure 2.5.1 – Study flow diagram.

Peripheral blood was collected by venepuncture. Routine laboratory haematology and biochemistry tests were performed immediately, and a sample of serum 1-1.5ml was stored at -20^{0}C for later analysis of vitamin D metabolites.

2.5.2 Biochemical measurements

25(OH)D_3, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ were measured using liquid chromatography tandem mass spectroscopy (LC/MS/MS) at Metabolomics Australia, University of Western Australia. Both 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ required derivatisation steps prior to LC/MS/MS analysis. VDBP was measured by immunonephelometry (Dade Behring, Liederbach, Germany) (inter-assay CV <6%), and albumin was measured by dye binding reaction to bromocresol green (inter-assay CV<5%). Free and bioavailable (free plus albumin-bound) concentrations of 25(OH)D were calculated using VDBP and albumin concentrations, according to modified "Vermeulen" equations previously published and validated.[2] Albumin, C-reactive protein, white cell count and platelet count were carried out through routine laboratory techniques.

2.5.3 Questionnaires

Within 2 weeks of blood draw at study entry, participants were asked to complete questionnaires (Appendix 1) including details of their demographics, ethnicity, smoking history, alcohol consumption, medication use, supplements, CD diagnosis and treatment. Validated questions to measure sunlight exposure and dietary vitamin D intake were included [28]. Numerous factors related to both the environment (e.g. solar zenith angle and weather) and the person (e.g. time outdoors and use of sun protection) affect the level of UVR reaching the skin. Most population studies depend on questionnaires to estimate UVR exposure, though the use of UV dosimeters worn on the wrist to directly measure UV exposure may be more accurate though not feasible in larger studies. Brief questionnaire-based measures were validated against objectively measured UVR exposure obtained from personal digital dosimeters [28]. Questions related to skin colour

and tanning characteristics, time spent outdoors between the hours of 10am and 3pm in the preceding month on weekdays and weekends, area of exposed skin and use of sun screen.

The sun exposure calculation was modified from the method described by Cargill et al [28]. The daily time outdoors was estimated by summing the median values in the range indicated as spent outdoors for each hour between 10am and 3pm (e.g. 0-15min=7.5min). If the diary was not completed for any period of time between the peak UVR hours of 10am and 3pm, the day was treated as a missing value for analysis. When fewer than 7 days of diary were available, data were averaged over the available number of days to derive weekday and weekend mean time outdoors. Analyses were computed separately for weekdays and weekends which were then summed, i.e ((weekday average X 5 + weekend average X 2). The calculated time outdoors in hours was then multiplied by a weighted UV multiplier taking the UV index in Sydney, Australia, during each month into account, where summer months had greatest weight and winter months the least (Table 2.5.1). These data were derived from the monthly UV index published by the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA) website (www.arpansa.gov.au/our-services/monitoring/ultraviolet-radiation-monitoring/ultraviolet-radiation-dose). Finally, the multiple of time outdoors and the UV multiplier, was then multiplied by a clothing multiplier to estimate body surface area exposed to sun (Table 2.5.2). The equation was therefore – Time outdoors as calculated above X UV multiplier X (1- (shirt clothing multiplier + pants clothing multiplier)).

Table 2.5.1 – UV multipliers

Month	UV Multiplier
January	12
February	11
March	9
April	7
May	6
June	5
July	5
August	7
September	9
October	10
November	11
December	12

Table 2.5.2 – Clothing multipliers

Long sleeve top (Shirt)	How often worn	Multiplier
	Never	0.1
	Less than half	0.2
	More than half	0.3
	Always	0.4
Long pants		
	Never	0.2
	Less than half	0.2

	More than half	0.3
	Always	0.4

Vitamin D intake was measured using a food frequency questionnaire of foods with highest vitamin D content (Appendix 1). Total vitamin D intake was then calculated based on the vitamin D content of foods as described by the Nutritional Tables (NUTTAB) 2010 produced by the Food Standards Australia New Zealand (http://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/default.aspx). At study exit a follow up questionnaire was administered including details of symptoms, medications, smoking, sun exposure and dietary intake.

2.5.4 Statistical analysis

The primary aim was to compare the vitamin D levels between the two groups at baseline. A sample size of 27 per group was selected to detect an 8% difference in $25(OH)D_3$ levels on cross sectional analysis with greater than 80% probability with a two-sided α of 0.05 whilst allowing for 10% drop out, using G*Power (version 3.1.9.3).

Summary statistics, including counts and percentages for categorical variables and mean +/- standard deviation (SD) or median and interquartile range (IQR) for continuous variables following testing for the presence of normal distribution were prepared. Group comparison of continuous variables were assessed using Student's unpaired t-test for parametric data, or Mann-Whitney *U* and Kruskall-Wallis testing for nonparametric data. Categorical variables were compared using Pearson chi-square test or Fisher's exact test.

The results of the dietary and sunlight questionnaires were combined with results from the UVR data as reported to by ARPANSA to create a score to represent the external determinants of vitamin D levels. Using the change over time in $25(OH)D_3$ from patients who remained in remission throughout the study period as

a control, a linear regression model was used to determine the relative impact of sunlight as compared with diet on serum 25(OH)D_3. This calculation of external determinants of vitamin D levels was then used in a multivariate linear regression model to control for the differing vitamin D determinants amongst participants.

All statistical analysis was completed on IBD® SPSS® Statistics version 25. A *P* value of <0.05 was considered statistically significant.

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Chapter 3

Ghaly S et al. **High Vitamin D-Binding Protein Concentration, Low Albumin, and Mode of Remission Predict Relapse in Crohn's Disease.** IBD. 2016;22(10):2456-64

High Vitamin D–Binding Protein Concentration, Low Albumin, and Mode of Remission Predict Relapse in Crohn's Disease

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Background: Vitamin D (25(OH)D) deficiency occurs in active Crohn's disease (CD) and may be secondary to reduced sunlight exposure and oral intake. Vitamin D–binding protein (VDBP) levels, however, fluctuate less with season and sunlight. The aim, therefore, was to examine patients with CD in remission and determine any associations between VDBP, serum 25(OH)D, and the calculated free 25(OH)D concentrations with the risk of disease flare.

Methods: Subjects were identified from prospectively maintained inflammatory bowel disease databases at 3 teaching hospitals in Australia. Patients were in steroid-free clinical remission at the time of blood draw and were followed for at least 12 months. Total and epimer-25(OH)D_3, VDBP concentrations, and genotypes were determined.

Results: A total of 309 patients with CD (46% men) met the inclusion criteria. A disease flare occurred in 100 (32.4%). Serum 25(OH)D_3 was deficient (<50 nmol/L) in 36 (12%) and insufficient (50–75 nmol/L) in 107 (35%) patients. Total, free, and epimer-25(OH)D_3 serum levels did not predict disease flare. Higher VDBP concentrations, however, significantly correlated with increased risk of disease flare (hazard ratio 1.2, 95% CI, 1.0–1.5). On multivariate analysis, VDBP concentration, low albumin, and medication-induced remission were significantly more associated with disease flare. VDBP genotypes were significantly associated with 25(OH)D and VDBP concentrations but not disease flare.

Conclusions: Vitamin D deficiency was uncommon in our patients with CD in remission, and serum 25(OH)D_3 did not predict disease flare, whereas higher VDBP concentrations were significantly associated with disease flare. Further investigations to explore the possible mechanisms for this association are warranted.

(Inflamm Bowel Dis 2016;22:2456–2464)

Key Words: vitamin D, vitamin D–binding protein, inflammatory bowel disease, Crohn's disease, colitis

Vitamin D deficiency is common among patients with inflammatory bowel diseases (IBD), Crohn's disease (CD), and ulcerative colitis. There is some controversy, however, about the definition of vitamin D status. The endocrine society defines vitamin D sufficiency as a serum 25(OH)D level ≥75 nmol/L, insufficiency as 50 to 75 nmol/L, and deficiency as <50 nmol/L.[1] In North American studies, 32% of patients with IBD have serum 25(OH)D_3 levels of <50 nmol/L, which has been associated with the onset of CD, increased levels of disease activity, the need for greater numbers of hospitalizations and surgery, as well as an increased risk of colorectal malignancy.[2] Vitamin D may also be a potential adjunctive treatment in active CD as the administration of 1,25 dihydroxyvitamin D (1,25$(OH)_2$D), and vitamin D analogs, can reduce colitis severity in mouse models of 2,4,6-Trinitrobenzenesulfonic acid (TNBS) colitis.[3,4]

Despite findings from observational studies, and the biological plausibility demonstrated by murine models, interventional studies involving vitamin D supplementation in other immune-related disorders, such as type 1 diabetes and multiple sclerosis, have been disappointing.[5] To date, only 1 placebo-controlled trial has evaluated the effect of vitamin D supplementation in patients with CD in remission and did not demonstrate a significant reduction in the number of relapses over 1 year.[3]

One possible explanation for the disparate findings between the association and interventional studies is reverse causality where a low serum 25(OH)D may be the result, rather than the cause, of acute inflammation. Although not universal, a number of studies, including uncontrolled studies after orthopedic surgery, and patients admitted with acute pancreatitis, support the theory

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that 25(OH)D is a negative acute phase reactant, as its circulating levels fall within 48 hours of these conditions.[6–9]

Circulating 25(OH)D is primarily bound (85%–90%) to vitamin D–binding protein (VDBP). The remaining non-VDBP fraction (bioavailable 25(OH)D) is then bound to albumin with less than 1% of 25(OH)D unbound or free.[10] VDBP binding inhibits some vitamin D actions as the bound fraction may be unavailable to act on target cells.[11] It has been proposed that the free or bioavailable 25(OH)D may be more physiologically relevant than total 25(OH)D.[10,12]The VDBP gene, however, is subject to considerable polymorphism leading to variants with differing affinity for both 25(OH)D and $1,25(OH)_2D$.[14] The VDBP variants have been consistently correlated with circulating 25(OH)D and VDBP concentrations. Thus, to interpret 25(OH)D levels accurately, the underlying VDBP genotype needs to be considered. Furthermore, VDBP has unique roles in actin scavenging and in neutrophil chemotaxis, and is less subject to seasonal variation compared with total 25(OH)D.[14] Thus, investigating VDBP is important when considering the effect of vitamin D metabolism in health and disease.

In this study, we aimed to determine whether serum 25(OH)D measured in patients with CD in clinical remission correlated with the risk of a clinical relapse. We also sought to determine whether VDBP and its specific genotypes, as well as free and bioavailable levels of 25(OH)D, were associated with the risk of a disease flare.

MATERIALS AND METHODS

Patients and Design

Prospectively collected patient data were retrospectively collected from 3 Gastroenterology units in Australia with a specialized interest in IBD. Most patients were included between April 2008 and April 2013, 2 patients were included outside this period from August 1996 to May 1997, respectively. The IBD centers included are as follows: (1) the Centre for Inflammatory Bowel Diseases, Fremantle Hospital, Fremantle, Western Australia/Centre for Inflammatory Bowel Diseases, Saint John of God Hospital, Subiaco, Western Australia; (2) Flinders Medical Centre, Adelaide, South Australia; and (3) the Inflammatory Bowel Disease unit, Royal Brisbane and Women's Hospital, Brisbane, Queensland.

All patients had confirmed CD in clinical remission and were phenotyped using the "Montreal Classification." CD was diagnosed according to standard clinical, endoscopic, and radiological criteria.[15] Data were collected at the time of first clinical visit and included disease activity, number, dates and types of CD-related surgeries, smoking, family history, and medication use. These data were updated for each patient at each subsequent visit to determine changes in disease activity and medication use. Blood was collected at the first visit, so that DNA and serum could be cryopreserved at −20°C.

Patients between age 15 and 78 and in clinical remission were included. Clinical remission was defined as disease controlled without any change in CD medications and off corticosteroids for at least 4 weeks before inclusion. Patients were not on vitamin D supplementation at inclusion or during the follow-up period. In addition, both physician and patient assessment identified no CD symptoms or evidence of active disease requiring escalation of therapy. Patients had not been hospitalized in the previous 12 weeks. Disease relapse was defined as worsening in CD symptoms where the physician deemed a change in CD medications to be necessary.

Biochemical Measurements

25(OH)D and C3-epimer-25(OH)D were measured using liquid chromatography tandem mass spectroscopy at the University of Western Australia, Centre for Metabolomics using 2 Agilent 1290 UPLC binary pumps coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA). This assay is certified by the Centre for Disease Control (NIH) for both precision and accuracy of 25 $(OH)D_3$ measurement with a correlation of R^2 0.9979, and also for 3-epi-25$(OH)D_3$ $R^2 = 0.9961$.[16] The endocrine society definitions of sufficiency (≥75 nmol/L), insufficiency (50–75 nmol/L), and deficiency (<50 nmol/L) were used in this study.[1] VDBP was measured by immunonephelometry (Dade Behring, Liederbach, Germany) (interassay coefficient of variation <6%), and albumin was measured by dye-binding reaction to bromocresol green (interassay coefficient of variation <5%). Free and bioavailable (free plus albumin-bound) concentrations of 25(OH)D were calculated using VDBP and albumin concentrations, according to modified "Vermeulen" equations previously published and validated.[17]

Deseasonalized 25(OH)D Measurements

Seasonal variation in circulating 25(OH)D concentrations can be large relative to mean values. As single measurements may misclassify annual exposure, a sinusoidal model incorporating month of blood collection was fitted to the actual vitamin D concentration for each subject to calculate "deseasonalized vitamin D" as previously described.[18]

Genotyping of the VDBP

The VDBP is encoded by the *GC* gene located on chromosome 4 (4q11-13).[19] Combinations of 2 well-studied non-synonymous single-nucleotide polymorphisms (SNPs) in the *GC* gene, rs4588 and rs7041, give rise to 3 major VDBP variants. These variants are distinguished by their amino acid composition and glycosylation patterns: GC-1f, GC-1s, and GC-2, which combine to give 6 diplotypes: 1f-1f, 1f-1s, 1s-1s, 1s-2, 1f-2, and 2-2.[14]

DNA samples from the participants were genotyped for the 2 common SNPs, rs4588 and rs7041 using TaqMan SNP Genotyping primers (C_3133594_30, C_8278879_10; Life technologies, Carlsbad, CA). All samples were genotyped using

the Rotor-Gene 3000 System (Qiagen, Hilden, Germany) and analyzed using Rotor-Gene 6 software (Corbett Research). PCR amplification was performed on 25 ng DNA using 2X TaqMan universal PCR master mix (Life Technologies). Amplification conditions on the Rotor-Gene Q were as follows: 1 cycle of 95°C for 10 minutes, followed by 50 cycles of 92°C for 15 seconds, and 60°C for 1 minute. Positive controls were used after sequencing. The genotype of a subset of samples was confirmed using Sanger sequencing with forward primer 5′agccaagttacaataacaccaggaa 3′ (C_A15629; Life Technologies) and reverse primer 5′ttccaattcagcagcgatttgt3′ (C_A15630; Life Technologies). Sequencing was performed on the 3730XL sequencer (Applied Biosystems, Foster City, CA), and the sequences obtained were aligned using the Assign software (Conexio Genomics, Fremantle, WA, Australia).

The allele at each gene locus on each chromosome determines the genotype for the rs4588 and rs7041 gene. The combination of alleles at the 2 loci determines the haplotype which in turn determines protein expression. Thus, if C at rs4588 combines with T at rs7041 GC1f results, A-T = GC2, C-G = GC1s. Where the genes are heterozygous at both sites, it was not possible to determine the subtype of the GC1 protein using standard allelic discrimination PCR method; thus, these are presented as GC 1-2.

Statistical Methods

Summary statistics, including counts and percentages for categorical variables and mean, SDs for continuous variables, have been presented overall and broken down by whether the patient flared. The time to flare was investigated using Cox proportional hazards models to describe the risk of flaring by the various predictors. Results from univariate (one predictor) have been provided for all potential predictor variables (age, sex, disease location, smoking status, mode of remission induction, season of blood draw, VDBP mg/L, total, bioavailable, and free 25(OH)D, C3-epimer-25(OH)D, and percentage epimer of total 25(OH)D and albumin), along with results from model selecting from this group of variables to determine one final multiple predictor model. Hazard ratios (HR) and 95% CIs are presented along with the Kaplan–Meier charts for the variables identified as statistically significant in this final model and for other nonsignificant comparisons of interest. Model selection was performed using a 0.05 significance level.

Genetic analysis was performed in 2 ways. First, analysis of variance models were used to determine whether there were differences in the levels of the 2 responses of VDBP and 25(OH)D_3 in 3 different ways, one for each factor, VDBP genotypes rs4588 (CC, CA, and AA), rs7041 (GG, GT, and TT), and diplotype (*GC* 1f-1f, 1f-1s, 1s-1s, 1-2, and 2-2). Pairwise comparisons were then undertaken between the levels to establish where the differences occurred. Second, Cox proportional hazards models were fitted, as previously described, using the genotypes as predictors of risk of flaring. All analyses were performed using the R language and Environment for Statistical Computing.[21]

Ethical Considerations

The study was approved by the human research ethics committee of the South Metropolitan Area Health Service, Western Australia (Ref: 07/589), with site-specific approvals at the secondary sites.

RESULTS

Patient and Disease Characteristics

Three hundred nine subjects meeting the inclusion criteria were identified and included in the final analysis. One hundred forty-three (46%) were men, mean age at inclusion was 40 ± 15 years with almost all of white background (92%) (Table 1). There was almost equal distribution between ileal (L1), colonic (L2), and ileocolonic (L3) disease, and most patients had a nonstricturing, nonpenetrating phenotype (55%). At the time of inclusion, 28% of patients were smoking. A family history for IBD was identified in 16% of cases. Many patients were on either a 5-aminosalicylate, or no therapy (45%), or on monotherapy with an immunomodulator (34%) with only 11% on anti–tumor necrosis factor therapy. Combination of immunomodulator and anti–tumor necrosis factor therapy was in use in 10% of patients. Surgical resection was used to induce remission in 102 (33%) patients, whereas the remaining patients achieved a medical-induced remission. The mean duration of remission before inclusion was 55.8 ± 156.4 months. The mean duration of remission among those with medical remission was 49.2 ± 134.9 months, compared with 69.3 ± 192.8 months among those with surgical remission. The difference is not statistically significant (P = 0.290, 95% CI, −17.2 to 57.3).

Vitamin D Data

25(OH)D_3 levels were analyzed in all 309 patients using LCMS/MS, and C3-epimer-25(OH)D_3 was available in 280 (91%), albumin in 307 (99%), and VDBP in 306 (99%) (Table 2). The free and bioavailable 25(OH)D_3 were calculated where the VDBP and albumin levels were available. At the time of inclusion, 166 (54%) patients were in the vitamin D sufficient range (≥75 nmol/L), 107 (35%) had vitamin D insufficiency (50–74 nmol/L), and surprisingly, only 36 (12%) were in the deficient range (<50 nmol/L). The deseasonalized 25(OH)D_3 levels were not significantly different.

Flare Characteristics

The mean duration of follow-up was 36.9 months (range 10.5–127.6) (data not shown). One hundred (32%) patients flared during the follow-up period, and the mean time to flare was 18.6 months (range 1–69). Significantly, more patients who had a surgery-induced remission remained in remission during follow-up (79% versus 62.0%, univariate HR = 2.29, 95% CI, 1.37–3.84, $P < 0.001$) (Table 1). Those who flared were more likely to have colonic disease compared with isolated ileal disease (Table 1). Those with isolated ileal disease, however, were significantly

TABLE 1. Baseline Characteristics and Univariate Results

	Overall, n = 309 (%)	No Flare, n = 209	Flare, n = 100	HR	95% CI
Sex					
Male	143 (46)	53 (37)	90 (63)	1.32	0.88–1.99
Age					
A1	49 (16)	31 (15)	18 (18)	1	
A2	176 (57)	114 (55)	62 (62)	1.25	0.64–2.45
A3	84 (27)	64 (31)	20 (20)	1.51	0.89–2.56
Location					
L1	100 (32)	24 (24)	76 (76)	1	
L2	94 (30)	35 (37)	59 (63)	1.93	1.12–3.31
L3	113 (37)	41 (36)	72 (64)	1.48	0.87–2.50
±L4	16 (5)	8 (50)	8 (50)		
Behavior					
B1	169 (55)	53 (31)	116 (69)	1	
B2	55 (18)	21 (38)	34 (62)	1.06	0.62–1.80
B3	82 (27)	24 (29)	58 (71)	0.79	0.48–1.32
N/A	3 (1)				
Smoking status					
Never	155 (50)	54 (35)	101 (65)	1	
Current	88 (28)	24 (27)	64 (73)	0.81	0.50–1.33
Ex-smoker	65 (21)	22 (34)	43 (66)	0.95	0.57–1.60
Ethnicity					
White	283 (92)	93 (33)	190 (67)	NA	NA
Asian	1 (0.3)	1 (100)	0 (0)		
Indian	5 (2)	1 (20)	4 (80)		
Middle East	2 (0.7)	0 (0)	2 (100)		
N/A	18 (6)				
Family history	49 (16)	21 (43)	28 (57)	1.36	0.82–2.25
Previous intravenous steroids	15 (4.9)	6 (40)	9 (60)	1.05	0.42–2.64
Treatment					
5ASA/no therapy	138 (45)	40 (29)	98 (71)	1	
Immunomodulator	105 (34)	34 (32)	71 (68)	1.02	0.63–1.64
Tumor necrosis factor	34 (11)	13 (38)	21 (62)	1.20	0.64–2.27
Immunomodulator+tumor necrosis factor	32 (10)	13 (41)	19 (59)	1.12	0.58–2.16
Mode of remission					
Surgical	101 (33)	21 (21)	80 (79)	1	
Medical	208 (67)	79 (38)	129 (62)	2.29	1.37–3.84

The HR and 95% CIs are calculated from univariate Cox proportional hazards models of variables impacting on the risk of disease flare.
ASA, 5 aminosalicylate.

more likely to have a surgery-induced remission (46% versus 26%, $P < 0.001$, data not shown). Oral steroids were most commonly used to treat the flare (49%) followed by an anti–tumor necrosis factor agent in 43%, whereas 4% of patients required surgery.

The $25(OH)D_3$ levels were not significantly different between patients who flared and those where clinical remission was maintained (HR 1.10, 95% CI, 0.9–1.35) (Table 2). Similarly, free, and bioavailable, $25(OH)D_3$ concentrations did not predict disease flare. The C3-epi-$25(OH)D_3$ has only recently been able to be measured and is believed to be less active than $25(OH)D_3$, although its physiological role remains unclear. Neither the absolute C3-epi-$25(OH)D_3$ level, nor the percentage epimer of the total $25(OH)D_3$, impacted the risk of disease flare (Table 2). Of note is that the mean VDBP concentration was significantly higher in patients who flared (310.9 ± 5.67 versus 295.2 ± 3.29 mg/L), and this was observed in both the univariate and multivariate models (HR 1.3, 95% CI, 1.08–1.57, $P < 0.001$) (Tables 2 and 3).

TABLE 2. Characteristics of Vitamin D Status, VDBP, Free, Bioavailable, and C3-epimer-25(OH)D Concentrations

	Overall, n = 309 (%)	No Flare, n = 209 (%)	Flare, n = 100 (%)	HR	95% CI
Season of bloods					
Summer	43 (14)	27 (63)	16 (37)	1	
Autumn	83 (27)	54 (65)	29 (35)	0.92	0.50–1.72
Winter	96 (31)	66 (69)	30 (31)	0.67	0.36–1.25
Spring	87 (28)	62 (71)	25 (29)	0.83	0.44–1.57
$25(OH)D_3$					
0–49	36 (12)	27 (75)	9 (25)	1	
50–74	107 (35)	72 (67)	35 (33)	1.79	0.74–4.31
75+	166 (54)	110 (66)	56 (34)	1.78	0.76–4.17
Deseasonalized $25(OH)D_3$					
0–49	34 (11)	27 (79)	7 (21)	1	
50–74	105 (34)	68 (65)	37 (35)	1.74	0.67–4.50
75+	170 (55)	114 (67)	56 (33)	1.66	0.66–4.12
Mean VDBP (mg/L ± SEM) HR for 50 mg/L increase		**295.2 ± 3.29**	**311.1 ± 5.73**	**1.23**	**1.03–1.46**
Mean bioavailable $25(OH)D_3$ (nmol/L ± SEM), HR for a 1-unit increase		6.45 ± 0.17	6.19 ± 0.23	0.98	0.89–1.07
Mean free $25(OH)D_3$ (pmol/L ± SEM), HR for a 3-unit increase		17.4 ± 0.44	17.0 ± 0.58	0.98	0.79–1.21
Mean C3-epi-$25(OH)D_3$ ± SEM, HR for a 1-unit increase		4.4 ± 0.15	4.4 ± 0.19	0.99	0.90–1.10
Mean % C3-epi-$5(OH)D_3$ ± SEM, HR for a 1-unit increase		5.6 ± 0.16	5.5 ± 0.20	0.96	0.87–1.06
Mean albumin ± SEM, HR for a 5-g/L increase		43.9 ± 0.27	43.2 ± 0.39	0.84	0.66–1.06

The HR and 95% CIs are calculated from univariate Cox proportion models of variables impacting on the risk of disease flare. Figures in bold signify statistically significant variables.

Multivariate modeling of the risk of flaring was determined including the following variables: age, sex, disease location, smoking status, mode of remission, season of blood collection, VDBP total $25(OH)D_3$, bioavailable 25(OH)D, free 25(OH)D, C3-epi-$25(OH)D_3$% C3-epi-$25(OH)D_3$, and albumin (Table 3).

TABLE 3. Multivariate Analysis

	HR	95% CI	*P*
VDBP, mg/L			
50 mg/L increase	1.30	1.08–1.57	<0.001
Mode of remission			
Medical versus surgical	2.46	1.44–4.18	<0.001
Albumin			
5 g/L increase	0.72	0.55–0.92	<0.001

Using Cox proportional hazard modeling, the following variables entered as potential predictors of disease flare: age, sex, location, smoking status, mode of remission, season of bloods, VDBP mg/L, total $25(OH)D_3$, bioavailable 25(OH)D, free 25(OH)D, VDBP, C3-epi-$25(OH)D_3$% C3-epi-$25(OH)D_3$, and albumin. Only the significant independent predictors are shown below.

Male gender, medically induced remission, and a higher VDBP concentration all independently were associated with an increased risk of disease flare, whereas a higher albumin concentration was protective (HR 0.77, 95% CI, 0.63–0.94, $P < 0.001$). Similarly, VDBP >320 mg/L and medically induced remission were associated with a shorter time to disease flare (Fig. 1), whereas no differences in the flare-free survival was observed with the total vitamin D concentration.

A multivariate sensitivity analysis was performed, where those patients who flared within the first 6 months of follow-up were excluded. The same predictors of a disease flare were observed in this model as for the total data set (data not shown).

Genetics

The major VDBP variants were genotyped in 240/309 (77%) patients. No significant deviations from Hardy Weinberg proportions ($P < 0.05$) were observed in either of the 2 polymorphisms (results not shown). The *GC* 1-1 diplotype was most common in this cohort overall (56%) with the most common subtype being *GC* 1s1s (35%). The *GC* 2-2 diplotype was the least common (8%). This was consistent with data from European populations.[19]

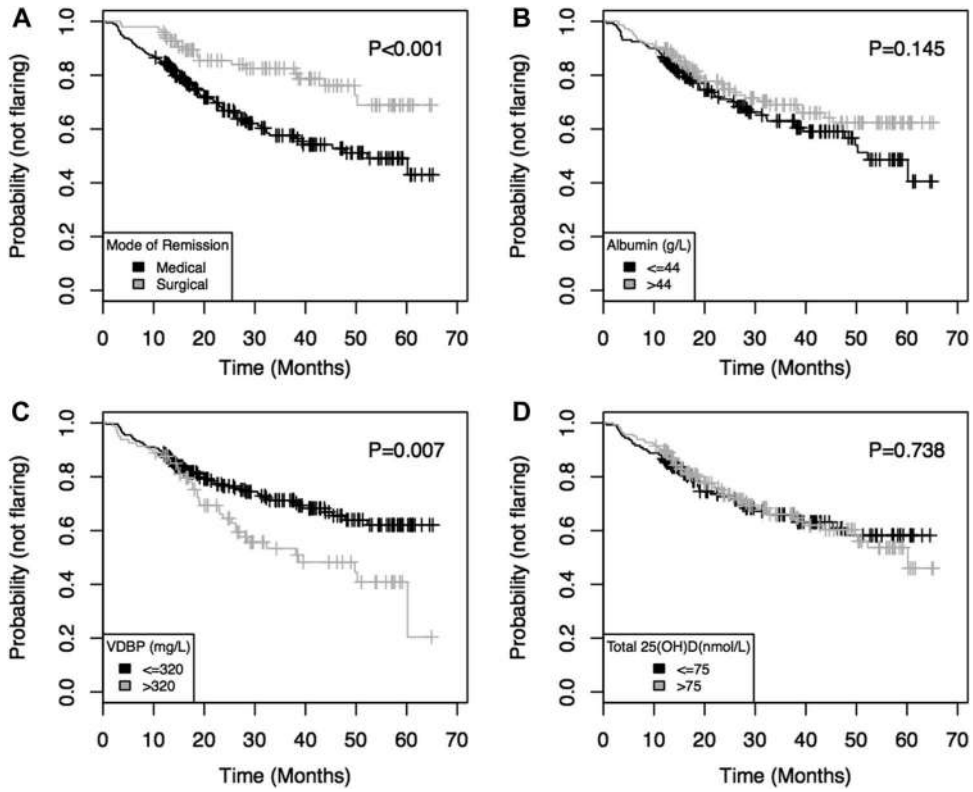

FIGURE 1. Flare-free survival Kaplan–Meier Curves. A, Model of remission (surgical versus medical); (B) serum albumin levels, low (≤44 g/L) versus high (>44 g/L); (C) VDBP concentration, low (≤320 mg/L) versus high (>320 mg/L); (D) serum total 25(OH)D, low (≤75 nmol/L) versus high (>75 nmol/L).

The rs4588 genotype had a statistically significant impact on the VDBP and 25(OH)D concentrations ($P < 0.001$ and $P = 0.014$, respectively, Fig. 2). Pairwise comparisons showed that CC was associated with the highest VDBP (mean 320.8 ± 51.0 mg/L) and 25(OH)D concentrations (mean 84.6 ± 28.4 nmol/L) compared with both CA (mean 290.9 ± 43.6 mg/L and 78.0 ± 29.8 nmol/L, respectively) and AA (mean 269.9 ± 53.3 mg/L and 65.2 ± 17.5 nmol/L), with the latter 2 not statistically significantly different from each other. When considering rs7041, a statistically significant difference was noted in VDBP and 25(OH)D concentrations ($P < 0.001$ for both) with GG associated with higher concentrations (322.0 ± 51.7 mg/L and 85.6 ± 26.5 mg/L,

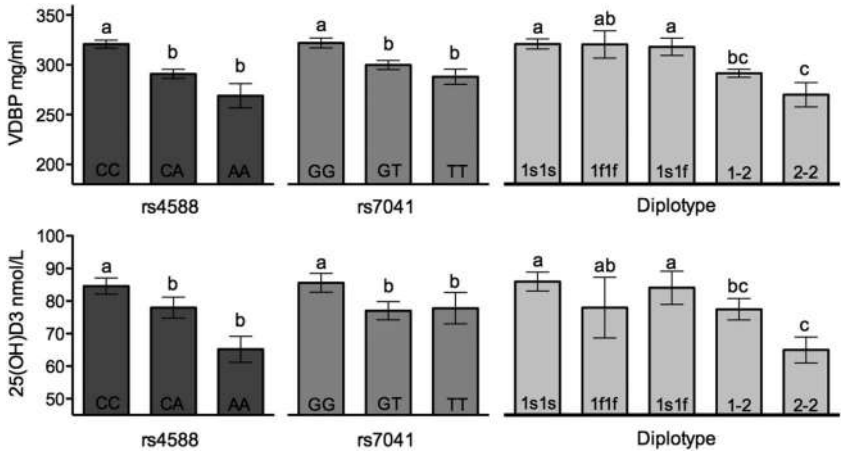

FIGURE 2. VDBP variance and 25(OH)D levels correlated with genetic group. Six analyses of VDBP and 25(OH)D levels by genetic groups are presented (mean ± standard error). Bars with the same letters above in each analysis were not significantly different.

respectively) than both GT (299.9 ± 48.0 mg/L, 77.7 ± 28.7 nmol/L) and TT (288.1 ± 51.4 mg/L, 77.8 ± 32.0 nmol/L). When comparing the diplotypes, the *GC* 1-1 diplotypes were associated with higher VDBP concentrations compared with *GC* 1-2 or 2-2 ($P < 0.001$), but there were no differences between the *GC* 1-1 subtypes, or between *GC* 1-2 and 2-2. The impact on 25(OH)D concentrations was similar, with the *GC* 1s1s and *GC* 1s1f associated with higher concentrations than the *GC* 2-2 and *GC* 1-2 diplotypes ($P = 0.004$ and $P = 0.019$, respectively). The VDBP genotypes and diplotypes were not associated with an increased risk of disease flare when examined by Cox proportion hazard modeling (data not shown).

DISCUSSION

This is the first study to correlate vitamin D status to the rate of disease flare in a cohort of patients with CD in remission. Vitamin D deficiency was uncommon in our cohort and represented only 12% of the population, although 35% of patients were in the insufficient range. By contrast, 33% of pediatric patients with CD in remission in a Canadian trial of vitamin D supplementation were vitamin D deficient, as where 29% to 33% of adult Danish patients with CD in remission.[3,22] The observed differences are likely due to the greater UV exposure of our patient population in the lower latitudes of Perth, Australia at 31.95°S where most cases were recruited. Furthermore, the liquid chromatography tandem mass spectroscopy assay used to analyze serum 25(OH)D in our patients, which is the current gold standard, has reported levels of between 11.61 and 26.05 nmol/L higher than the commonly used immunoassays such as the DiaSorin Liaison used in the Canadian study.[23]

In our study, we predicted that patients with lower serum 25(OH)D levels would be at a greater risk of disease flare. Surprisingly, no increased risk was observed with either lower total, free, or bioavailable, 25(OH)D levels. The findings were the same using deseasonalized 25(OH)D levels. Most studies that associate vitamin D deficiency with more flares are retrospective, or cross sectional, in design, and thus, a causal relationship cannot be established. To date, only 1 placebo-controlled trial has evaluated the effect of vitamin D in CD and demonstrated a 13% reduction in flare rate, but this did not reach statistical significance ($P = 0.06$).[3] An open-label pilot study also examined 18 CD patients with mild-to-moderate activity, and plasma 25(OH)D levels <100 nmol/L, received vitamin D_3 to achieve plasma 25(OH)D levels of >100 nmol/L. This was associated with a reduction in the unadjusted mean Crohn's disease activity index by 112 ± 81 points ($P < 0.01$), but no significant change in any inflammatory marker was observed. The lack of objective endpoints, and the small study population, however, limits the generalizability of the finding.

One explanation for the disparate findings between our current study, and the controlled trials, when compared with the retrospective, cross-sectional, and uncontrolled studies, is that vitamin D deficiency could be the result, rather than cause, of active inflammation in CD. There are also a number of confounding factors in patients with active CD, including a reduction in time spent outdoors, reduced oral intake, and potential changes in vitamin D absorption in the presence of widespread small bowel disease or surgery. Furthermore, 25(OH)D seems to be a negative acute phase reactant, as demonstrated by a reduction in serum 25(OH)D by 17.8% 48 hours after knee, or hip, arthroplasty that corresponded to an increase in the C-reactive protein (CRP).[6] Similarly, vitamin D deficiency is prevalent among the critically ill in the intensive care setting and corresponds to disease severity.[24,25] Finally, although this study only included patients who had no record of starting vitamin D supplementation during the follow-up period, we cannot exclude the possibility that patients failed to report or clinicians did not record the use of vitamin D. So, it is possible that supplementation in the small group with vitamin D deficiency or insufficiency prevented the detection of a difference in relapse rates.

VDBP levels in our study were, however, positively associated with disease flare. A 50 mg/L increase in VDBP concentration increased the rate of flaring by 30% (HR 1.3, 1.08–15.7, $P < 0.001$). This was independent of season of blood collection, sex, age, ethnicity, treatment type, smoking status, and mode of remission induction. This has the potential clinical implication of being used as a biomarker to identify a group of patients at risk of disease flare who may require close monitoring or optimization of therapy. The physiological role of VDBP, however, is incompletely understood, but in addition to its role in vitamin D transport, it participates in the actin scavenging system by sequestering G-actin released into the circulation from damaged tissues. VDBP also enhances the chemotactic activity of complement 5a and contributes to inflammation by being the precursor of macrophage activation factor.[26–28] Elevated VDBP levels occur in patients with stable multiple sclerosis and in the cerebral spinal fluid of patients with secondary progressive multiple sclerosis.[28,29] In vitro studies also demonstrate that $CD4^+$ T lymphocytes cultured with high VDBP levels in the medium have a reduced capacity to produce $1,25(OH)_2D_3$, which normally suppresses T-cell proliferation and determines the T-cell phenotype.[30–32] Similarly, monocytes cultured in medium containing serum from VDBP-knockout mice produce greater human antimicrobial peptide cathelicidin after stimulation with $25(OH)D_3$, while VDBP added to serum-free medium attenuated the $25(OH)D_3$ responses.[11] It is thus possible that higher circulating concentrations of VDBP may attenuate any favorable effect that $25(OH)D_3$ has on the innate, and adaptive, immune responses.

There are limited data on the effect of VDBP in IBD. A cross-sectional study found a negative correlation between total, free, and bioavailable 25(OH)D with the fecal inflammatory marker calprotectin.[33] The prevalence of VDBP isoforms in CD and ulcerative colitis has also been compared with non-IBD controls and, as we observed, the GC-2 haplotype was less common in the IBD population.[34] In our study, the concentration of both 25(OH)D and VDBP decreased according to the phenotype: GC 1-1>GC 1-2>GC 2-2 consistent with the findings from

a cross-sectional study of 595 postmenopausal women.[35] These isoforms differ in their affinity for 25(OH)D, with GC2 having the lowest affinity and GC1f the highest. Thus, when interpreting serum $25(OH)D_3$ levels, the VDBP phenotype should be taken into account with lower $25(OH)D_3$ levels being considered as "normal" in those with GC 2-2.[36]

Intriguingly, we observed that those patients with medication-induced remissions were more likely to flare compared with those with a surgery-induced remission (HR 2.46, 95% CI, 1.44–4.18, $P < 0.0001$). Notably, patients who underwent surgery were more likely to have disease localized to the terminal ileum, and this is associated with good outcomes with only 40% of patients requiring further surgery by 10 years.[37] To date, however, there have not been direct comparisons between medical and surgical treatment, and such comparisons should not be inferred from our data as there was a bias toward surgery in the group with localized ileal disease. An albumin level <44 g/L was also associated with disease relapse (Fig. 1). Studies examining predictors of disease relapse have identified biomarkers, such as CRP, the erythrocyte sedimentation rate, and fecal calprotectin to predict disease relapse, but to our knowledge, this is the first report of albumin also being a predictor of disease relapse.[38–41] Albumin is a negative acute phase reactant, and this finding is likely due to subclinical inflammatory activity in those that subsequently flare. As albumin was required for the calculation of free and bioavailable vitamin D, it was specifically measured in the stored serum of all patients, we did not measure CRP or erythrocyte sedimentation rate.

There are several limitations to our study. As a retrospective analysis, disease remission was defined by the stable use of medication, remaining corticosteroid free, and the clinician's clinical assessment of the patient remaining in remission. We did not have clinical indices such as the CD activity index or the Harvey Bradshaw index recorded. Similarly, we did not have systemic inflammatory markers, or endoscopy results, available in all patients at the time of study inclusion. The same issues apply to the clinical definitions of relapse. Within these limitations, it should be noted that patients were recruited from high-volume tertiary centers where clinicians have extensive experience in the management of CD. Furthermore, more than a third of patients had their remission induced by surgical resection, which is not vulnerable to subjective assessment of remission.

The second limitation is the small number of patients with vitamin D deficiency (12%), limiting the power to detect an effect of vitamin D deficiency on disease flare. As previously mentioned, this may be in part due to the higher levels of 25(OH)D that are measured using liquid chromatography tandem mass spectroscopy, but even when examining the level of 25(OH)D as a continuous variable, there was no signal of an increased risk of disease flare with the lower concentrations. Perhaps the most pertinent point is that vitamin D deficiency is uncommon in CD remission. The study's strengths, however, include the use of a single, high-quality, and internationally validated 25(OH)D assay for all samples leading to reliable results. It is also the first study to examine the relationship between VDBP, its genetic variants, and the subsequent disease course in CD.

In conclusion, for patients with CD in remission, vitamin D deficiency was uncommon, and the serum $25(OH)D_3$ did not predict subsequent disease flare. By contrast, higher VDBP concentration was significantly associated with disease flare, which may reflect its function in mediating the inflammatory response. The VDBP concentration is largely determined by genetic factors. Further investigations to explore the possible mechanisms for this association are thus warranted.

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Chapter 4

Ghaly S et al. **High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis.** Sci Rep. 2018;8(1):11511

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High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis

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Vitamin D has been suggested as a possible adjunctive treatment to ameliorate disease severity in human inflammatory bowel disease. In this study, the effects of diets containing high (D++, 10,000 IU/kg), moderate (D+, 2,280 IU/kg) or no vitamin D (D−) on the severity of dextran sodium sulphate (DSS) colitis in female C57Bl/6 mice were investigated. The group on high dose vitamin D (D++) developed the most severe colitis as measured by blinded endoscopic ($p < 0.001$) and histologic ($p < 0.05$) assessment, weight loss ($p < 0.001$), drop in serum albumin ($p = 0.05$) and increased expression of colonic TNF-α ($p < 0.05$). Microbiota analysis of faecal DNA showed that the microbial composition of D++ control mice was more similar to that of DSS mice. Serum 25(OH)D_3 levels reduced by 63% in the D++ group and 23% in the D+ group after 6 days of DSS treatment. Thus, high dose vitamin D supplementation is associated with a shift to a more inflammatory faecal microbiome and increased susceptibility to colitis, with a fall in circulating vitamin D occurring as a secondary event in response to the inflammatory process.

Vitamin D is recognized as a regulator of both innate and adaptive immune responses[1], and vitamin D deficiency has been associated with the development of a number of immune mediated disorders including the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC).

Several confounding factors, such as reduced sunlight exposure, low dietary intake and reduced intestinal absorption, limit the ability to draw conclusions about the causality of the observed link between vitamin D deficiency and active IBD. As a result, investigators have turned to mouse models of IBD such as the dextran sodium sulphate (DSS)-induced model of colitis[2]. Vitamin D receptor (VDR) knockout (KO), CYP27B1 KO, as well as dietary vitamin D-deficient mouse models are more susceptible to colitis[3–5]. In an IL-10-KO mouse model, which typically develop spontaneous colitis, administration of 1,25$(OH)_2$D ameliorated the severity of colitis[6]. Thus, in select animal models, vitamin D deficiency increases susceptibility to colitis, and restoring vitamin D sufficiency may ameliorate colitis.

There are few studies exploring the effect of high vitamin D levels on immune regulation. Population studies describe a reverse 'J' or 'U' phenomenon where both vitamin D deficiency and high vitamin D are associated with increased all-cause and cardiovascular specific mortality[7–9]. Higher vitamin D levels at birth have also been linked to the development of allergy[10,11], and genetic analysis within these cohorts identify epigenetic changes in a number of genes including the thymic stromal lymphopoietin (*TSLP*) gene that may explain the immune mechanism for predisposition to allergy with elevated vitamin D levels[12]. The effect of higher vitamin D levels in clinical IBD or animal models of IBD have not been explored.

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Vitamin D-related changes to gut microbiota are a possible mechanism for altering susceptibility to colitis. Vitamin D-deficient mice developed elevated bacterial counts in colonic tissue and greater susceptibility to DSS colitis[3]. Dietary-induced vitamin D deficiency alters the composition of the faecal microbiome of C57Bl/6 mice, with an increase in the relative quantities of Bacteroidetes, Firmicutes, Actinobacteria, and Gamma-Proteobacteria in naïve, non-colitic mice[13]. The effect of vitamin D on microbiota, however, is not limited to the gastrointestinal tract with an inverse correlation between circulating 25(OH)D levels and *Pseudomonas* operating taxonomic units (OTU) observed in the lungs of naïve female BALB/c mice[14]. Thus, vitamin D may regulate the microbiome at different sites and this could be due to its effect on innate immune responses, in particular the expression of antimicrobial peptides, such as the cathelicidins and β-defensins[15].

The aim of this study was, therefore, to determine the effect of diets supplemented with high doses of vitamin D, compared to standard diets sufficient in vitamin D and diets deficient in vitamin D with no supplementation, on the susceptibility to DSS-induced colitis. Further, we sought to investigate the effect of different doses of vitamin D on the faecal microbiota and how this correlated with susceptibility to colitis.

Materials and Methods

Mice and Diet. Female 6 week-old C57Bl/6 mice were fed semi-pure diets supplemented with higher than usual doses of vitamin D (SF14-069, Specialty Feeds, Perth, Western Australia, 10,000 IU/kg vitamin D_3, 0.5% calcium), standard doses of vitamin D to achieve vitamin D sufficiency similar to standard chow (SF05-34, Specialty Feeds, 2,280 IU/kg vitamin D_3, 1% calcium) or no added vitamin D to induce vitamin D deficiency (SF05-033, Specialty Feeds, 0 IU/kg vitamin D_3, 2% calcium). All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia with the approval from the Telethon Kids Institute Animal Ethics Committee (AEC #276). Mice were purchased from the Animal Resources Centre, Western Australia.

Mice were housed under perspex-filtered fluorescent lighting, which emitted no detectable UVB radiation as measured using a UV radiometer (UVX Digital Radiometer, Ultraviolet Products Inc., Upland, CA, USA).

Colitis Model. After 4 weeks of being fed the respective diets, colitis was induced by the addition of DSS [3% (wt/vol)(MP Biomedicals LLC, OH)] to the drinking water for 6 days. Control mice received water without DSS. As the efficacy of DSS varies between batches, the experiments were conducted using the same batch[16]. In preliminary experiments, 3% DSS induced adequate colitis with peak weight loss ranging between 0.3% to 10.3% at day 7. Following induction of colitis, mice recovered over a period of 0–4 weeks without ongoing DSS treatment (Fig. 1). Mouse body weight was assessed daily during DSS treatment and weekly during recovery. The experiment was repeated, with a total of 35 mice per group. Mice were sacrificed at day 7, 14, 21 and 35.

Murine colonoscopy. A high-resolution mouse video endoscopic system was used to assess the level of colitis. All mice were scoped on day 6 after commencing DSS treatment and then at the time of sacrifice. Mice were anaesthetised using isofluorane unless the colonoscopy was being performed at the end-point when ketamine 20 mg/ml and xylazine 2 mg/ml by intraperitoneal injection was used. All procedures were digitally recorded then scored in a blinded fashion. The experimental endoscopy setup consisted of a miniature endoscope (1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Karl Storz, Germany) to achieve regulated inflation of the mouse colon.

The severity of colitis was determined using the modified '**m**urine **e**ndoscopic **i**ndex of **c**olitis **s**everity' (MEICS)[16,17]. The MEICS system consists of 5 parameters: thickening of the colon wall, changes of the normal vascular pattern, presence of fibrin, mucosal granularity and stool consistency. Endoscopic grading was performed for each parameter (scored between 0 and 3) leading to a cumulative score of between 0 (no signs of inflammation) and 15 (endoscopic signs of severe inflammation). Healthy mice had a score of 0–3.

UV Radiation. For UV experiments, a bank of six 40 W lamps (Philips TL UV-B, Eindhoven, The Netherlands) emitting broadband UVR, 250–360 nm, with 65% of the output in the UVB range (280–315 nm), was used to irradiate mice and to deliver 1 kJ/m^2 of UVR onto clean-shaven 8 cm^2 dorsal skin. A new sheet of PVC plastic film (0.22 mm) was taped to the top of each Perspex cage immediately before irradiation to screen wavelengths $<$290 nm. Sunlamps were held 20 cm above the cages.

Histological Assessment of Colitis. Colons were removed with the rectum discarded as this has a different tissue fibro-structure. The distal 1 cm of colon was dissected, cleaned, formalin-fixed and embedded in paraffin wax. Sections were stained with haemotoxylin and eosin (H&E). All H&E sections were assessed blindly by a specialist gastroenterological histopathologist (CF) according to the scoring system by Dieleman *et al.*[18]. In this scoring system, the severity and depth of inflammation as well as the level of crypt damage and regeneration are scored.

Measurement of serum metabolites. At the time of sacrifice, blood was drawn by cardiac puncture. Levels of 25(OH)D_3 were measured in the serum by liquid chromatography tandem mass spectroscopy (LC/MS/MS) (Centre for Metabolomics, UWA)[19]. Levels of 1,25$(OH)_2D_3$ were measured using IDS EIA ELISA kits (Immunodiagnostic Systems Ltd, Fountain Hills, AZ) as described by the manufacturer.

The serum vitamin D binding protein (VDBP) concentration was measured in duplicate using a Quantikine ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer's instructions. The results were verified by a radial immunodiffusion method as previously published[20].

Serum calcium and albumin were measured by standard colorimetric reactions using the Architect c16000 Analyzer (Abbott Diagnositcs, Abbott Park, IL) by PathWest, Royal Perth Hospital, WA.

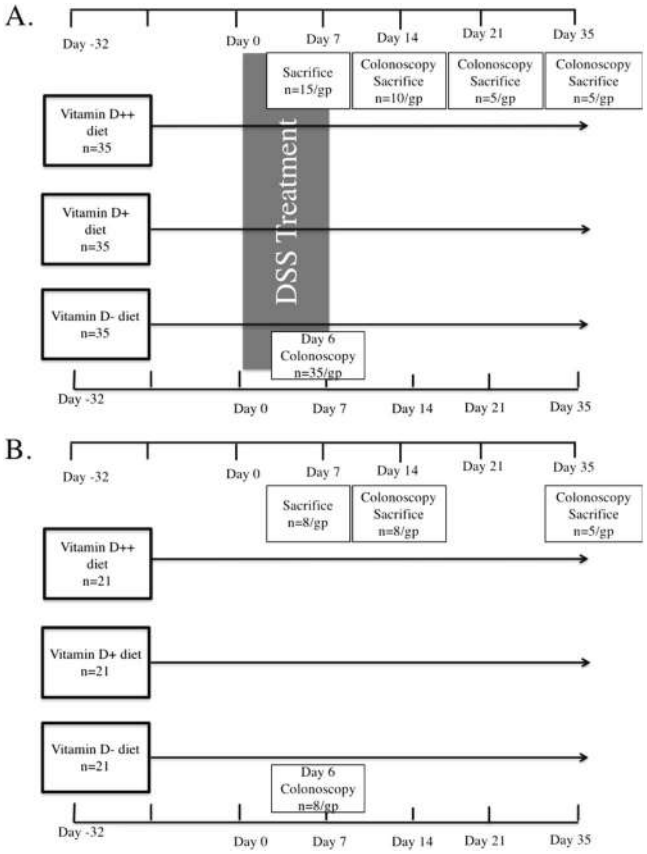

Figure 1. The experimental approach. 6-week-old C57BL/6 female mice were fed diets with high (D++), moderate (D+) or no (D−) vitamin D3. (**A**) After 4 weeks of being fed the respective diets, mice were treated with DSS for 6 days to induce colitis, and then were maintained without DSS and continued their respective vitamin D diet until day 35. All mice underwent colonoscopy at day 6, and were sacrificed at day 7, 14, 21 or 35. An additional colonoscopy was performed on day 14, 21 or 35 when mice were sacrificed. (**B**) A control group of mice fed the three different diets followed the same protocol but were not treated with DSS and the day 21 timepoint is not included. The experiment was performed twice.

Serum cytokines were measured using Bio-Plex Pro™ Mouse Cytokine 23-plex panel (Bio-Rad Laboratories, Hercules, CA) as per the manufacturer's instructions. The cytokines analysed included interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-17, eotaxin (CCL11), G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α.

Real-time PCR. Messenger RNA was extracted from snap-frozen colon, liver and kidney tissue with cDNA synthesised and real-time assays performed as previously described[21]. Real-time PCR primers were *CYP27B1* cat # 301447280210/0&1, *CYP24A1* cat # 3014472802-20/0&1, *CYP2R1* cat # QT0005750 (Sigma-Aldrich co., St. Louis, MO), and for TNFα Qiagen Quantitect Primer Assay (Qiagen, Hilden, Germany). Housekeeping genes included elongation factor 1α (eEF1α) for kidney and liver tissue, TATA-box-binding protein for colonic tissue (Sigma-Aldrich co., St. Louis,MO). Quantitect SYBRGreen was used for qPCR on the AB17900HT instrument. Fold-change was determined by using the $2^{-\Delta\Delta Ct}$ method.

Faecal Microbiota analysis. The faecal microbiome was analysed by sequencing the V3-V4 segment of the 16S ribosomal RNA (rRNA) gene using Illumina MiSeq chemistry. Faecal pellets were collected and stored at −20 °C. Bacterial DNA was extracted using the PowerSoil® DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA). PCR amplification (341F/806F primer pair) and sequencing was performed by the Australian Genome Research Facility on the Illumina MiSeq (San Diego, CA) with 2 × 300 bp paired-end chemistry. Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5)[22]. Primers were trimmed using Seqtk (version 1.0)[23]. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8)[24] USEARCH (version 7.1.1090)[25,26] and UPARSE[26] software. Using QIIME, taxonomy was assigned using Greengenes database (Version 13_8, Aug 2013)[27].

The biom file, the OTU table, the taxonomic assignments and associated sample data were imported into R to create a phyloseq object. For all beta diversity analyses, OTUs for which the variance across all samples were very low, were filtered out. For testing a single categorical experimental condition, exact tests for differences in the means between two groups of negative-binomially distributed counts were computed. Data were normalized

	D++	D+	D−	p-value D++ vs D+	p-value D+ vs D−
25(OH)D nmol/L	100.8 ± 4.6	41.2 ± 2.0	12.0 ± 4.0	<0.001	0.001
$1,25(OH)_2D$ pmol/L	119.9 ± 17.7	140.3 ± 26.3	85.6 ± 8.6	NS	NS
Calcium mmol/L	2.2 ± 0.04	2.3 ± 0.1	1.9 ± 0.1	NS	NS

Table 1. Serum 25 (OH)D, $1,25(OH)_2D$ and calcium levels after 5 weeks on vitamin D diets. Data are shown as mean ± SEM for $n = 7–8$ for 25(OH)D levels, $n = 3–5$/group for $1,25(OH)_2D$, $n = 4–5$/group for calcium. *P values* reflect differences in mean between vitamin D groups calculated by independent student t-test. Results pooled from two independent experiments. NS = not significant.

using the RLE scaling factor method and dispersions estimated. The counts were extracted and ranked by p-value, applying a false discovery rate cut-off of less than 0.001.

Statistical Analysis. Statistical significance was calculated using IBM® SPSS® Statistics Version 22 (IBM Corp. Armonk, NY). All graphs and comparison of differences between groups were assessed using Student's unpaired t-test or ANOVA with post hoc LSD analysis for multiple group analysis. Non-parametric data using Mann-Whitney U and Kruskall-Wallis testing. Microbiome statistical analysis was undertaken using the programming language R, specifically the *phyloseq* and *edgeR* packages available through Bioconductor, a project providing tools for the analysis and comprehension of high-throughput genomic DNA. LEfSe (Linear discriminant analysis effect size) was used to identify differentially abundant microbial taxa[28].

Results

Vitamin D_3 diets. Six-week-old C57Bl/6 female mice were fed diets with high (D++), moderate (D+) or no (D−) vitamin D_3 for four weeks. At ten weeks of age, the mice in the three groups were not significantly different in weight (data not shown). D++ mice had significantly higher serum $25(OH)D_3$ compared to D+ mice which was in turn higher than D− (Table 1). The serum concentration of activated vitamin D, $1,25(OH)_2D_3$, was not significantly different between the D++ and D+ groups. As previously observed[29], $1,25(OH)_2D$ concentrations in the D− mice were lower than in the other groups, though this did not reach statistical significance ($p = 0.087$). There was no difference in serum calcium levels between the three groups.

Vitamin D and DSS-induced colitis. Body weight loss and liquid stools were observed between 2–7 days after commencing the DSS treatment. All DSS-treated mice lost weight compared to pre-DSS measures. The peak percentage weight loss occurred at day 7 (Fig. 2A). At day 6 and 7, the D++ mice lost significantly more weight than D+ mice ($p < 0.001$), and at day 7 and 8, D− mice lost significantly more weight than D+ mice ($p < 0.01$) (Fig. 2A,B).

At the day 6 colonoscopy, control mice in all groups had solid stool, preserved mucosal vascularity, normal colonic translucency with a mean MEICS of 0.6 ± 0.17 (n = 23, range 0–3), consistent with no colitis (Fig. 2C). DSS-treated mice demonstrated endoscopic inflammation with loose stools, loss of intestinal wall translucency and mucosal bleeding with a mean MEICS 4.8 ± 0.2 (n = 102, range 0–10). This was significantly greater than control mice ($p < 0.001$, 95% CI 3.6–4.7).

At day 6, MEICS was significantly greater in D++ (6.3 ± 0.30, n = 33) than in the D+ (4.1 ± 0.30, n = 34, $p < 0.001$) and D− (3.9 ± 0.33, n = 35, $p < 0.001$) groups (Fig. 2C). There was no difference in colitis severity observed in mice from the D+ and D− groups ($p = 0.65$). At day 14, a higher MEICS was observed in the D++ group compared to the D+ group ($p < 0.01$), but this difference resolved by day 21 and 35 as recovery was almost complete (Fig. 2C).

The histological grading of colitis at the distal colon on day 14 was greater in mice from the D++ (8.22 ± 2.53, n = 9) than D+ (1.42 ± 0.57, n = 7, $p < 0.05$) or D− (1.43 ± 0.57, n = 8, $p < 0.05$) groups. At Day 7 there was a trend for higher inflammation in D++ and D− compared to D+ mice though this did not reach statistical significance ($p = 0.25$) (Supplementary Fig. 1A,B). Among all groups, there was positive correlation between MEICS and day 7 weight loss ($r = 0.60$, n = 253, $p < 0.001$), day 7 histological score ($r = 0.51$, n = 86, $p < 0.001$) and day 14 histological score ($r = 0.59$, n = 56, $p < 0.001$) (data not shown).

At day 7, the mean serum albumin was less in all groups with colitis (Fig. 2D) than corresponding controls. Among DSS-treated mice, the mean albumin level was significantly lower among the D++, compared to the D+ ($p < 0.05$) and D− ($p < 0.05$) group, consistent with a worse colitis seen in the D++ group.

Gene expression of TNF-α in colon tissue by RT-PCR at day 7, was increased in all DSS-treated groups compared to controls. This was again greatest in the D++ group compared to D− (6.5 ± 3.1 vs 2.2 ± 0.36 fold, $p < 0.05$) with a trend to be greater than that measured in the D+ mice (4.1 ± 0.11, $p = 0.08$) (Fig. 2D).

Serum cytokines TNF-α, IFN-γ, IL-10, IL-6, IL-12p40 and IL-1β at day 7 were greater among all DSS mice compared to control mice ($p < 0.05$ for all cytokines, data not shown). When comparisons are stratified by vitamin D groups, the DSS group had higher levels than controls though this was not always statistically significant (Supplementary Fig. 2). IL-12p40 was highest among D++ compared to D− groups ($p < 0.01$), but similar changes were not seen with other cytokines (Supplementary Fig. 2).

25(OH)D, $1,25(OH)_2D$ and VDBP concentrations. At day 7, serum 25(OH)D reduced by greater than 60% among DSS mice from the D++ group (Fig. 3A). This difference remained at day 35 ($p = 0.053$, n = 5). At day 7 a similar but smaller reduction in serum 25(OH)D levels was observed in the D+ group (Fig. 3B).

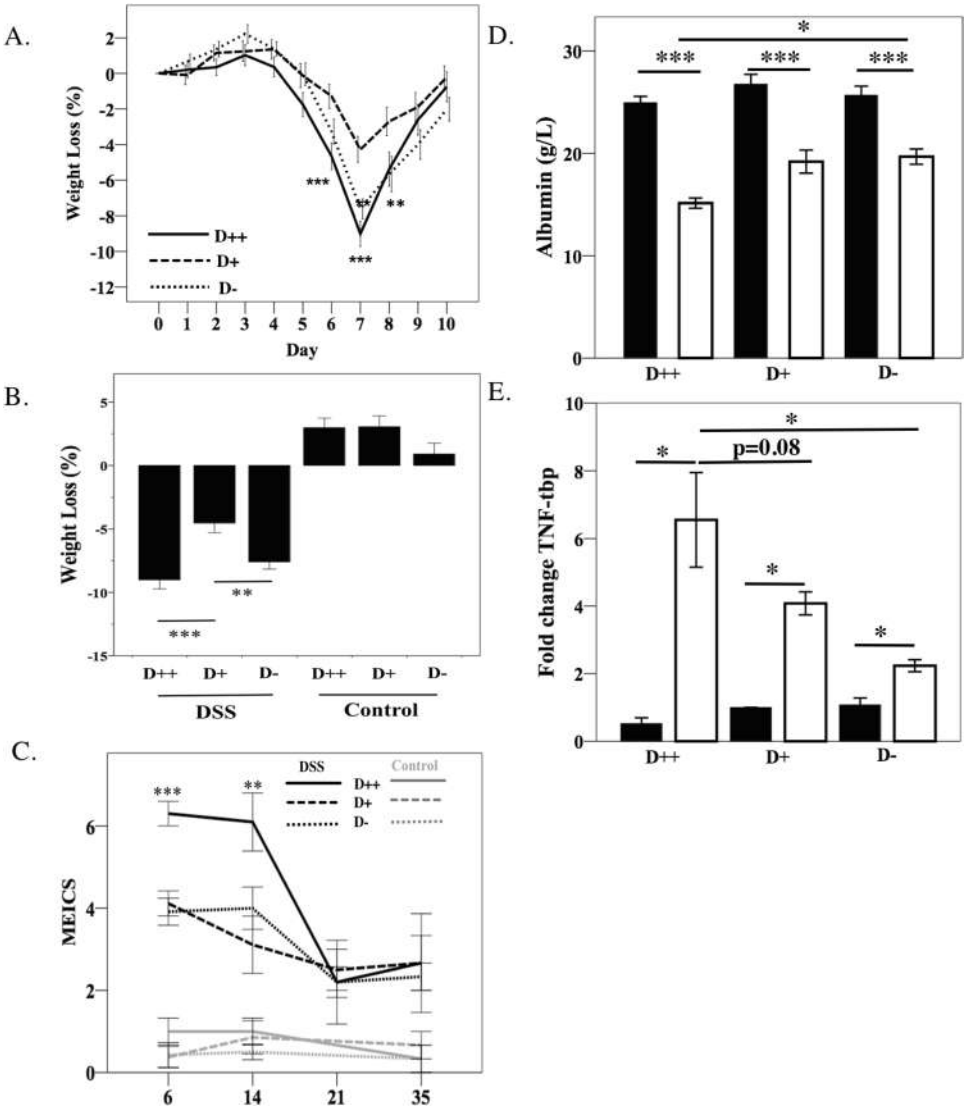

Figure 2. Outcomes of colitis. 6-week old female C57Bl/6 mice were established on diets with high (D++), moderate (D+) or no (D−) vitamin D3, before being treated with DSS for 6 days. Mice were regularly weighed and underwent colonoscopy procedures at regular intervals. (**A**) The percentage weight loss from baseline to day 10 post-DSS treatment. Comparisons are made to group D+ as the reference group. (**B**) Weight loss at day 7. (**C**) Endoscopic severity over time measured by murine endoscopic index of severity (MEICS). Comparisons are made to group D+ as the reference group. n = 35/group for day 6 and 7 assessments, n = 10/group day 14, n = 5/group for days 21 and 35. (**D**) Serum albumin at day 7, n = 5–10/group. (**E**) Colonic TNF-α gene expression after 6 days DSS treatment, fold change using the $2^{-\Delta\Delta T}$ method with TATA-box-binding protein as housekeeping gene, n = 4–5/group. Solid bars for control mice, open bars for DSS mice. Data are shown as mean ± SEM, from two experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Differences were not seen at the later time points. In mice from the D− group, given that 25(OH)D levels were already low at baseline, no reduction in 25(OH)D was detectable (Fig. 3C).

As the large decrease in 25(OH)D may have been due to an increase in its conversion to $1,25(OH)_2D$, changes in the levels of $1,25(OH)_2D$ were investigated. A significant decrease at day 7 in $1,25(OH)_2D$ concentrations (relative to control mice), was detected in both the D++ and D+ groups suggesting that increased conversion was not the cause of 25(OH)D reductions ($p < 0.001$) (Fig. 3D).

As 25(OH)D, and to a lesser extent $1,25(OH)_2D$, are mostly bound to the VDBP, we questioned if the large decrease in both these vitamin D metabolites was due to a loss of VDBP. As described previously (Fig. 2D), serum albumin dropped with colitis. Surprisingly, the VDBP levels, measured by ELISA, increased with colitis in all groups and this was statistically significant in the D++ and D− groups (Fig. 3E). The increase in VDBP with DSS colitis was also seen when VDBP was measured by radial immunodiffusion, though this was significant among the D+ and D− group and there was a trend to significance among the D++ group ($p = 0.076$) (Supplementary Fig. 1C). These data suggest that the induction of colitis increases circulating VDBP levels.

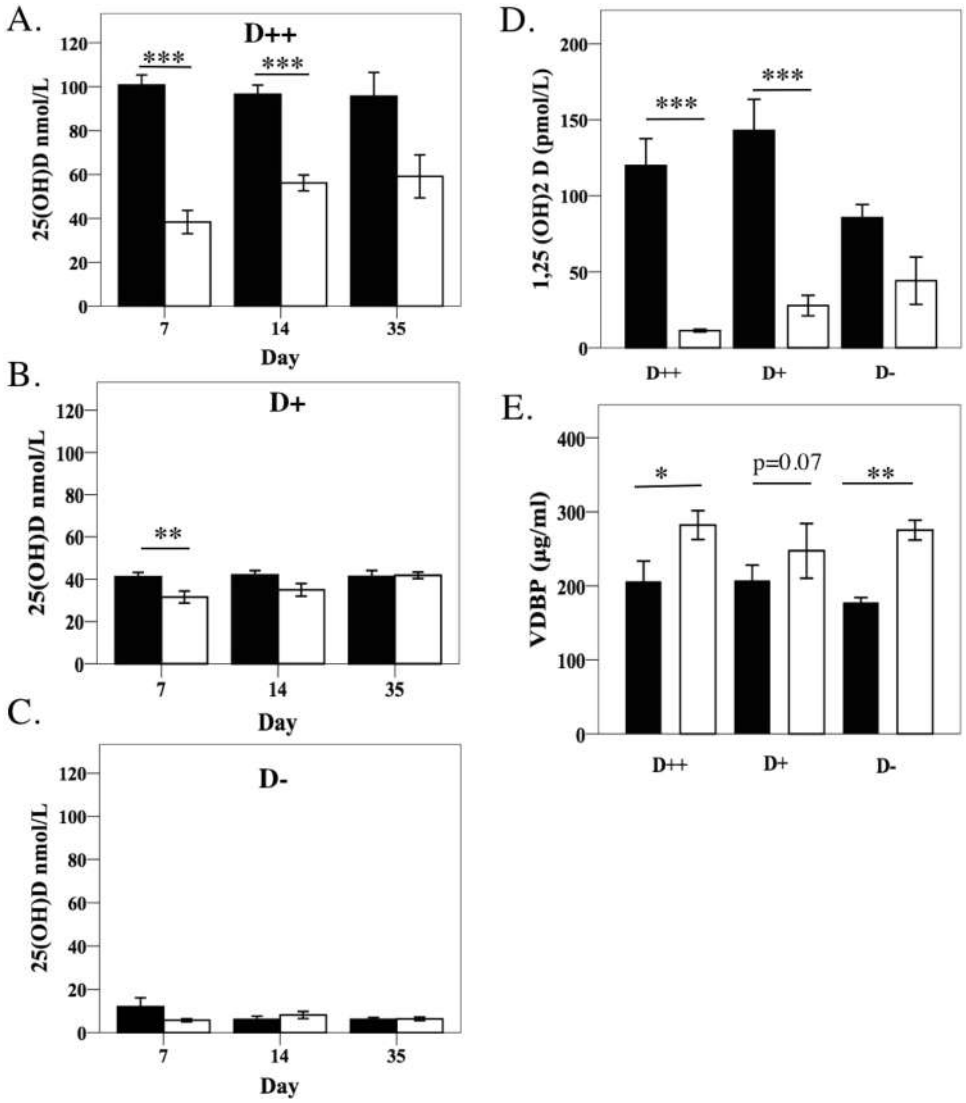

Figure 3. Vitamin D and vitamin D binding protein levels. Serum concentrations of 25(OH)D_3 at day 7, 14 and 35 among DSS-treated mice and controls, (**A**) D++, (**B**) D+, (**C**) D−, $n = 7$–8/group at day 7 and day 14, $n = 5$/group at day 35. (**D**) 1,25(OH)$_2$D and (**E**). Vitamin D binding protein concentrations in serum at day 7, $n = 3$–5/group. Solid bars for control mice, open bars for DSS mice. Values are expressed as mean ± SEM, from at least two experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Kidney *CYP24A1* gene expression in DSS colitis. In an attempt to explain the reduced 25(OH)D and 1,25(OH)$_2$D at day 7 post-DSS treatment, changes in the level of expression of mRNA of enzymes involved in vitamin D metabolism were explored. Neither liver *CYP2R1* nor kidney *CYP27B1* mRNA levels changed significantly with the induction of colitis (Fig. 4A,B). There was 5.5 ± 1.3 fold more *CYP24A1* mRNA in the kidneys of DSS-treated mice on D+ diets compared to their corresponding control group ($p < 0.01$) (Fig. 4C). Similarly, kidney *CYP24A1* mRNA was expressed 4.3 ± 0.6 fold more among DSS-treated mice on D− diets compared to corresponding controls ($p < 0.001$). Kidney *CYP24A1* was up-regulated 4.5 ± 0.9 fold among D++ controls compared to D+ controls ($p < 0.05$) as an appropriate homeostatic mechanism, but there was no further increase with the induction of colitis. Thus, increased kidney metabolism may help to explain the reduced 25(OH)D and 1,25(OH)$_2$D at day 7 in the D+ group, though a yet to be identified mechanism must exist to explain the drop in the D++ group.

UV radiation-induced 25(OH)D and colitis. The reduced circulating levels of 25(OH)D and 1,25(OH)$_2$D observed in mice with DSS-induced colitis could be caused by decreased intestinal absorption of vitamin D. If so, then 25(OH)D derived from skin exposed to UVB radiation should not fall with inflammation. To test this, mice fed vitamin D-deficient diets for 4 weeks were treated with daily UV radiation (1 kJ/m^2) for 4 days followed by biweekly UV (1 kJ/m^2) for the remainder of the experiment (D−UV+). After the 4 days of UV pretreatment, mice were treated with DSS for a further 6 days.

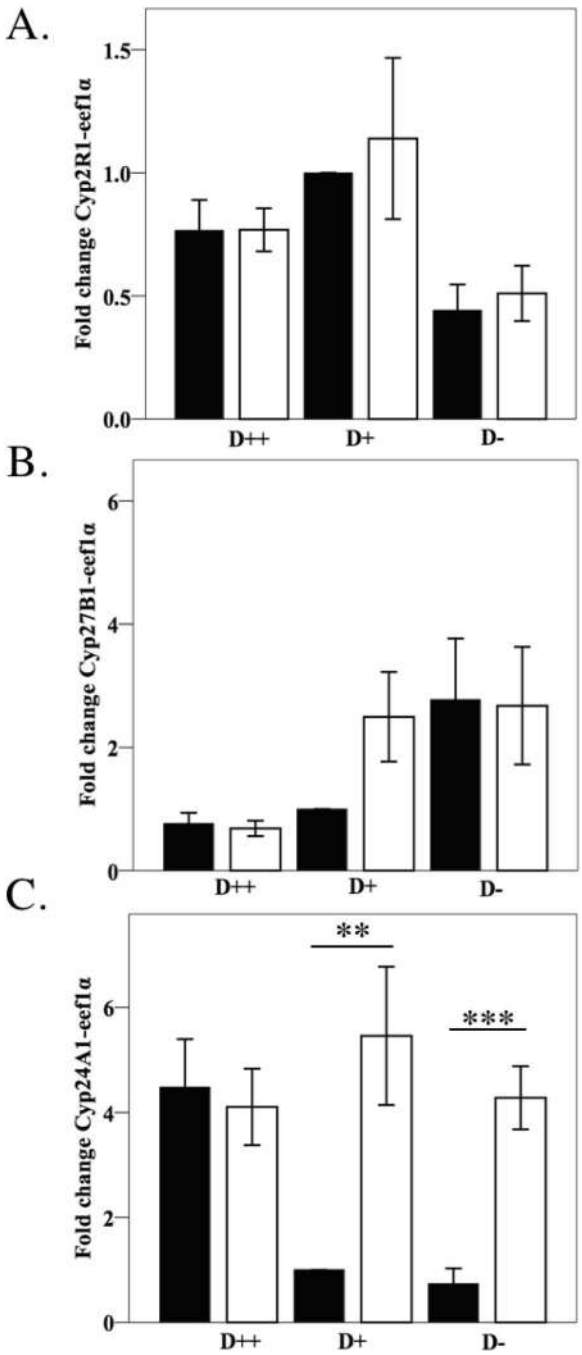

Figure 4. Kidney Cyp24A1 gene expression is upregulated with DSS colitis. Female C57Bl/6 mice were established on three vitamin D diets for 4 weeks before treatment with DSS. On day 7 mice were sacrificed with livers and kidneys harvested to determine (**A**) Liver Cyp2R1, (**B**) Kidney Cyp 27B1, and (**C**). Kidney Cyp24A1 gene expression. mRNA gene expression by qtPCR was calculated using the $2^{-\Delta\Delta CT}$ method with eEF1α as the housekeeping gene, $n = 5$–10/group. Solid bars for control mice, open bars for DSS mice. Values are expressed as mean ± SEM, from at least two experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

After 4 doses of UV irradiation, serum levels of 25(OH)D in mice from the D−UV+ treatment was 58.0 ± 2.49 nmol/L compared to 4.8 ± 0.15 nmol/L among D− mice without UV treatment (D−UV−) (n = 4/group, $p < 0.001$), Fig. 5A. The 25(OH)D concentrations among D−UV+ mice were similar to the D+ without UV treatment (D+UV−).

After 6 days of DSS treatment, there was no significant difference in endoscopic severity of colitis between vitamin D-deficient mice (D−) exposed (UV+) or not exposed (UV−) to UV radiation (MEICS 4.3 ± 0.45 vs 3.9 ± 0.33, n = 35/gp, p = 0.42), nor was there a difference compared to D+UV− mice (MEICS 4.3 ± 0.45 vs 4.12 ± 0.45, n = 35/group, p = 0.64), Supplementary Fig. 1D. By day 7, the 25(OH)D (Fig. 5B) and $1,25(OH)_2D$ concentrations (not shown) were significantly lower among UV-irradiated vitamin D-deficient, DSS-treated mice

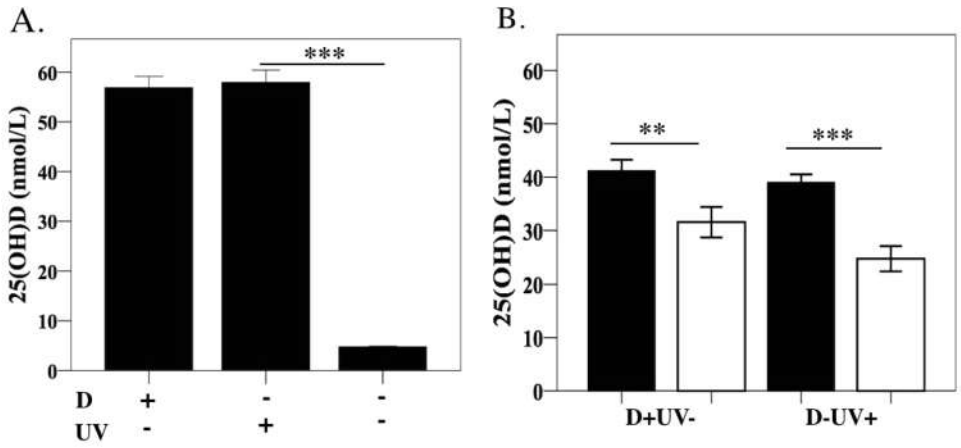

Figure 5. Serum levels of 25(OH)D_3 in mice treated with and without UV radiation. The shaved dorsal surfaces of female C57Bl/6 mice on vitamin D deficient diets (D−) were irradiated with 1 kJ/m^2 ultraviolet radiation daily for 4 days before undergoing DSS treatment. (**A**) 25(OH)D_3 levels 24 h after four doses of UVB irradiation. (**B**) 25(OH)D levels after 6 days of DSS treatment in a separate group of mice. Solid bars control mice, open bars DSS mice. Values are expressed as mean ± SEM, from two experiments. $n = 4$/group day 0 and $n = 7$–8/group day 7. $^{**}P < 0.01$, $^{***}P < 0.001$.

as compared to corresponding controls. Thus, these data suggest that the drop in circulating 25(OH)D in mice where vitamin D is acquired only through irradiation of the skin, cannot be due to malabsorption.

Effect of vitamin D on faecal microbiota. Microbiota analysis was performed on 42 faecal samples, comprised of 5 samples from each of the vitamin D groups among controls and DSS mice at day 7 and 4 samples per group among controls at day 35. One control mouse was considered an outlier and excluded from further analyses (Supplementary Fig. 3A).

Effect of Vitamin D on faecal microbiota from control, non-DSS mice. There were no differences seen in α-diversity as measured by species richness, evenness or Shannon's diversity in day 7 samples collected from plain water-treated control mice, and the result was reproducible for day 35 samples (Fig. 6A, Supplementary Fig. 3B,C). Similarly, no significant differences in β-diversity were noted between day 7 and day 35 samples from control mice (data not shown). Further analysis was carried out only on day 7 samples.

Comparisons between vitamin D groups found increasing vitamin D doses did not affect species richness as measured by chao1 among control mice (Fig. 6A), but it did reduce Shannon's diversity between D++ controls compared to D− controls (Fig. 6B). No significant difference was noted between D− and D+ control groups; however, PERMANOVA analysis, a measure of global β-diversity, confirmed significant differences between D− and D++ controls ($p = 0.012$, $t = 1.68$, Permutations = 126) and D+ and D++ controls ($P = 0.01$, $t = 1.76$, Permutations = 126).

To examine the effect of vitamin D grouping on individual taxa, linear discriminant analysis (LEfSe) was performed. Forty microbial taxa at all taxonomic levels were found to be significantly different between the three vitamin D groups, of which 37 showed strong associations (linear discriminant analysis score > 3) (Supplementary Table Ia).

To determine the effect of measured serum 25(OH)D_3 levels on individual taxa, correlation analysis was measured using distance based linear modelling (DistLM) analysis between serum 25(OH)D3 levels (Euclidean distance resemblance matrix) and relative abundances of microbial taxa. This identified a significant correlation with four taxa (>0.1% average relative abundance) which included: *Paulidibacter*|OTU46 (Pseudo-F: 4.6, P = 0.04, Df: 26); Bacteroidales|S24-7|OTU58 (Pseudo-F: 6.7, P = 0.02, Df: 26); *Sutterella*|OTU174 (Pseudo-F: 5.1, P = 0.038, Df: 26); and *Coprococcus*|OTU118 (Pseudo-F: 4.8, P = 0.02, Df: 26). To further inform our 25(OH) D3 correlation analyses and establish the response of these four taxa to vitamin D intake, the relative abundance of these four taxa across each vitamin D diet group were plotted (Fig. 6C). The relative abundance of *Paulidibacter*|OTU46, Bacteroidales|S24-7|OTU58, and *Sutterella*|OTU174 increased with vitamin D intake, while *Coprococcus*|OTU118 decreased.

Effect of DSS colitis on faecal microbiota. Treatment with DSS reduced the number of operational taxonomic units (OTUs) within samples analysed at day 7 from the D− ($P = 0.09$) and D+ ($P = 0.04$) but not D++ group relative to D− controls (not shown), but there was no significant difference between the DSS groups (Fig. 7A). DSS did not affect other measures of α-diversity, in particular species evenness and Shannon's diversity (Fig. 7B, Supplementary Fig. 3D). However, DSS had a significant impact on overall microbial composition (β-diversity) at day 7 (Fig. 7C). Further, 111 microbial taxa at all taxonomic levels were found to be differentially abundant between controls and DSS mice using LEfSe analysis (Supplementary Table Ib). There was enrichment with DSS of disease-associated Proteobacteria and a reduction in taxa belonging to Firmicutes.

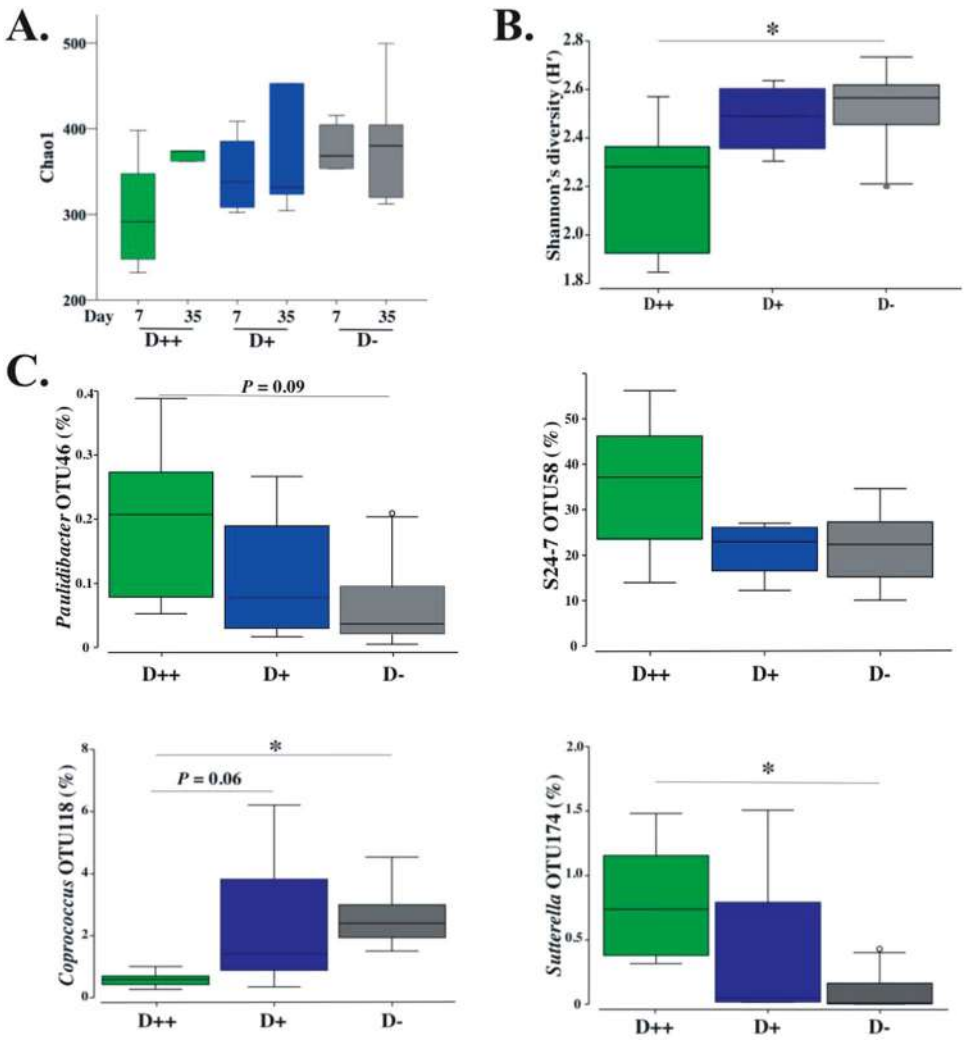

Figure 6. Microbial composition of faecal samples from control mice. Faecal pellets were collected control mice from each of the vitamin D dietary groups (D++, D+ and D−). (**A**) Comparison of day 7 and day 35 species richness for samples from control mice (measured by chao1). (**B**) Day 7 Shannon's diversity (H′). (**C**) Day 7, Relative abundance (%) of OTUs that correlated with serum vitamin D levels among controls using distance based linear modeling (DistLM) analysis. P-values calculated by PERMANOVA on Euclidean distance resemblance matrices generated from square root transformed relative abundance from each OTUs. n = 5/group for day 7 analyses, n = 4/group at day 35. $^{*}P < 0.05$.

Notably, *Sutterella* OTU174 increased in relative abundance in DSS mice as compared to controls (LDA score: 4.27, $p < 0.0001$) (Fig. 7D, Supplementary Table Ia). This is relevant given similar rise of *Sutterella* in non-DSS treated controls from the D++ group, Fig. 6C, suggesting a shift in faecal microbiome of D++ controls to that of DSS mice. Further, when examining the overall microbiome composition there is a clear shift for D++ mice towards that of DSS mice (Fig. 7E).

Discussion

This is the first study to examine the effect of high dose vitamin D supplementation in an IBD model. High dose vitamin D supplementation led to more severe DSS colitis as measured by blinded endoscopic and histologic assessment, weight loss and fall in serum albumin.

The development of colitis was associated with an acute drop in serum 25(OH)D and $1,25(OH)_2D$ levels by a mean of 62% among the high vitamin D group and 23% among the vitamin D sufficient group. Other groups have demonstrated a drop in $1,25(OH)_2D_3$ with DSS colitis but not in $25(OH)D_3$[5]. This is most likely because the change in $25(OH)D_3$ was most apparent in the high vitamin D group where the baseline $25(OH)D_3$ level was significantly greater, this diet has not been examined in the prior studies of DSS colitis. The drop in $25(OH)D_3$ was associated with a greater than five-fold increase in gene expression of kidney CYP24A1 among D+ mice, an enzyme responsible for the catabolism of both 25 and $1,25(OH)_2D$. This suggests that vitamin D metabolites drops in response to inflammation. We did not observe this increase in kidney CYP24A1 expression in the D++ group which had the greatest drop in 25(OH)D, likely due to increased expression at baseline as a

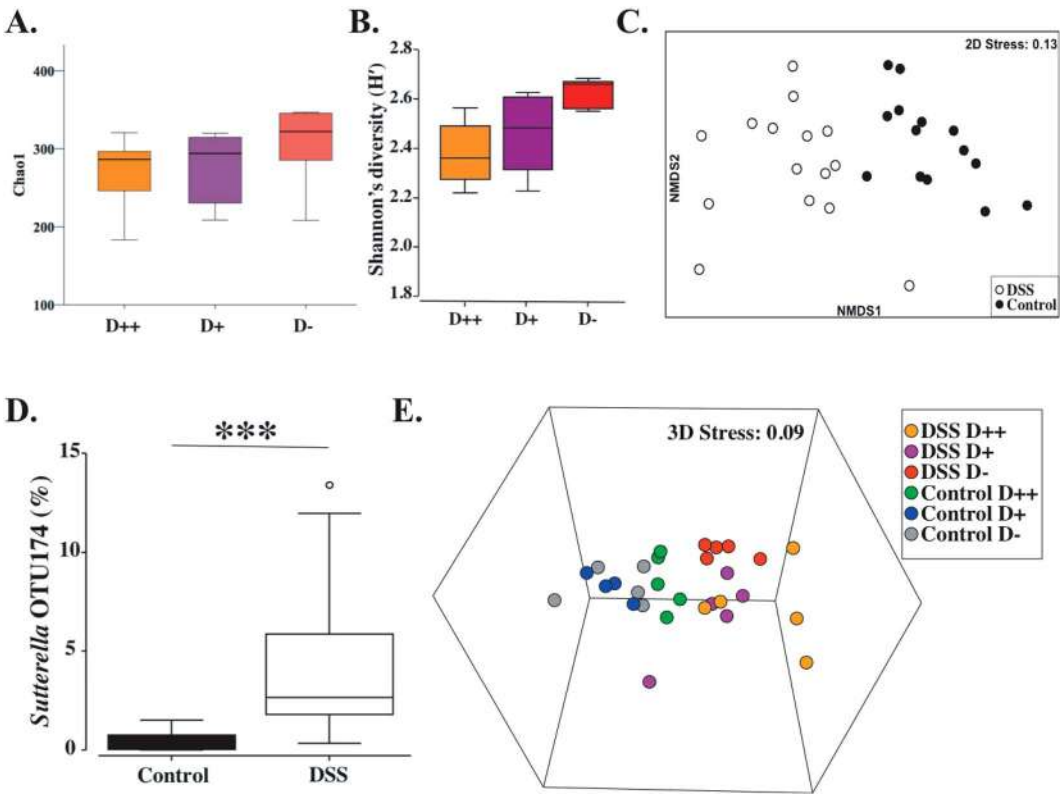

Figure 7. Microbial composition of faecal samples at Day 7 from DSS-treated mice. (**A**) Species richness (measured by chao1), n = 5/group. (**B**) Shannon's diversity (H′), n = 5/group. (**C**) Non-metric multidimensional scaling (NMDS) plot of the Bray-Curtis resemblance matrix following square-root transformation of relative abundance data showing the impact of DSS on the overall microbial composition, confirmed by pair-wise PERMANOVA (Control vs DSS): t = 3.34, p = 0.01, permutations = 999, n = 15/group (with and without nesting for vitamin D subgrouping). (**D**) Relative abundance (%) of *Sutterella* OTU174 between control and DSS mice (LDA 4.27, P < 0.0001), p-value derived from Linear discriminant analysis effect size (LEfSe), n = 15/group. (**E**) NMDS plot of the Bray-Curtis resemblance matrix following square-root transformation of relative abundance data demonstrating a significant shift of the control D++ microbial composition (green) towards that of the DSS group, n = 5/group. $^{***}P < 0.001$.

counter-regulatory mechanism to the higher 25(OH)D levels. An alternative mechanism must exist to explain the fall in vitamin D levels in this group. It is well established that $1,25(OH)_2D$ can be produced by colonic epithelial cells[30] and monocytes[31], and this is also regulated by local CYP27B1 and CYP24A1. In fact, a study by Liu *et al.*, demonstrated increased Cyp24A1 expression from the proximal, but not distal, colon with DSS colitis[15]. Thus, it is possible that with the colon inflammation, colonic epithelial cell and monocyte CYP24A1 expression is upregulated leading to the fall in serum 25(OH)D levels. The recovery of $25(OH)D_3$ was slow and did not return to baseline by day 35 in the D++ group despite resolution of colitis. This is likely due to this group having the greatest drop in $25(OH)D_3$, thus the time taken for $25(OH)D_3$ to return to baseline would be expected to take the longest even after the colitis has resolved.

In critical illness, reduced 25(OH)D and $1,25(OH)_2D$ levels have been observed, however this has been in association with reduced concentrations of the VDBP[32]. In our model we observed an increase in VDBP levels. While this could simply be a discrepancy between mouse and human vitamin D metabolism, it may also be unique to intestinal inflammation. In our previous work examining over 300 patients with CD in remission, high VDBP concentrations were independently associated with a 20% increased risk of subsequent clinical relapse of disease[33]. Thus, subclinical inflammation may lead to increased VDBP levels and subsequent disease flare. VDBP has an important role in actin scavenging such that in the case of acute tissue damage, cellular actin will bind plasma VDBP for subsequent rapid clearance of the complex[34]. It is possible that in response to intestinal tissue damage, VDBP was upregulated and thus is a biomarker of subclinical disease activity.

Contrary to previous animal studies, vitamin D deficiency did not predispose mice to worse colitis, though the measurement of colitis severity by endoscopy in this study differed to the clinical measurements reported in other studies. We did however observe transient weight loss and a trend for increased histological inflammation of the colon in the vitamin D-deficient group consistent with other reports. Previously reported rodents with increased susceptibility to DSS colitis have included vitamin D receptor- and Cyp27B1- KO mice. However, they had absolute vitamin D deficiency and numerous complicating skeletal abnormalities such that they may not be a good representation of the human condition[4,5]. Animal models of dietary-induced vitamin D deficiency better

mimic clinical deficiency observed in humans; however, mixed results have been reported in these vitamin D deficient mice[3,35].

Serum cytokines, TNF-α, IFN-γ, IL-10, IL-6, IL-12p40 and IL-1β, increased with the induction of DSS colitis, and is consistent with previous work from our group[16]. No differences were seen in serum cytokine levels between the different vitamin D groups, with the exception of IL-12p40 which was higher among colitic mice from the D++ group compared to the vitamin D-deficient group. It is possible that other cytokine levels reached a plateau level in all DSS groups, making it impossible to detect differences between the different vitamin D dietary groups. We have previously reviewed the effect of vitamin D supplementation *in-vivo* and *in-vitro* on innate and adaptive immune cells responses, particularly with respect to gastrointestinal inflammation[36]. In summary, vitamin D supplementation can have favourable effects in preserving intestinal epithelial barrier function, production of anti-microbial peptides, attenuating capacity for antigen presentation, reducing dendritic cell maturation and T-cell proliferation. The effect of different doses of vitamin D on these responses was not examined in this study, but it would be important in future work to identify those responses that reversed with high vitamin D supplementation.

The mechanism by which high dose vitamin D supplementation increased colitis susceptibility remains unclear, but we did observe that vitamin D had a potent effect on the microbial composition of faeces from plain-water treated, control mice. Interestingly, the microbial composition of faeces from D++ control mice approached that of DSS mice, suggesting a shift to a more pro-inflammatory microbiome even before starting DSS treatment. Some of the changes seen across vitamin D categories were confirmed by correlation with serum $25(OH)D_3$ levels. Most notably there was a consistent increase in *Sutterella* spp. This same organism was also enriched in mice with colitis. *Sutterella* spp. have been reported to be enriched in human subjects with inflammatory bowel diseases[37] and a recent study of faecal microbiota transplantation in ulcerative colitis found recipients with increases in *Sutterella* spp. were consistently less likely to respond to the treatment[38]. Similarly, the group Bacteroidales S24-7 showed a strong positive correlation with $25(OH)D_3$ levels but was depleted in the DSS mice. It is possible that the significant bloom in this taxon in D++ mice impacts microbiome stability and, in turn, susceptibility to the effects of DSS, thus potentially predisposing these mice to more severe colitis.

It remains unclear if the observed changes in faecal microbiota are a direct response to changes in vitamin D, or mediated indirectly through changes in mucosal immune responses. While our findings demonstrate an association between vitamin D dosing, faecal microbiota changes and susceptibility to colitis, we acknowledge that a specifically designed study would be needed to draw definitive conclusions about cause and effect.

In conclusion, high dose vitamin D worsens the severity of murine colitis induced by DSS, and is associated with distinct changes in microbial composition that may be a direct dietary effect or as a result of dysregulation of the gut mucosal immune response. Future work needs to further explore the effects of high levels of vitamin D on gut mucosal immunity to better understand if high as well as low vitamin D levels lead to a dysregulation.

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Author Contributions

P.H.H. and S.G. conceived and conducted the experiments, data analysis and drafting of manuscript. I.C.L. conceived experiment. R.B., F.L., T.M., D.M., A.B., B.K., S.Go. and C.F. conducted experimental work. N.O.K. and L.G. performed data analysis. All authors reviewed the manuscript.

Additional Information

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SUPPLEMENTARY FILE

High Dose Vitamin D supplementation alters faecal microbiome and predisposes mice to more severe colitis

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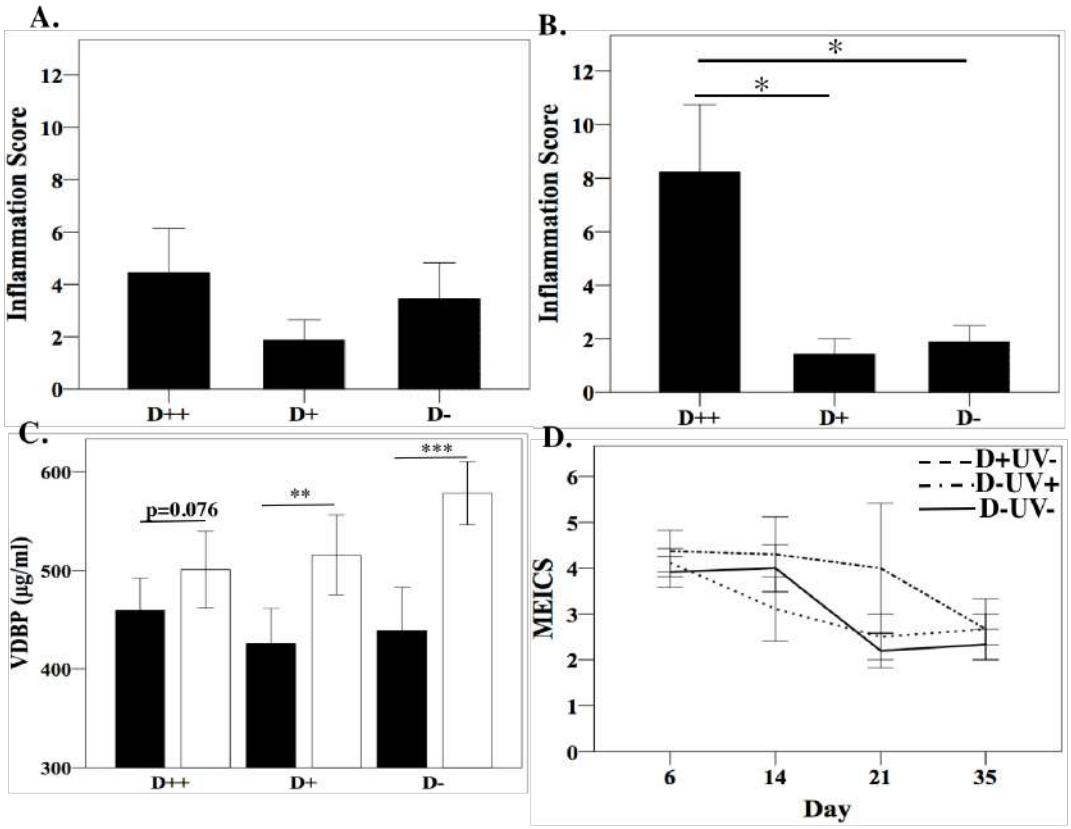

Figure 1 The extent of histological inflammation as determined by grading the distal colons at **A.** Day 7 and **B.** Day 14, post-initial DSS treatment. *n*=7-9/grp. **C.** Vitamin D binding protein levels measured on day 7 by the radial immunodiffusion method**.** Solid bars for control mice, open bars for DSS mice. N=6-8/group **D.** Endoscopic severity of colitis over time among DSS-treated mice ± UV treatment. The murine endoscopic index of severity (MEICS) was measured after 6, 14, 21 and 35 days, n=35/group. Data are shown as mean ± SEM from two experiments $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

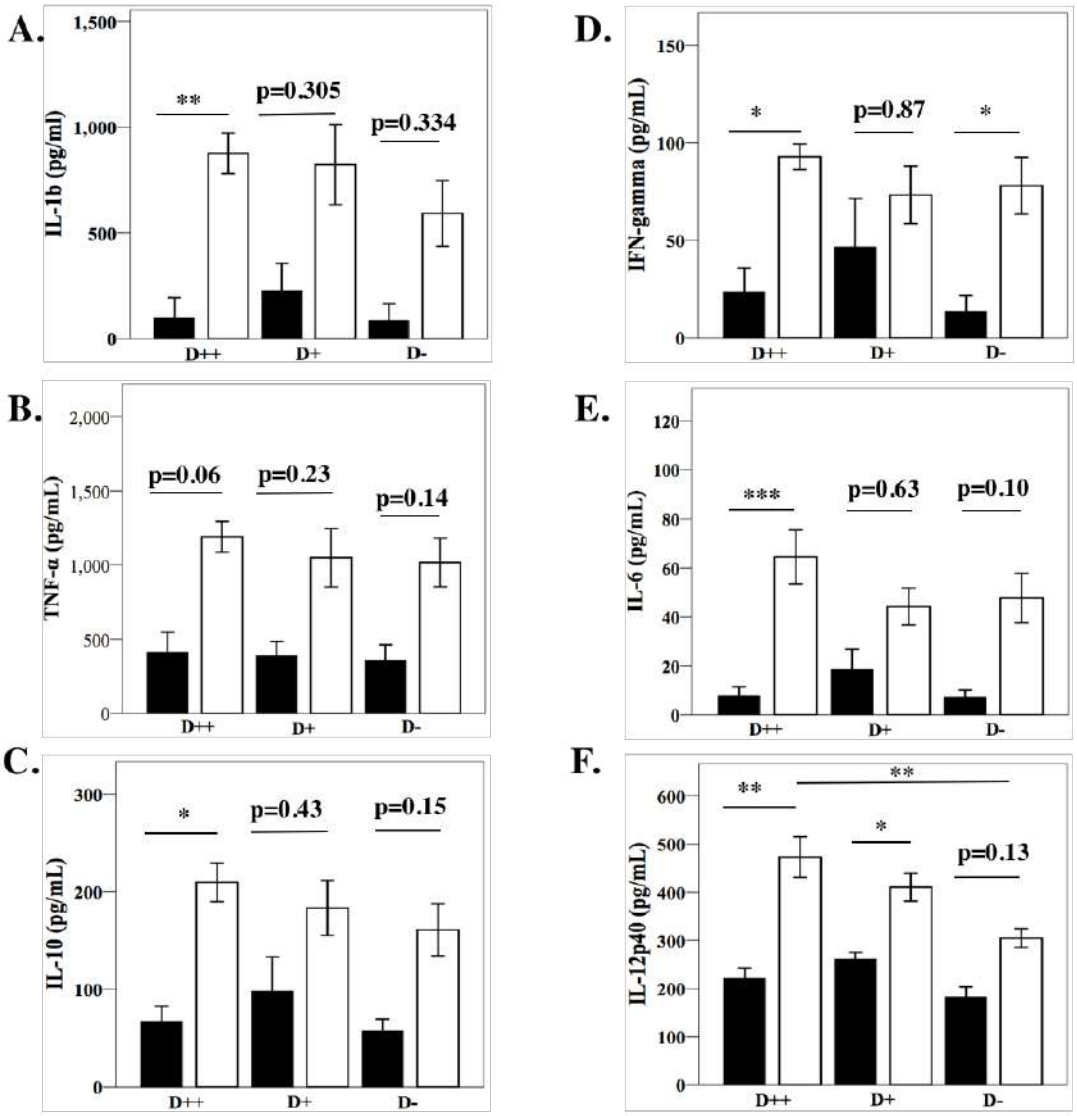

Figure 2 Serum cytokine levels at day 7. A. IL-1b, B. TNF-α, C. IL-10, D. IFN-γ, E. IL-6, F IL-12p40. Solid bars for control mice, open bars for DSS mice. Values are expressed as mean ± SEM, from two experiments. *n*=3-4/group for controls, *n*=7-9/group for DSS-treated mice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$

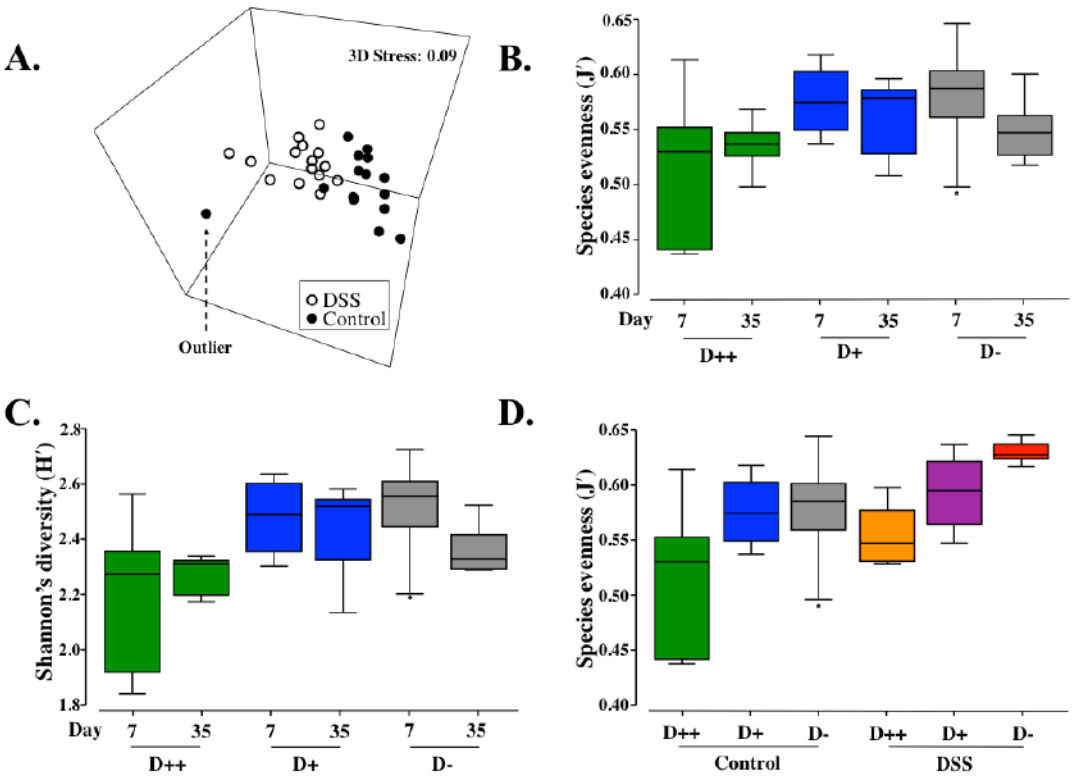

Figure 3. Microbial composition of faecal samples. Faecal pellets were collected from both DSS and control mice from each of the vitamin D dietary groups (D++, D+ and D-). **A.** Non-metric multidimensional scaling (NMDS) plot of the Bray-Curtis resemblance matrix following square-root transformation of relative abundance data showing the existence of an outlier mouse which was removed from all further analyses. **B.** Comparison of day 7 and day 35 species evenness. **C.** Shannon's diversity (H′) between day 7 and day 35 mice. n=5/group. **D.** Species evenness between control and DSS groups. n=5/group.

Microbial taxa	Group	LDA Score	P-value
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified.Actinomycetales_unclassified.OTU18	D+	3.96	0.047
Bacteria.Actinobacteria.Coriobacteriia	D-	3.33	0.016
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales	D-	3.33	0.016
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae	D-	3.33	0.016
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Coriobacteriaceae_unclassified	D-	3.30	0.011
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Coriobacteriaceae_unclassified.OTU30	D-	3.30	0.011
Bacteria.Bacteroidetes	D++	5.16	0.011
Bacteria.Bacteroidetes.Bacteroidia	D++	5.17	0.011
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales	D++	5.16	0.011
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	D++	3.96	0.020
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.PW3	D++	4.10	0.008
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.PW3.OTU57	D++	4.10	0.008
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.Rikenellaceae_unclassified	D++	3.97	0.020
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.Rikenellaceae_unclassified.OTU55	D++	3.95	0.020
Bacteria.Bacteroidetes.Flavobacteriia	D++	4.16	0.014
Bacteria.Bacteroidetes.Flavobacteriia.Flavobacteriales	D++	4.17	0.014
Bacteria.Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriales_unclassified	D++	4.22	0.014
Bacteria.Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriales_unclassified.Flavobacteriales_unclassified	D++	4.20	0.014
Bacteria.Bacteroidetes.Flavobacteriia.Flavobacteriales.Flavobacteriales_unclassified.Flavobacteriales_unclassified.OTU64	D++	4.21	0.020
Bacteria.Firmicutes	D+	5.21	0.009
Bacteria.Firmicutes.Clostridia	D+	5.14	0.010
Bacteria.Firmicutes.Clostridia.Clostridiales	D+	5.13	0.010
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified	D+	4.95	0.020
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified.Clostridiales_unclassified	D+	4.94	0.020
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified.Clostridiales_unclassified.OTU105	D+	4.88	0.021
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae	D+	2.92	0.031
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae.Dehalobacterium	D+	2.94	0.031
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae.Dehalobacterium.OTU114	D+	2.93	0.031
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	D-	4.47	0.010
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Coprococcus	D-	4.01	0.024
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Coprococcus.OTU118	D-	4.01	0.024
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified	D-	4.29	0.023
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified.OTU115	D-	4.19	0.024
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified.OTU116	D-	3.61	0.034
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	D+	4.48	0.030
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Oscillospira	D+	4.37	0.024
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Oscillospira.OTU127	D+	4.41	0.024
Bacteria.Proteobacteria.Alphaproteobacteria	D++	3.49	0.048
Bacteria.TM7.TM7_3.TM7_3_unclassified.TM7_3_unclassified.TM7_3_unclassified.OTU230	D+	3.89	0.044
Bacteria.TM7.TM7_3.TM7_3_unclassified.TM7_3_unclassified.TM7_3_unclassified.OTU231	D+	4.09	0.047

Table Ia LEfSe analyses comparing control mice from different vitamin D groups.
Threshold used was linear discriminant analysis (LDA) score >2 and P<0.05. The results provide a list of bacterial taxa at all taxonomic levels that are differentially abundant between the three vitamin D groups in control mice.

Microbial taxa	Group	LDA Score	P-value
Bacteria.Actinobacteria.Actinobacteria_unclassified	DSS	2.65	0.003
Bacteria.Actinobacteria.Actinobacteria_unclassified.Actinobacteria_unclassified	DSS	2.65	0.003
Bacteria.Actinobacteria.Actinobacteria_unclassified.Actinobacteria_unclassified.Actinobacteria_unclassified	DSS	2.65	0.003
Bacteria.Actinobacteria.Actinobacteria_unclassified.Actinobacteria_unclassified.Actinobacteria_unclassified.Actinobacteria_unclassified	DSS	2.65	0.003
Bacteria.Actinobacteria.Actinobacteria_unclassified.Actinobacteria_unclassified.Actinobacteria_unclassified.Actinobacteria_unclassified.OTU14	DSS	2.65	0.003
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified	Control	3.27	0.011
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified.Actinomycetales_unclassified	Control	3.27	0.011
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified.Actinomycetales_unclassified.OTU17	Control	3.22	0.003
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Adlercreutzia	Control	2.61	0.021
Bacteria.Actinobacteria.Coriobacteriia.Coriobacteriales.Coriobacteriaceae.Adlercreutzia.OTU31	Control	2.61	0.021
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales._Paraprevotellaceae_	Control	3.36	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales._Paraprevotellaceae_.YRC22	Control	3.36	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales._Paraprevotellaceae_.YRC22.OTU62	Control	3.36	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae	DSS	4.78	0.002
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidaceae.Bacteroides	DSS	4.78	0.002
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Bacteroidales_unclassified.Bacteroidales_unclassified.OTU35	DSS	4.12	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae	DSS	4.65	0.002
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Parabacteroides	DSS	4.61	0.003
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Parabacteroides.OTU48	DSS	4.00	0.002
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Parabacteroides.OTU49	DSS	4.49	0.013
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Porphyromonadaceae_unclassified	DSS	2.99	0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Porphyromonadaceae.Porphyromonadaceae_unclassified.OTU43	DSS	2.99	0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae	Control	3.78	0.023
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.Rikenellaceae_unclassified	Control	3.78	0.023
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.Rikenellaceae_unclassified.OTU55	Control	3.79	0.023
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7	Control	4.94	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7.S24_7_unclassified	Control	4.94	<0.001
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7.S24_7_unclassified.OTU58	Control	4.94	<0.001
Bacteria.Deferribacteres	DSS	3.24	0.001
Bacteria.Deferribacteres.Deferribacteres	DSS	3.24	0.001
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales	DSS	3.24	0.001
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae	DSS	3.24	0.001
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae.Mucispirillum	DSS	3.24	0.001
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae.Mucispirillum.OTU81	DSS	3.24	0.001
Bacteria.Firmicutes	Control	4.96	0.001
Bacteria.Firmicutes.Bacilli	Control	4.10	<0.001
Bacteria.Firmicutes.Bacilli.Bacillales	Control	3.01	0.012
Bacteria.Firmicutes.Bacilli.Lactobacillales	Control	4.11	<0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Enterococcaceae	Control	2.75	0.015
Bacteria.Firmicutes.Bacilli.Lactobacillales.Enterococcaceae.Enterococcus	Control	2.75	0.015
Bacteria.Firmicutes.Bacilli.Lactobacillales.Enterococcaceae.Enterococcus.OTU95	Control	2.75	0.015
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae	Control	4.08	0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.Lactobacillus	Control	4.08	0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.Lactobacillus.OTU97	Control	4.07	<0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae	Control	2.56	0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae.Streptococcus	Control	2.56	0.001
Bacteria.Firmicutes.Bacilli.Lactobacillales.Streptococcaceae.Streptococcus.OTU100	Control	2.56	0.001
Bacteria.Firmicutes.Clostridia	Control	4.95	0.001
Bacteria.Firmicutes.Clostridia.Clostridiales	Control	4.95	0.001

Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiaceae	DSS	3.38	0.016
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified	Control	4.75	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified.Clostridiales_unclassified	Control	4.75	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified.Clostridiales_unclassified.OTU105	Control	4.75	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae	Control	2.58	0.009
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae.Dehalobacterium	Control	2.58	0.009
Bacteria.Firmicutes.Clostridia.Clostridiales.Dehalobacteriaceae.Dehalobacterium.OTU114	Control	2.58	0.009
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae	Control	4.18	0.008
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Butyrivibrio	Control	2.74	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Butyrivibrio.OTU117	Control	2.75	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified	Control	4.13	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified.OTU115	Control	3.98	0.002
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified.OTU116	Control	3.60	<0.001
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae	Control	4.23	0.004
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Oscillospira	Control	4.16	0.002
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Oscillospira.OTU127	Control	4.16	0.002
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcaceae_unclassified.OTU125	Control	2.79	0.005
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcus	Control	3.06	0.011
Bacteria.Firmicutes.Clostridia.Clostridiales.Ruminococcaceae.Ruminococcus.OTU129	Control	3.06	0.011
Bacteria.Firmicutes.Erysipelotrichi	DSS	3.99	0.008
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales	DSS	3.99	0.008
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae	DSS	3.99	0.008
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae_unclassified	DSS	3.96	0.001
Bacteria.Firmicutes.Erysipelotrichi.Erysipelotrichales.Erysipelotrichaceae.Erysipelotrichaceae_unclassified.OTU147	DSS	3.98	<0.001
Bacteria.Proteobacteria	DSS	4.67	<0.001
Bacteria.Proteobacteria.Alphaproteobacteria.Alphaproteobacteria_unclassified	Control	2.62	0.002
Bacteria.Proteobacteria.Alphaproteobacteria.Alphaproteobacteria_unclassified.Alphaproteobacteria_unclassified	Control	2.62	0.002
Bacteria.Proteobacteria.Alphaproteobacteria.Alphaproteobacteria_unclassified.Alphaproteobacteria_unclassified.Alphaproteobacteria_unclassified	Control	2.62	0.002
Bacteria.Proteobacteria.Alphaproteobacteria.Alphaproteobacteria_unclassified.Alphaproteobacteria_unclassified.Alphaproteobacteria_unclassified.OTU159	Control	2.62	0.002
Bacteria.Proteobacteria.Betaproteobacteria	DSS	4.22	<0.001
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales	DSS	4.22	<0.001
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae	DSS	4.22	<0.001
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae.Sutterella	DSS	4.22	<0.001
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Alcaligenaceae.Sutterella.OTU174	DSS	4.22	<0.001
Bacteria.Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrionaceae_unclassified	Control	3.02	<0.001
Bacteria.Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrionaceae_unclassified.OTU186	Control	3.02	<0.001
Bacteria.Proteobacteria.Epsilonproteobacteria	DSS	3.11	<0.001
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales	DSS	3.11	<0.001
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae	DSS	3.11	<0.001
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae.Flexispira	DSS	3.10	<0.001
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae.Flexispira.OTU194	DSS	3.10	<0.001
Bacteria.Proteobacteria.Gammaproteobacteria	DSS	4.39	0.001
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales	DSS	4.39	0.001
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	DSS	4.39	0.001
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Enterobacteriaceae_unclassified	DSS	4.39	0.001

Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Enterobacteriaceae_unclassified.OTU 203	DSS	4.39	0.001
Bacteria.Proteobacteria.Gammaproteobacteria.Xanthomonadales	Control	2.94	<0.001
Bacteria.Proteobacteria.Gammaproteobacteria.Xanthomonadales.Sinobacteraceae	Control	2.86	0.010
Bacteria.Proteobacteria.Gammaproteobacteria.Xanthomonadales.Sinobacteraceae.Sinobacteraceae_unclassified	Control	2.92	0.010
Bacteria.Proteobacteria.Gammaproteobacteria.Xanthomonadales.Sinobacteraceae.Sinobacteraceae_unclassified.OTU219	Control	2.89	0.010
Bacteria.Tenericutes	Control	2.69	<0.001
Bacteria.Tenericutes.Mollicutes	Control	2.69	<0.001
Bacteria.Tenericutes.Mollicutes.RF39	Control	2.69	<0.001
Bacteria.Tenericutes.Mollicutes.RF39.RF39_unclassified	Control	2.69	<0.001
Bacteria.Tenericutes.Mollicutes.RF39.RF39_unclassified.RF39_unclassified	Control	2.69	<0.001
Bacteria.Tenericutes.Mollicutes.RF39.RF39_unclassified.RF39_unclassified.OTU234	Control	2.69	<0.001
Bacteria.TM7	Control	3.50	0.007
Bacteria.TM7.TM7_3	Control	3.50	0.006
Bacteria.TM7.TM7_3.CW040	Control	3.50	0.005
Bacteria.TM7.TM7_3.CW040.F16	Control	3.50	0.005
Bacteria.TM7.TM7_3.CW040.F16.F16_unclassified	Control	3.50	0.005
Bacteria.TM7.TM7_3.CW040.F16.F16_unclassified.OTU232	Control	3.50	0.005

Table Ib. LEfSe analyses comparing DSS to control mice.
Dietary vitamin D grouping was nested as a subclass. Threshold used was linear discriminant analysis (LDA) score >2 and P<0.05. The results provide a list of bacterial taxa at all taxonomic levels that are differentially abundant between DSS and control mice.

Chapter 5

Ghaly S et al. **Ultraviolet Irradiation of Skin Alters the Faecal Microbiome Independently of Vitamin D in Mice.** Nutrients. 2018;10(8)

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Article

Ultraviolet Irradiation of Skin Alters the Faecal Microbiome Independently of Vitamin D in Mice

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Abstract: Reduced sunlight exposure has been associated with an increased incidence of Crohn's disease and ulcerative colitis. The effect of ultraviolet radiation (UVR) on the faecal microbiome and susceptibility to colitis has not been explored. C57Bl/6 female mice were fed three different vitamin D-containing diets for 24 days before half of the mice in each group were UV-irradiated (1 kJ/m^2) for each of four days, followed by twice-weekly irradiation of shaved dorsal skin for 35 days. Faecal DNA was extracted and high-throughput sequencing of the 16S RNA gene performed. UV irradiation of skin was associated with a significant change in the beta-diversity of faeces compared to nonirradiated mice, independently of vitamin D. Specifically, members of phylum Firmicutes, including *Coprococcus*, were enriched, whereas members of phylum Bacteroidetes, such as Bacteroidales, were depleted. Expression of colonic *CYP27B1* increased by four-fold and *IL1β* decreased by five-fold, suggesting a UVR-induced anti-inflammatory effect. UV-irradiated mice, however, were not protected against colitis induced by dextran sodium sulfate (DSS), although distinct faecal microbiome differences were documented post-DSS between UV-irradiated and nonirradiated mice. Thus, skin exposure to UVR alters the faecal microbiome, and further investigations to explore the implications of this in health and disease are warranted.

Keywords: ultraviolet radiation; microbiome; vitamin D; inflammatory bowel disease

1. Introduction

The health of the gastrointestinal tract is dependent on the bidirectional interaction between gut microbial antigens and the intestinal immune system to maintain homeostasis or "physiological inflammation". In inflammatory bowel disease (IBD), Crohn's disease (CD), and ulcerative colitis (UC), there is a dysregulated immune response against luminal antigens leading to uncontrolled inflammation. It remains unclear if the primary problem is the dysregulated immune response or change in luminal antigens, or as is most likely the case, a combination of both. Ultraviolet (UV) irradiation of skin has both vitamin D-dependent and -independent effects on systemic immunity, and interestingly, IBD is increased in areas of low sun exposure and higher latitudes [1–5].

Diet-induced vitamin D deficiency is associated with altered faecal microbial composition in C57Bl/6 mice, with an increase in the relative quantities of Bacteroidetes, Firmicutes, Actinobacteria, and Gammaproteobacteria in naïve, noncolitic mice [6]. This group has recently demonstrated reduced global β-diversity in faeces from mice fed diets with high vitamin D content compared to no vitamin D, as well as 40 microbial taxa that were significantly different between the groups [7]. The vitamin D-independent effect of skin exposure to UV radiation (UVR) on the composition of intestinal microbiota remains unexplored and is important in understanding whether sun exposure is equivalent to a vitamin D tablet in its impact in both health and disease.

The vitamin D-independent pathways by which UVR may suppress immunity have yet to be fully elucidated; however, a number of mechanisms have been proposed and reviewed elsewhere [8,9]. Two studies have examined the effect of phototherapy in an animal model of IBD using oral dextran sodium sulfate (DSS), which causes a chemical injury to the gastrointestinal tract that is then repaired by innate immune mechanisms [10]. These studies reported a reduction in disease severity with light therapy, but the findings are limited by the subjective measures of colitis severity, the small numbers of mice used, and most importantly, the lack of definition and consistency of the light sources used.

In this current study, the effect of UVR on the faecal microbiome was explored in the setting of high dietary vitamin D (D++), vitamin D sufficiency (D+), and vitamin D deficiency (D−). Furthermore, the effect of UVR on the severity of DSS colitis was studied.

2. Materials and Methods

2.1. Mice and Diets

Female 6-week-old C57Bl/6 mice were fed semipure diets containing high levels of vitamin D (SF14-069, Specialty Feeds, Perth, Western Australia, 10,000 IU/kg vitamin D_3, 0.5% calcium), moderate levels of vitamin D to maintain vitamin D sufficiency similar to standard chow (SF05-34, Specialty Feeds, 2280 IU/kg vitamin D_3, 1% calcium), or no vitamin D to induce vitamin D deficiency (SF05-033, Specialty Feeds, 0 IU/kg vitamin D_3, 2% calcium). Female mice were used as we have previously shown that acute erythemal or short-term suberythemal UVR does not increase $25(OH)D_3$ levels in male mice [11,12]. All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia with the approval from the Telethon Kids Institute Animal Ethics Committee (AEC #276). Mice were purchased from the Animal Resources Centre, Western Australia.

Mice were housed under perspex-filtered fluorescent lighting, which emitted no detectable UVB radiation as measured using a UV radiometer (UVX Digital Radiometer, Ultraviolet Products Inc., Upland, CA, USA).

2.2. UV Radiation

A bank of six 40 W lamps (Philips TL UV-B, Eindhoven, The Netherlands) emitting broadband UVR, 250–360 nm, with 65% of the output in the UVB range (280–315 nm), was used to irradiate mice and to deliver 1 kJ/m^2 of UVR onto clean-shaven 8 cm^2 dorsal skin. This dose of UVR is approximately 50% of the minimal erythemal dose for C57Bl/6 mice, i.e., 50% of the lowest amount of UVR causing just-perceptible erythema after 24 h. A new sheet of polyvinyl chloride (PVC) plastic film (0.22 mm) was taped to the top of each perspex cage immediately before irradiation to screen wavelengths <290 nm. Sunlamps were held 20 cm above the cages. UV irradiation was performed consistently between the hours of 08:00 and 11:00 h. The dorsal skin of mice not treated with UVR was also shaved and the mice were handled in an identical fashion to UV-irradiated mice, including being placed in the UV irradiation room for the same duration.

2.3. Faecal Microbiota Analysis

The faecal microbiome was analysed by sequencing the V3–V4 segment of the 16S ribosomal RNA (rRNA) gene using Illumina MiSeq chemistry. Faecal pellets were collected and stored at −20 °C for up to six months. Bacterial DNA was extracted using the PowerSoil® DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA, USA). PCR amplification (341F/806F primer pair) and sequencing was performed by the Australian Genome Research Facility on the Illumina MiSeq (San Diego, CA, USA) with 2 × 300 bp paired-end chemistry. DNA extraction controls were used to account for any contaminants. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5) [13]. Primers were trimmed using Seqtk (version 1.0) [14]. Trimmed sequences were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) [15] USEARCH (version 7.1.1090) [16,17] and UPARSE [17] software. Using QIIME, taxonomy was assigned using Greengenes database (Version 13_8, 2013) [18].

Microbiome statistical analysis was undertaken using the programming language R, specifically the *phyloseq* and *edgeR* packages available through Bioconductor, a project providing tools for the analysis and comprehension of high-throughput genomic DNA. The biom file, operating taxonomy unit (OTU) table, taxonomic assignments, and associated sample data were imported into R to create a *phyloseq* object. For all beta-diversity analyses, OTUs for which the variance across all samples was very low were filtered out. For testing a single categorical experimental condition, exact tests for differences in the means between two groups of negative-binomially distributed counts were computed. Data were normalised using the run-length encoding (RLE) scaling factor method and dispersions estimated. The counts were extracted and ranked by *p* value, applying a false discovery rate cutoff of less than 0.001. LEfSe (linear discriminant analysis effect size) was used to identify differentially abundant microbial taxa [19].

2.4. Real-Time PCR

Messenger RNA was extracted from snap-frozen colon and kidney with cDNA synthesised and real-time assays performed as previously described [20]. Real-time PCR primers were *CYP27B1* cat # 301447280210/0&1, *VDR* cat# KSPQ12012G (Sigma-Aldrich, St. Louis, MO, USA), *CAMP* cat# QT00241003, *IL-1β* cat# QT01048355 (Qiagen, Hilden, Germany). Housekeeping genes used were TATA-box-binding protein for colonic tissue (Sigma-Aldrich) and elongation factor 1α for kidney tissue (Sigma-Aldrich). Quantitect SYBRGreen was used for qPCR on the AB17900HT instrument. Fold-change was determined by using the $2^{-\Delta\Delta Ct}$ method.

2.5. Colitis Model

After 28 days on respective diets, with or without UVR exposure on days 24–28, half of the mice underwent treatment to induce colitis by the addition of DSS (3% (wt/vol) (MP Biomedicals LLC, OH)) to the drinking water for 6 days (Supplementary Figure S1). As the efficacy of DSS varies between batches, all experiments were conducted using the same batch [21]. In preliminary experiments, 3% DSS induced adequate colitis with peak weight loss ranging between 0.3% and 10.3% after 6 days of DSS treatment. Following induction of colitis, mice recovered over a period of 0–4 weeks without ongoing DSS treatment. Mouse body weight was assessed daily during DSS treatment and weekly during recovery. The experiment was repeated, with a total of 35 mice per group. Mice were sacrificed at days 35, 42, 49, and 63.

2.6. Murine Colonoscopy

A high-resolution mouse video endoscopic system was used to assess the level of colitis. All mice were colonoscoped after 6 days of DSS treatment and then at the time of sacrifice. Mice were anaesthetised using isofluorane unless the colonoscopy was being performed at the end-point, when ketamine 20 mg/mL and xylazine 2 mg/mL by intraperitoneal injection was used. All procedures

were digitally recorded and then scored in a blinded fashion. The experimental endoscopy setup consisted of a miniature endoscope (1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Karl Storz, Germany) to achieve regulated inflation of the mouse colon.

The severity of colitis was determined using the modified Murine Endoscopic Index of Colitis Severity (MEICS) [21,22]. The MEICS system consists of five parameters: thickening of the colon wall, changes of the normal vascular pattern, presence of fibrin, mucosal granularity, and stool consistency. Endoscopic grading was performed for each parameter (scored between 0 and 3) leading to a cumulative score of between 0 (no signs of inflammation) and 15 (endoscopic signs of severe inflammation). Healthy mice had a score of 0–3.

2.7. Histological Assessment of Colitis

Colons were removed with the rectum discarded as this has a different tissue fibro-structure. The distal 1 cm of colon was dissected, cleaned, formalin-fixed, and embedded in paraffin wax. Sections were stained with haemotoxylin and eosin (H&E). All H&E sections were assessed blindly by a specialist gastroenterological histopathologist (CF) according to the scoring system by Dieleman et al. [23]. In this scoring system, the severity and depth of inflammation as well as the level of crypt damage and regeneration are scored.

2.8. Measurement of Serum Metabolites

At the time of sacrifice, blood was drawn by cardiac puncture. Levels of $25(OH)D_3$ were measured in the serum by liquid chromatography tandem mass spectroscopy (LC/MS/MS) [24]. Levels of $1,25(OH)_2D_3$ were measured using IDS EIA ELISA kits (Immunodiagnostic Systems, Fountain Hills, AZ, USA) as described by the manufacturer.

Serum cytokines were measured using Bio-Plex Pro™ Mouse Cytokine 23-plex panel (Bio-Rad Laboratories, Hercules, CA, USA) as per the manufacturer's instructions. The cytokines analysed included interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-17, eotaxin (CCL11), G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.

2.9. Statistical Analyses

Statistical significance was calculated using IBM® SPSS® Statistics Version 22 (IBM Corp. Armonk, NY, USA). All graphs and comparison of differences between groups were assessed using Student's unpaired *t*-test or ANOVA with post-hoc least significant difference (LSD) analysis for multiple group analysis. Nonparametric data were analysed using Mann–Whitney *U* and Kruskall–Wallis testing.

3. Results

3.1. Microbiome Changes in UV-Irradiated Mice

After being established on the respective vitamin D diets for 24 days (days 0 to 24), half of the mice in each group received daily UVR, 1 kJ/m^2, for four days (days 24 to 28), followed by twice-weekly treatment with 1 kJ/m^2 UVR in an attempt to mimic physiological UVR exposure. Microbiota analysis was performed on 60 faecal samples, comprising five samples from each group (D++UVR+, D++UVR−, D+UVR+, D+UVR−, D-UVR+, D-UVR−) at day 35 and day 63. Nonmetric multidimensional scaling (NMDS) plot of the Bray–Curtis resemblance matrix following square-root transformation of relative abundance data showed an outlier control mouse in the D+UVR− group which was removed from all further analyses. Among mice irradiated with UV, there was no difference in alpha-diversity as measured by Chao1 in faecal samples harvested at both days 35 and 63, compared to the corresponding nonirradiated group (Figure 1).

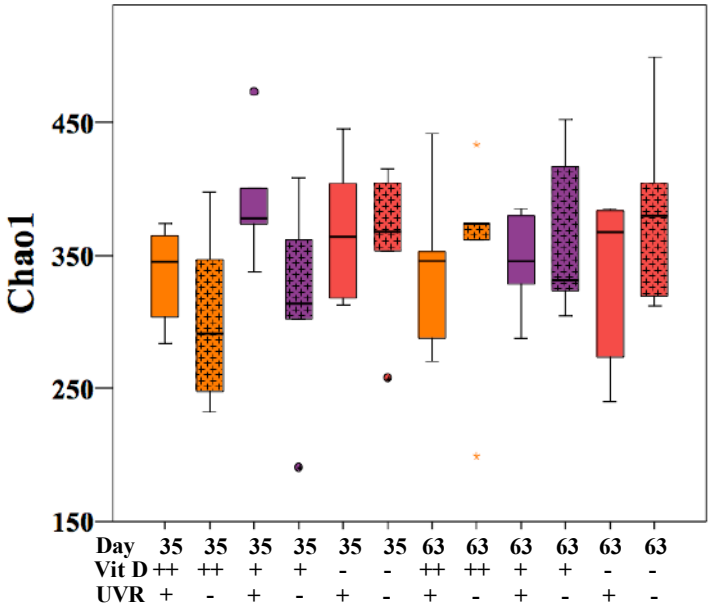

Figure 1. Alpha diversity of faecal samples. Faecal pellets were collected from UV-irradiated and nonirradiated mice that had been given either a high-dose (D++), standard-dose (D+), or no vitamin D (D−) diet. Mice were first established on diets for 24 days (days 0–24), then half were UV-irradiated daily with 1 kJ/m^2 UVR for 4 consecutive days (days 24 to 28), followed by biweekly exposures. Samples were collected after 6 UV treatments (day 35) and at the end of follow-up (protocol day 63). After faecal DNA was extracted, the V3–V4 segment of 16S rRNA was sequenced using the Illumina MiSeq. Alpha diversity is represented by Chao1. n = 5/group. UVR = ultraviolet radiation.

The effects of UVR on the beta-diversity of individual vitamin D groups were examined at day 35 and day 63 by PERMANOVA, but no significant differences were seen (Table 1).

Table 1. Effect of UV irradiation of skin on overall beta-diversity in faecal samples.

Day/Treatment	Group 1	Group 2	*t*-Value	*p*-Value
Day 35	D++ UVR+	D++ UVR−	1.24	0.15
	D+ UVR+	D+ UVR−	0.79	0.84
	D− UVR+	D− UVR−	0.89	0.67
	D− UVR+	D+ UVR−	0.86	0.67
Day 63	D++ UVR+	D++ UVR−	1.09	0.30
	D+ UVR+	D+ UVR−	1.03	0.39
	D− UVR+	D− UVR−	0.79	0.84
	D− UVR+	D+ UVR−	0.82	0.79

PERMANOVA was used to calculate the effect of UVR on overall beta-diversity in faecal samples from mice within each vitamin D group. A separate analysis of mice acquiring vitamin D solely through UVR (D−UVR+) versus diet (D+UVR−) is shown in the fourth line of each group. n = 5/group, degrees of freedom (df) = 7–8 in all analyses, and permutations = 126. "t" = t-statistic.

When all groups were analysed together using multifactor PERMANOVA (n = 60), there was a significant difference in overall beta-diversity with UV irradiation, independent of the effect of vitamin D (t = 1.7, p = 0.009, Perms = 999, degrees of freedom (df) = 53). Linear discriminant analysis (LDA) scores were calculated for individual taxa that significantly change in relative abundance with UVR compared to no UVR exposure (Figure 2). An enrichment of *Coprococcus* (LDA score 4.01, p = 0.04) and *Mucispirillum* (LDA score 3.17, p = 0.04) with UVR exposure was observed. Conversely, Bacteroidales were more abundant in the faeces from the nonirradiated group, including an unclassified species (OTU 36, LDA = 4.38, p = 0.02) and *Parabacteroides* (LDA = 4.13, p = 0.04).

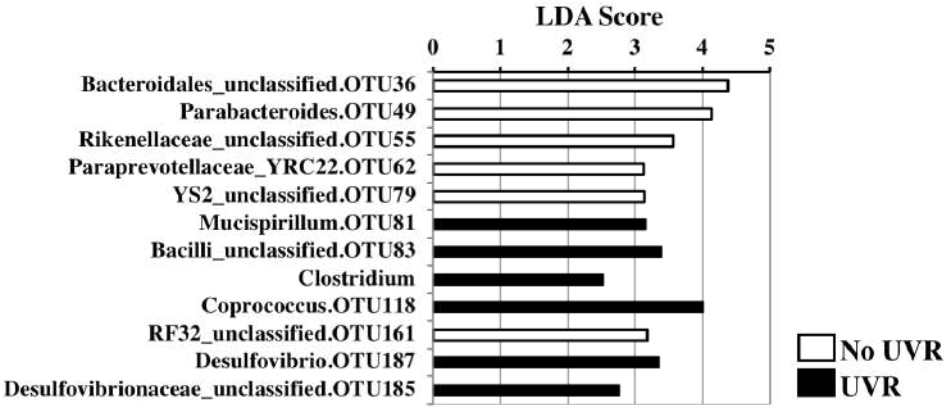

Figure 2. Faecal microbial composition of UV-irradiated vs nonirradiated mice. The effect of UV irradiation of skin on faecal microbial composition was examined using two-factor PERMANOVA controlling for vitamin D group. Linear discrimination analysis effect size (LDA score) was used to determine significant differences in relative abundance of individual taxa with UV treatment. Only taxa where a significant change ($p < 0.05$) was observed are illustrated. $n = 44$/group.

3.2. Effect of UV Irradiation of Skin on Serum Vitamin D and Cytokine Levels

After being fed the vitamin D diets for 35 days (day 0 to day 35) and exposure to daily UVR between days 24 to 28 and biweekly irradiation thereafter, blood was collected by cardiac puncture at protocol day 35. There was a trend towards higher serum 25(OH)D_3 levels in the D+ group with UV irradiation ($p = 0.06$), but not in the D++ group (Supplementary Figure S2). The vitamin D-deficient group (D−) had the lowest serum 25(OH)D_3 levels and, as expected, their levels increased significantly with UVR exposure to levels comparable to the vitamin D-sufficient (D+) group (Supplementary Figure S2).

UV irradiation of the mice caused a decrease in circulating IL-17 levels among D+ mice that trended to statistical significance ($p = 0.05$) (Table 2). Similar changes were not seen among D++ or D− mice. IL-17 levels, however, were lower among mice exclusively deriving vitamin D from UVR (D−UVR+; mean ± SEM = 121.5 ± 12.0 pg/mL, $n = 4$) compared to those deriving vitamin D from diet (D+UVR−; 207.9 ± 72.7 pg/mL, $n = 3$; $p = 0.07$). Serum protein levels of interleukin (IL)-1β, TNF, IL-10, and IL-6 did not change with UVR exposure.

Table 2. Serum cytokines and skin exposure to UV irradiation.

	D++UVR+	D++UVR−	*p*	D+UVR+	D+UVR−	*p*	D−UVR+	D−UVR−	*p*
IL-1β (pg/mL)	35.0 ± 70.0	97.3 ± 194.6	ns	0	226.0 ± 226.0	ns	0	86.9 ± 78.0	ns
TNF-α (pg/mL)	335.4 ± 120.6	411.3 ± 274.2	ns	301.6 ± 99.5	392.2 ± 161.2	ns	230.2 ± 19.1	356.4 ± 107.6	ns
IL-10 (pg/mL)	53.8 ± 20.1	66.9 ± 31.9	ns	51.9 ± 3.8	98.1 ± 60.8	ns	40.4 ± 3.3	57.2 ± 12.2	ns
IL-6 (pg/mL)	7.9 ± 3.6	7.8 ± 7.2	ns	6.7 ± 1.0	18.5 ± 14.5	ns	6.1 ± 1.6	7.2 ± 3.1	ns
IL-17 (pg/mL)	146.2 ± 38.5	177.6 ± 53.2	ns	116.7 ± 30.1	207.9 ± 72.7	0.05	121.5 ± 12.0	159.7 ± 24.8	ns

The effect of UVR of skin on serum IL-1β, TNF-α, IL-10, IL-6, and IL-17 was examined in mice on the D++, D+, and D− diets. Values are represented as mean ± SD. $n = 3$–4/group. ns = not significant.

3.3. Effect of UV Irradiation on Colonic and Kidney Vitamin D Pathway Gene Expression

Given the change in microbiota with UVR treatment, changes were sought in the gene expression of colonic *CYP27B1*, vitamin D receptor (*VDR*), cathelcidin antimicrobial peptide (*CAMP*), and *IL-1β*. Specifically, colons from mice from the D+UVR- and D-UVR+ groups were examined as these groups had similar circulating 25(OH)D_3 levels, but vitamin D was acquired either exclusively from diet or UVR exposure. CYP27B1 is the 1-alpha hydroxylase responsible for the local activation of 25(OH)D to 1,25$(OH)_2D_3$. Expression of *CYP27B1* was four-fold greater among UV-irradiated mice compared to nonirradiated mice in the proximal but not distal colon ($p = 0.007$) (Figure 3A,E). Vitamin D receptor

gene expression did not differ between groups (Figure 3B,F). As $1,25(OH)_2D_3$ induces the cathelcidin gene to produce antimicrobial peptides (CAMP), the gene expression of *CAMP* was examined and found to be similar among mice from the UVR and non-UVR groups (Figure 3C,G). The mRNA expression of *IL-1β*, encoding the proinflammatory cytokine IL-1β, was reduced almost five-fold among UV-irradiated compared to the nonirradiated mice in the proximal ($p = 0.011$) but not distal colon (Figure 3D,H). Gene expression of *CYP27B1* in kidney tissue was 1.8-fold greater among D−UVR+ compared to D+UVR− mice, but this was not statistically significant ($p = 0.2$), and serum $1,25(OH)_2D_3$ levels were similar between groups (Supplementary Figure S3).

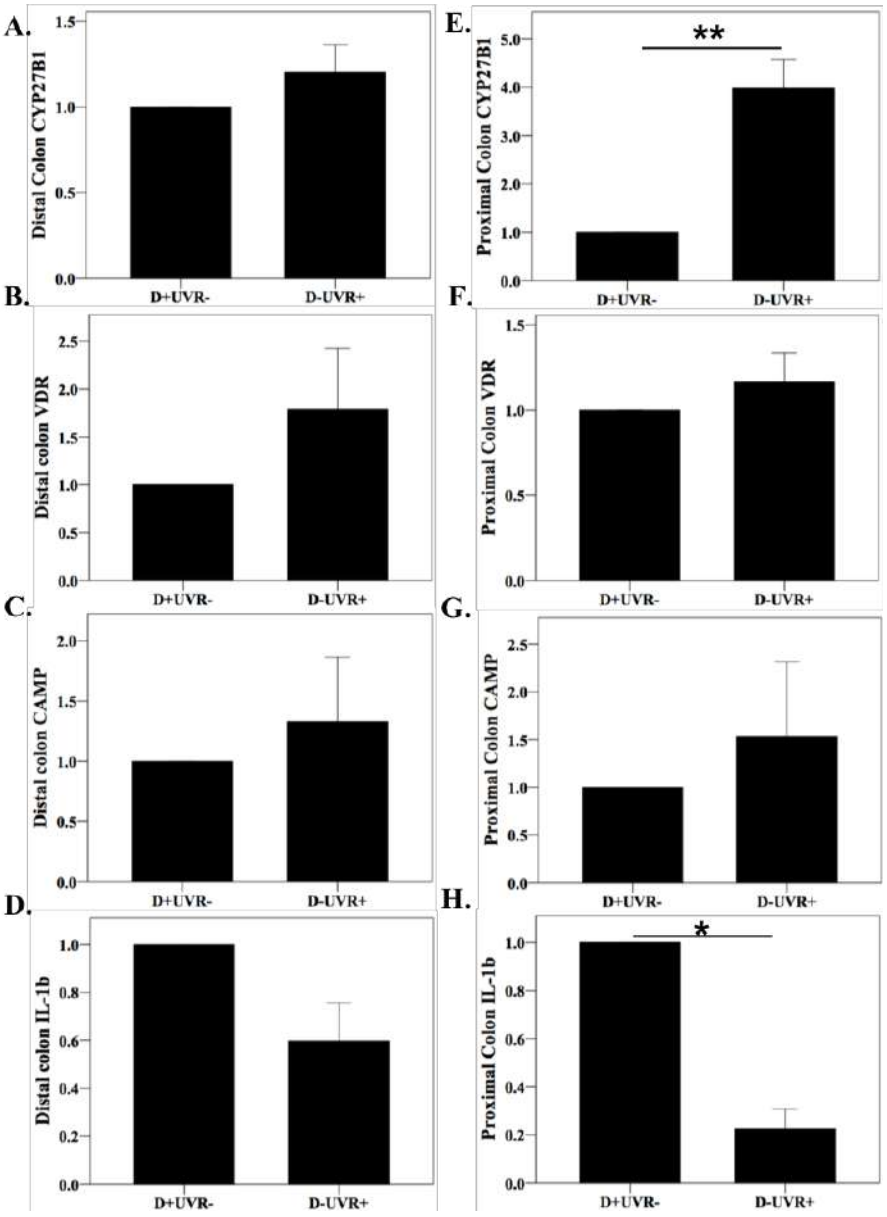

Figure 3. Colon *CYP27B1*, vitamin D receptor, cathelcidin, and *IL-1β* gene expression. At day 35, mice were sacrificed and colonic tissue harvested to determine gene expression in the distal (**A–D**) and proximal (**E–H**) colon from mice acquiring vitamin D exclusively through diet (D+UVR−) or UV irradiation (D−UVR+). Data are expressed as fold-change with the D+UVR− group as the control. mRNA gene expression by qPCR was calculated using the $2^{-\Delta\Delta CT}$ method with TATA-box-binding protein as the housekeeping gene, $n = 3$/group. Values are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$.

3.4. The Effect of UV Irradiation of Skin on DSS Colitis

After six days of DSS treatment (days 28 to 34), all mice underwent colonoscopy (n = 35/group) and were sacrificed at days 35, 42, 49, and 63 (Supplementary Figure S1). As expected, all DSS-treated mice lost weight and liquid stools were observed. We previously reported worse colitis in D++ compared to D+ mice as measured by colonoscopy, weight loss, and histology, and there was no improvement in colitis severity among D− mice exposed to UVR [7].

In the current study, D++ mice treated with or without UVR demonstrated significantly greater endoscopic evidence of colitis (MEICS) and greater weight loss at days 34 and 35, compared to their D+ counterparts (Figure 4A,B). Among the D++ and D+ groups, UV irradiation of the mice did not alter the initial severity of colitis or the speed of recovery over time compared to mice without UVR exposure, with similar MEICS scores at all timepoints. At day 34, greater weight loss was recorded among D+UVR+ mice compared to D+UVR− mice (p = 0.04). At day 38, there was a trend for D++UVR+ mice to have regained less weight compared to D++UVR− with persisting 5.3 ± 1.7% versus 1.2 ± 1.4% weight loss compared to baseline (p = 0.07).

Histological colitis was more severe among D++ mice compared to D+ mice irrespective of UVR exposure; these changes were statistically significant at day 42 (Figure 4C,D). At day 35, there was a trend for worse colitis among UVR-irradiated D++ mice compared to D++ UV nonirradiated mice (p = 0.08). At day 42, there were no differences detected between UV-irradiated and nonirradiated groups.

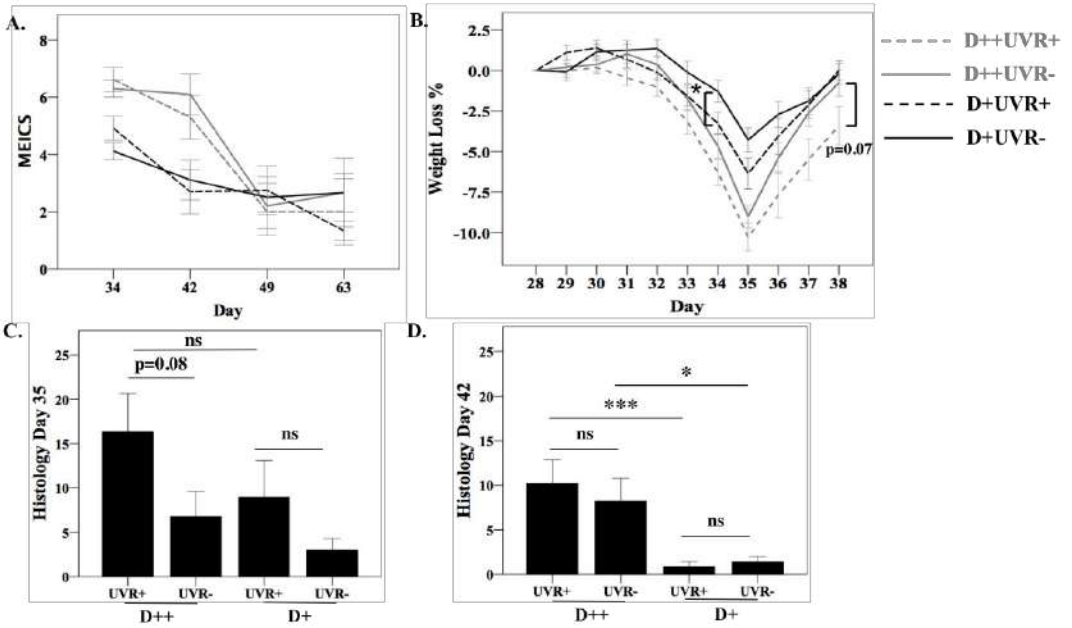

Figure 4. Effect of UV irradiation of skin on dextran sodium sulfate (DSS) colitis. After mice were established on respective diets for 28 days and half of each group received 4 daily doses of 1 kJ/m^2 UVR, mice were treated orally with DSS 3% for 6 days. Colitis was measured by (**A**) colonoscopy calculating the murine endoscopic index of severity (MEICS), with n = 35/group for day 34, n = 10/group for day 42, and n = 5/group for assessments at days 49 and 63; (**B**) percentage weight loss; p-value comparison was for UVR vs no UVR within the same vitamin D diet group; and (**C**) histological severity score at day 35 and (**D**) day 42, n = 7–10/group. Values are shown as mean ± SEM. * $p < 0.05$, *** $p < 0.001$.

3.5. Microbiome in Faecal Samples from DSS Mice

The effect of UV irradiation on the faecal microbiome from all groups treated with DSS was explored. Faecal samples from 28 mice were collected at day 35 (seven days after DSS initiation).

These comprised five samples from each of the six groups, except D++UVR+ and D+UVR+, where four samples were collected. No differences were seen in alpha-diversity between groups.

The overall beta-diversity was examined by PERMANOVA (Table 3). Significant differences were detected with UV irradiation of vitamin D-deficient mice (D−UVR+ vs D−UVR−, t = 1.42, p = 0.034). Furthermore, when comparing mice that exclusively derived vitamin D through UVR exposure (D−UVR+) to mice receiving only dietary vitamin D (D+UVR−), a significant difference was seen (t = 1.52, p = 0.021) despite the mice having similar serum 25(OH)D and 1,25$(OH)_2D_3$ levels (Supplementary Table S1 and Supplementary Figure S3).

Table 3. Effect of UV irradiation of skin on overall beta-diversity in faecal samples from DSS mice at day 35.

Group 1	Group 2	*t*	*p*-Value
D++ UVR+	D++ UVR−	0.92	0.61
D+ UVR+	D+ UVR−	1.17	0.26
D− UVR+	D− UVR−	1.42	0.034
D− UVR+	D+ UVR−	1.52	0.021

PERMANOVA was used to calculate the effect of UVR on overall beta-diversity in faecal samples from mice within each vitamin D group after treatment for 6 days with DSS (day 35). A separate analysis of mice acquiring vitamin D solely through UVR (D−UVR+) versus diet (D+UVR−) is shown in the fourth line. n = 4–5/group, degrees of freedom (df) = 7–8 in all analyses, and permutations = 126.

Changes in individual taxa were also examined in faecal pellets from the DSS mice. In vitamin D-deficient mice exposed to UVR (D−UVR+), an overall shift from phylum Bacteroidetes to phyla Firmicutes and Verrucomicrobia was detected in faecal pellets compared to those from nonirradiated counterparts (D−UVR−) at day 35 using linear discriminant analysis (LEfSe) (Supplementary Table S1). Comparing the faecal microbiome of mice deriving vitamin D from UVR (D−UVR+) compared to diet (D+UVR−), there were a number of taxa reaching LDA scores greater than 3.5, including Actinomycetales (p = 0.005), *Mucispirillum* (p = 0.047), *Lactobacillus* (p = 0.009), *Methylibium* (p = 0.005), *Flexispira* (p = 0.028), *Enterobacteraceae* (p = 0.047), and *Photobacterium* (p = 0.019) (Figure 5). Conversely, the faecal microbiome of mice in the D+UVR− group had greater relative abundance of *Rickenellaceae* (p = 0.005), S24_7 (p = 0.047), and *Akkermansia* (p = 0.028), which all had LDA scores >4.

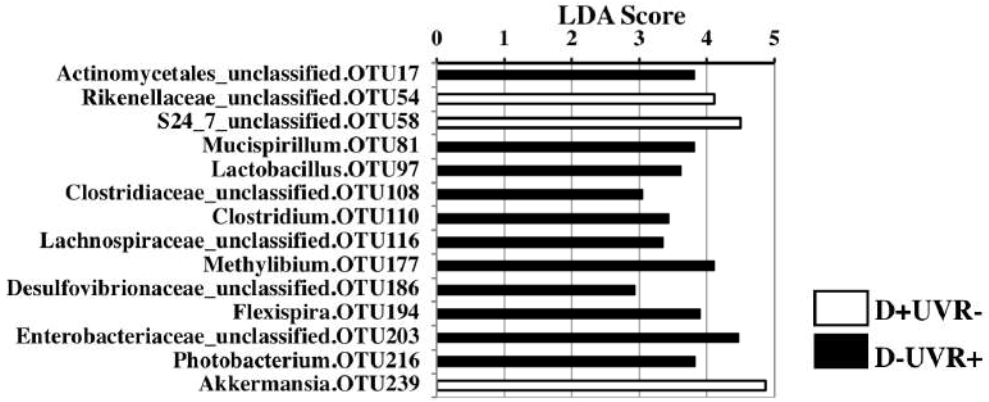

Figure 5. The effect of UV irradiation of skin versus dietary vitamin D on microbial composition of faecal samples post-DSS. Linear discrimination analysis effect size (LDA score) was determined for evaluation of significant differences in the relative abundance of individual taxa in faeces from DSS mice (day 35) that acquired vitamin D following skin exposure to UVR (D−UVR+) versus those mice acquiring vitamin D through diet alone (D+UVR−). Only taxa where a significant change (p < 0.05) was observed are illustrated; n = 5/group.

4. Discussion

Dietary interventions including oral vitamin D supplementation can have significant effects on the intestinal microbiome [25]. To our knowledge, this is the first study to explore the effect of UVR on the faecal microbiome. When the effect of UV irradiation within each vitamin D group was examined, there were no significant changes in alpha- or beta-diversity measures, but this is likely due to the study being underpowered to detect this effect. When the faecal microbiomes of UV-irradiated mice were collectively examined, significant differences were observed compared to those of nonirradiated mice, even after controlling for vitamin D group and time of sacrifice. At the phylum level, there was a shift from Bacteroidetes to Deferribacteres and Firmicutes. The genus *Mucispirillum* was enriched with UVR exposure ($p = 0.04$): *Mucispirillia* are abundant inhabitants of the intestinal mucus layer of rodents and other animals which possess mucolytic activity and are increased during inflammation [26,27]. A loss of mucus secretion due to *Mucispirillia* may increase sensitivity to chemical adjuvants, and in some cases, induce spontaneous colitis [28–30]. Among Firmicutes, the genus *Coprococcus* was enriched with UVR exposure. This bacterium produces butyric acid, a short-chain fatty acid with known anti-inflammatory effects which is depleted in paediatric patients with active inflammatory bowel disease [31]. Interestingly, we recently reported the depletion of the genus *Coprococcus* with high vitamin D diets, which was also associated with more severe DSS colitis [7], again suggesting different effects of UVR versus dietary vitamin D on specific bacterial taxa. Members of the genus *Clostridium* were also enriched in the faecal samples from UV-irradiated mice; these species are commensals within the gastrointestinal tract, however they include well-recognised pathogens such as *Clostridium difficile*. Among the phylum Proteobacteria, there was a shift from Alphaproteobacteria to Deltaproteobacteria, with the genus *Desulfovibrio* in particular well represented with UVR exposure. *Desulfovibrio* spp. have the capacity to metabolise colonic mucin. These bacteria may contribute to mucosal inflammation in UC through production of potentially toxic hydrogen sulfide, released as a by-product of the metabolism of sulfated mucin [32]. As is evident here, there were mixed changes in the faecal microbiome and according to current knowledge, some may be beneficial and others detrimental.

Vitamin D-sufficient (D+) mice exposed to UVR had a lower level of serum IL-17 compared to nonirradiated mice, with a trend to statistical significance ($p = 0.05$). This effect of UVR was also noted among mice on high vitamin D diets (D++), although this was again not statistically significant, likely due to the high level of variability. This is consistent with the current understanding that UVR can inhibit T-cell proliferation and suppress antigen-specific responses involving Th1, Th17, and Th2 cells [33,34]. IL-17 is increased in human inflammatory bowel disease [35]. IL-17A knockout (KO) mice were protected against 2,4,6-Trinitrobenzenesulfonic acid solution (TNBS)-induced colitis, and similarly, IL-17F KO mice had less severe DSS-colitis; however, interestingly, IL-17A KO mice had worse DSS-induced inflammation [36]. Furthermore, a clinical trial of the anti-IL-17 monoclonal antibody secukinumab led to worse outcomes in Crohn's disease [37]. As a result, no further trials were carried out for this drug in IBD, but it remains a proven treatment option in other autoimmune conditions such as psoriasis [37,38].

Mice deriving vitamin D through exposure to UVR (D−UVR+) had greater *CYP27B1* gene expression in the proximal colon than was measured in vitamin D-sufficient mice not exposed to UVR (D+UVR−). This suggested that UVR may have stimulated increased $1,25(OH)_2D_3$ synthesis in the colon and may explain local effects on luminal microbiota. To our knowledge, prior studies have not examined colonic gene expression of *CYP27B1* after UV irradiation of shaved skin. In a previous study, greater *CYP27B1* expression was measured in the distal rather than the proximal colon, but treatment with DSS induced greater expression of the enzyme in the proximal rather than the distal colon [39]. While not specific to UVR exposure, these previous findings support the concept of differential *CYP27B1* expression in the proximal compared to the distal colon with different environmental exposures. Increased *CYP27B1* expression is expected to cause higher $1,25(OH)_2D_3$ levels and activation of downstream pathways to promote the intracellular killing of bacteria, attenuation of dendritic cell capacity for antigen presentation, promotion of IL-10, and inhibition of IL-12 expression [40].

Recent data support a fundamental role for IL-1β in Th17 modulation, with IL-1β able to induce the expression of transcription factors necessary for Th17 development [41]. Thus, the reduced proximal colon expression of *IL-1β* in UV-irradiated mice is consistent with the lower levels of circulating IL-17 also measured in the UV-irradiated mice.

UVR exposure did not protect against DSS colitis. There was greater weight loss in the D+UVR+ versus D+UVR− group at day 34, but not at other timepoints, and there was a nonsignificant increase in histological colitis severity in the D++UVR+ versus D++UVR− group. In contrast, results from two other studies of light therapy in DSS colitis showed a protective effect. In the first, unshaven mice were treated either with no phototherapy, low light (1000 lux), or high light (2500 lux) phototherapy for one week starting on the second day of DSS colitis [42]. Phototherapy was delivered for 12 h/day, but the wavelengths used were not described. They found reduced colitis severity in the low but not the high light group, though $1,25(OH)_2D$ was only increased in the high light group [42]. The second study irradiated C57BL/6 shaven mice (eight/group) with 1.5 kJ/m^2 broadband UVR (280–350 nm) daily for four days prior to treatment with 2.5% DSS for eight days [43]. There was significantly less weight loss, reduced faecal hemoccult blood, and histological colitis scores in UV-irradiated mice. The reason for the conflicting results is not clear; there were differences in the measurement of colitis severity and the doses of UVR given (1.5 kJ/m^2 as opposed to 1 kJ/m^2 in our study). The strength of our results is that greater numbers of mice were examined and that colitis was assessed objectively with blinded endoscopic and histologic measurement. Overall, the DSS model of colitis may not be the ideal experimental model to determine the benefits of UVR exposure. DSS induces chemical trauma to the colonic mucosa to generate colitis, and thus is a better model for studying innate immune responses in IBD. UVR, on the other hand, has effects on both innate and adaptive immune responses, but its effect on T cells and their involvement in the adaptive immune system plays a major role in its ability to suppress systemic immunity. Thus, models such as the IL-10 KO and $TNF^{\Delta\Delta RE}$, which develop intestinal inflammation due to defects in adaptive immunity, may be better suited to exploring the benefits of skin exposure to UVR [10].

The impact of vitamin D on DSS colitis and the faecal microbiome has been previously reported by us [7] and others [44,45], and recently also in a small series of IBD patients [46,47]. In the current study, UVR was associated with changes in the faecal microbial composition after DSS exposure that differed from the changes described in non-DSS mice. Notably, there was a significant change in beta-diversity between D−UV+ compared to the D+UV− group despite similar serum 25(OH)D levels. This difference could be attributed to the vitamin D-independent effects of UVR, or alternatively, it may reflect the mode of acquiring vitamin D. Dietary vitamin D, by virtue of direct contact with the intestinal mucosa and microbiota, may have a different impact on the intestinal microbial composition as opposed to vitamin D acquired via UVR/dermal synthesis and reaching the intestinal mucosa through the systemic circulation. Interpreting the wide range of changes in specific taxa after DSS treatment is challenging, but notably in mice acquiring vitamin D only through diet (D+UVR−) compared to UVR (D−UVR+), there was enrichment of *Verrucomicroiaceae*, *Akkermansia*, and the recently described Bacteroidales S24-7. A recent study comparing the effects of *Salmonella* infection and DSS colitis demonstrated enrichment of these taxa in mice with low-level inflammation secondary to *Salmonella* infection or DSS [48]. *Akkermansia* are known to degrade mucin as their sole carbon and nitrogen source, and these bacteria increased after low levels of inflammation, when mucin may be produced. S24-7 have similar mucin degradation capacity. Both *Akkermansia* and members of family S24-7 encode the capacity for propionate production, which may stabilise inflammation in the gut, forming a positive feedback loop [49]. Thus, while induced by inflammation, their metabolic activity may act to limit colitis. Conversely, the D−UVR+ faecal microbiome was enriched with members of Clostridia, Helicobacter, and Enterobacteriaceae, which may potentiate inflammation, as well as Lactobacilli, which are often used as a probiotic. The 'net effect' of these microbial changes on inflammation is difficult to determine, but these data suggest that when faced with an insult such

as DSS colitis, there are changes in the faecal microbiome of UV-irradiated mice that are different to those measured in mice acquiring vitamin D exclusively through dietary sources.

Some study limitations are noteworthy. There was a relatively limited sample size per group examined for faecal microbiota analysis at each timepoint (n = 5/group); this was due to the large number of study groups—12 in total—necessitating fewer samples per group for feasibility. A sample size of five per group, however, is in keeping with previously published and frequently cited murine microbiome studies [28]. Notwithstanding this, in the absence of colitis, the sample size limited the ability to detect changes in faecal microbial composition from mice on individual diets +/− UVR. As described, however, when the groups were analysed together (n = 60), clear differences in microbial composition were seen with UV-irradiation and controlling for vitamin D groups. Furthermore, as the study was only conducted in female mice, the study findings may not be generalisable to male mice. We acknowledge the impact gender may have on vitamin D metabolism as described in both human and animal studies, and future studies should aim to replicate these findings in both male and female mice [11,50].

In conclusion, both diet and UVR (as in sunlight) were effective methods of acquiring vitamin D; however, they were associated with different effects on the faecal microbiome in the healthy state and after an insult such as DSS colitis. These differential effects may have significance in health and disease, including inflammatory bowel disease, and further experimentation with alternative animal models are warranted.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/10/8/1069/s1, Figure S1: The experimental approach, Figure S2: Serum 25(OH)D_3 levels, Figure S3: Kidney *CYP27B1* gene expression and 1,25$(OH)_2D_3$ levels, Table S1: LEfSe analyses comparing mice acquiring vitamin D either exclusively through diet versus UV-irradiation.

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nutrients

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Supplementary Materials: Ultraviolet Irradiation of Skin Alters the Faecal Microbiome Independently of Vitamin D in Mice

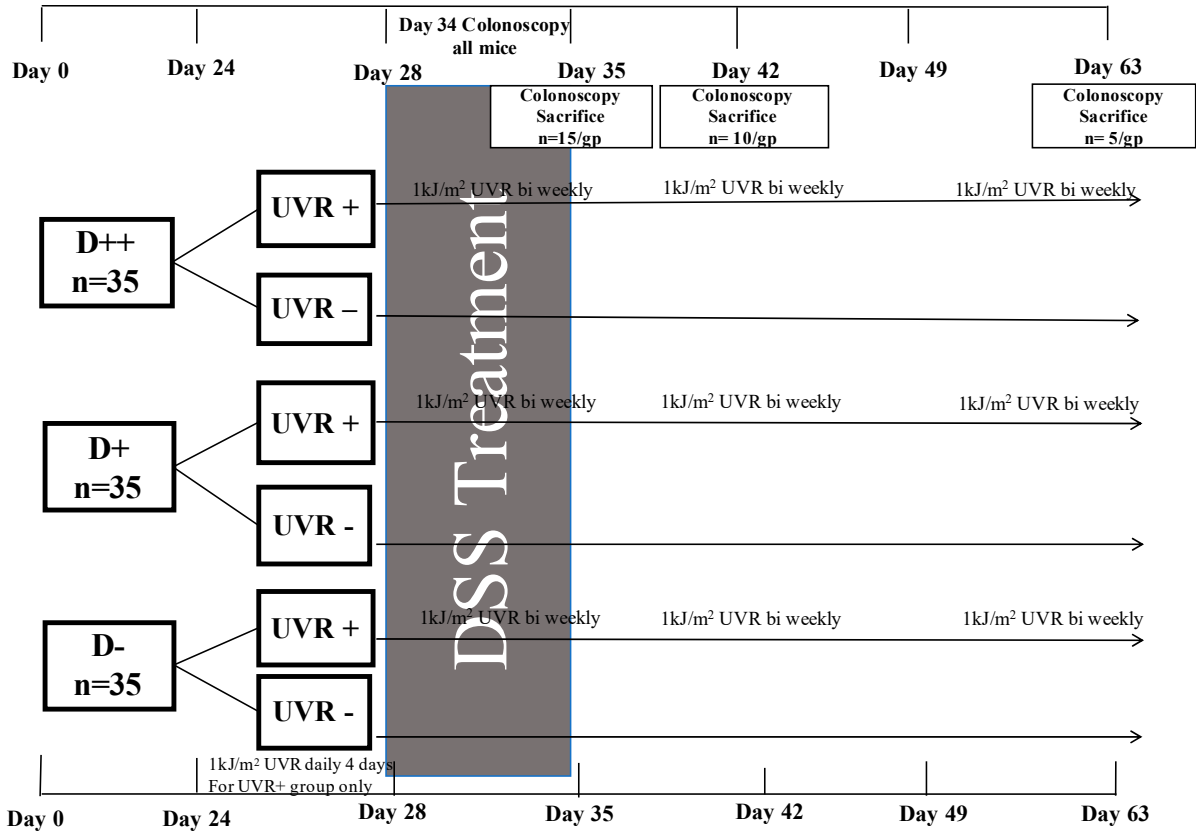

Figure S1. The experimental approach. C57BL/6 female mice (6-weeks-old) were fed diets with high (D++), moderate (D+) or no (D−) vitamin D3. After 24 days, half of the mice in each group were irradiated with 1kJ/m^2 UVR daily for 4 days, and then were maintained with twice weekly exposure to UVR for the remainder of the study. In the treatment group, after 28 days of dietary supplements, with or without UVR exposure (designated day 0), mice were given DSS orally for 6 days to induce colitis, and then were maintained without DSS and continued their respective vitamin D diet until day 63. All mice underwent colonoscopy at day 34, and were sacrificed at days 35, 42, 49 or 63. An additional colonoscopy was performed on days 42, 49 or 63 when mice were sacrificed.

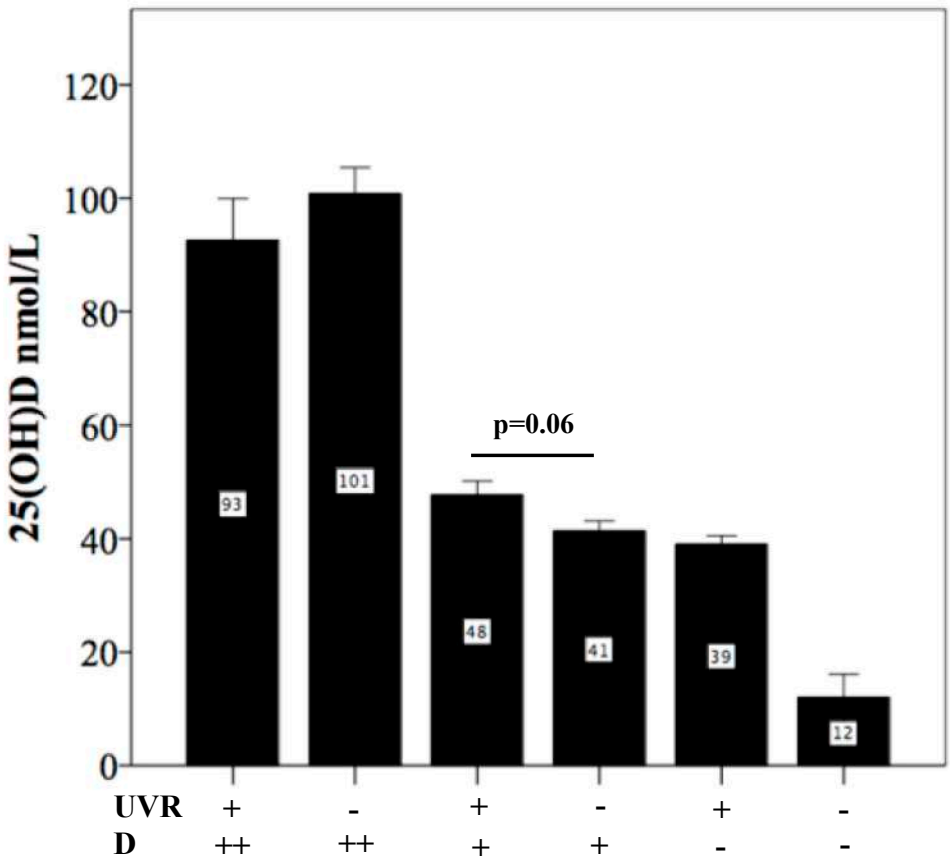

Figure S2. Serum 25(OH)D3 levels. Serum concentrations of 25(OH)D3 after 5 weeks on vitamin D diets and completing a total of 6 exposures to UVR (protocol day 7), n = 7–8/group. Values are expressed as mean ± SEM, from at least two experiments with the values shown in the bars. * $p < 0.05$, *** $p < 0.001$.

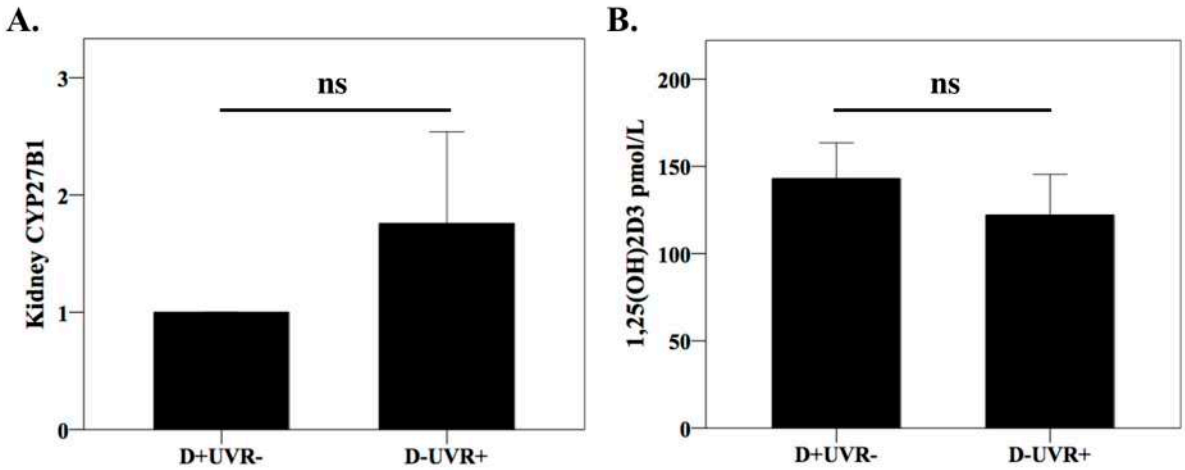

Figure S3. Kidney *CYP27B1* gene expression and 1,25(OH)2D3 levels. (**A**) Kidney tissue was harvested at day 35 to determine gene expression of CYP27B1. Data expressed as fold-change with the D+UVR− group as control. mRNA gene expression by qtPCR was calculated using the 2-ΔΔCT method with elongation factor 1 (eef1) as housekeeping gene. *n* = 3/group. (**B**) Serum 1,25(OH)2D3 measured at day 35, *n* = 5/group. Values are expressed as mean ± SEM, ns = not significant..

Supplementary Table S1. LEfSe analyses comparing mice acquiring vitamin D either exclusively through diet versus UV-irradiation.

Taxa	Group	LDA Score	*P*-Value
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales	D-UVR+	4.06	0.047
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified	D-UVR+	3.98	0.013
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified.Actinomycetales_unclassified	D-UVR+	3.97	0.013
Bacteria.Actinobacteria.Actinobacteriia.Actinomycetales.Actinomycetales_unclassified.Actinomycetales_unclassified.OTU17	D-UVR+	3.82	0.005
Bacteria.Deferribacteres	D-UVR+	3.82	0.047
Bacteria.Deferribacteres.Deferribacteres	D-UVR+	3.82	0.047
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales	D-UVR+	3.82	0.047
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae	D-UVR+	3.82	0.047
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae.Mucispirillum	D-UVR+	3.82	0.047
Bacteria.Deferribacteres.Deferribacteres.Deferribacterales.Deferribacteraceae.Mucispirillum.OTU81	D-UVR+	3.82	0.047
Bacteria.Firmicutes.Bacilli	D-UVR+	3.82	0.028
Bacteria.Firmicutes.Bacilli.Lactobacillales	D-UVR+	3.61	0.009
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae	D-UVR+	3.60	0.009
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.Lactobacillus	D-UVR+	3.60	0.009
Bacteria.Firmicutes.Bacilli.Lactobacillales.Lactobacillaceae.Lactobacillus.OTU97	D-UVR+	3.61	0.009
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiaceae.Clostridiaceae_unclassified.OTU108	D-UVR+	3.05	0.007
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiaceae.Clostridium	D-UVR+	3.44	0.007
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiaceae.Clostridium.OTU110	D-UVR+	3.44	0.007
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified	D-UVR+	4.58	0.047
Bacteria.Firmicutes.Clostridia.Clostridiales.Clostridiales_unclassified.Clostridiales_unclassified	D-UVR+	4.58	0.047
Bacteria.Firmicutes.Clostridia.Clostridiales.Lachnospiraceae.Lachnospiraceae_unclassified.OTU116	D-UVR+	3.35	0.016
Bacteria.Proteobacteria	D-UVR+	4.72	0.016
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Comamonadaceae	D-UVR+	3.77	0.005
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Comamonadaceae.Methylibium	D-UVR+	4.12	0.005
Bacteria.Proteobacteria.Betaproteobacteria.Burkholderiales.Comamonadaceae.Methylibium.OTU177	D-UVR+	4.11	0.005
Bacteria.Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrionaceae_unclassified	D-UVR+	2.91	0.0283

Bacteria.Proteobacteria.Deltaproteobacteria.Desulfovibrionales.Desulfovibrionaceae.Desulfovibrionaceae_unclassified.OTU186	D-UVR+	2.93	0.0283
Bacteria.Proteobacteria.Epsilonproteobacteria	D-UVR+	3.90	0.0283
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales	D-UVR+	3.90	0.0283
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae	D-UVR+	3.90	0.0283
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae.Flexispira	D-UVR+	3.90	0.0283
Bacteria.Proteobacteria.Epsilonproteobacteria.Campylobacterales.Helicobacteraceae.Flexispira.OTU194	D-UVR+	3.90	0.0283
Bacteria.Proteobacteria.Gammaproteobacteria	D-UVR+	4.46	0.0472
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales	D-UVR+	4.46	0.0472
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae	D-UVR+	4.46	0.0472
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Enterobacteriaceae_unclassified	D-UVR+	4.46	0.0472
Bacteria.Proteobacteria.Gammaproteobacteria.Enterobacteriales.Enterobacteriaceae.Enterobacteriaceae_unclassified.OTU203	D-UVR+	4.46	0.0472
Bacteria.Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae	D-UVR+	3.85	0.0343
Bacteria.Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.Photobacterium	D-UVR+	3.82	0.0186
Bacteria.Proteobacteria.Gammaproteobacteria.Vibrionales.Vibrionaceae.Photobacterium.OTU216	D-UVR+	3.82	0.0186
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.Rikenellaceae.Rikenellaceae_unclassified.OTU54	D+UVR-	4.11	0.008
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7	D+UVR-	4.49	0.047
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7.S24_7_unclassified	D+UVR-	4.49	0.047
Bacteria.Bacteroidetes.Bacteroidia.Bacteroidales.S24_7.S24_7_unclassified.OTU58	D+UVR-	4.49	0.047
Bacteria.Verrucomicrobia	D+UVR-	4.87	0.0283
Bacteria.Verrucomicrobia.Verrucomicrobiae	D+UVR-	4.87	0.0283
Bacteria.Verrucomicrobia.Verrucomicrobiae.Verrucomicrobiales	D+UVR-	4.87	0.0283
Bacteria.Verrucomicrobia.Verrucomicrobiae.Verrucomicrobiales.Verrucomicrobiaceae	D+UVR-	4.87	0.0283
Bacteria.Verrucomicrobia.Verrucomicrobiae.Verrucomicrobiales.Verrucomicrobiaceae.Akkermansia	D+UVR-	4.87	0.0283
Bacteria.Verrucomicrobia.Verrucomicrobiae.Verrucomicrobiales.Verrucomicrobiaceae.Akkermansia.OTU239	D+UVR-	4.87	0.0283

Threshold used was linear discriminant analysis (LDA) score > 2 and $P < 0.05$. The results provide a list of bacterial taxa at all taxonomic levels that are differentially abundant between the D−UVR+ and D+UVR− groups at Day 35.

Chapter 6

Ghaly S et al. **Vitamin D C3-epimer levels are proportionally higher with oral vitamin D supplementation compared to ultraviolet irradiation of skin in mice but not humans**. J Steroid Biochem Mol Biol. 2018. Oct 5.

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Vitamin D C3-epimer levels are proportionally higher with oral vitamin D supplementation compared to ultraviolet irradiation of skin in mice but not humans

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ABSTRACT

A proportion of circulating 25-hydroxy vitamin D_3 ($25(OH)D_3$) undergoes epimerization to form C3-epi 25(OH) D_3 and C3-epi $1,25(OH)_2D_3$. These epimers have less calcaemic activity than non-epimerized metabolites and are not differentiated by many immunoassays when reporting total $25(OH)D_3$ levels. This study aimed to compare the effect of exposure to ultraviolet radiation (UVR) and oral vitamin D_3 supplementation on vitamin D C3-epimer levels. C57Bl/6 female mice were fed either vitamin D-sufficient (vitamin D_3 2000 IU/kg) or -deficient diets (no vitamin D_3) for 4 weeks. Among the vitamin D-deficient group, the shaved backs of half were irradiated daily for 4 days with 1 kJ/m^2 UVR, followed by twice weekly irradiation for 4 weeks. Despite similar $25(OH)D_3$ levels, the UV-irradiated group had a lower proportion of C3-epi $25(OH)D_3$ at week 7 ($p < 0.05$) and week 9 ($p < 0.01$). C3-epimer concentrations and %C3-epi $25(OH)D_3$ were also analysed in serum samples from two human clinical trials. These trials investigated the effect of high dose oral vitamin D_3 supplementation and narrowband UVB phototherapy, respectively. Serum $25(OH)D_3$ and the %C3-epi $25(OH)D_3$ levels measured at 12 months after oral vitamin D_3 supplementation were not significantly different to those measured at the time of maximal effect of phototherapy (2 months). Thus, the proportion of $25(OH)D_3$ that undergoes epimerization is greater with oral vitamin D_3 supplementation than exposure to UVR in mice, but not in humans. This important difference between human and murine vitamin D metabolism warrants consideration when interpreting animal studies.

1. Introduction

Vitamin D deficiency and insufficiency are global health issues, yet it remains unknown if oral vitamin D supplementation is as effective as cutaneous production of vitamin D after sunlight exposure for both skeletal and non-skeletal actions. Exposure to ultraviolet radiation (UVR) has benefits that are independent of vitamin D, including reducing cardiovascular disease, obesity and modulation of systemic immune responses [1–3].

The major source of vitamin D_3 in humans is via skin exposure to sunlight. People living in far northern and southern latitudes with less UV exposure rely more on dietary sources of vitamin D [4]. In Australia, traditionally 80–90% of vitamin D is obtained through sunlight exposure, though special populations, such as those in residential care, are more dependent on oral vitamin D [5]. In humans and many animals, 7-dehydrocholesterol (provitamin D_3) is converted into previtamin D_3 in the skin by UVR. Previtamin D_3 is thermodynamically unstable and is rapidly transformed to vitamin D_3. Vitamin D_3 whether from diet or cutaneous production, undergoes 25-hydroxylation in the liver by the cytochrome p450 enzyme, CYP2R1, to produce $25(OH)D_3$ which is then

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further hydroxylated in the kidney and extra-renal tissues by CYP27B1 to produce the metabolically active $1,25(OH)_2D_3$ [6]. $1,25(OH)_2D_3$ exerts its actions through binding to the nuclear vitamin D receptor (VDR). These main metabolites also undergo epimerization to produce C3-epi $25(OH)D_3$ and C3-epi $1,25(OH)_2D_3$ [7].

Vitamin D epimers have traditionally been considered to be inactive, but recent data suggest that C3-epi $25(OH)D_3$ has positive effects on growth and bone mineral density [8]. Sprague Dawley rats supplemented with two different doses of C3-epi $25(OH)D_3$ for 8 weeks demonstrated similar growth and bone mineral density to control mice supplemented with vitamin D_3 [8]. The authors concluded that C3-epi $25(OH)D_3$ clearly has biological activity, warranting further exploration. C3-epi $1,25(OH)_2D_3$ can stimulate gene transcription through the vitamin D receptor (VDR), although it appears to have weaker binding affinity for the VDR than $1,25(OH)_2D_3$ [9].

In humans, C3-epi $25(OH)D_3$ contributes substantially to the circulating level of total $25(OH)D_3$ in newborns and infants but decreases in the first year after birth [10]. The clinical significance of this remains unknown. Both dietary and cutaneous production of vitamin D_3 appear to contribute to the concentration of C3-epi $25(OH)D_3$ in human serum [11]. Supplementation with vitamin D at 800 IU/day in both men and women ≥ 50yrs for 15 weeks increased C3-epi $25(OH)D_3$ by week 7 and these levels were maintained until week 15 [11]. The reported proportion of epimer to total 25(OH)D varies widely from 0 to 26% [12–14]. The physiological factors influencing epimer production remain unknown, including the impact of the source of vitamin D; i.e., dietary versus cutaneous production.

Using an experimental mouse model and samples from participants in two clinical trials, the production of C3-epi $25(OH)D_3$ from dietary vitamin D_3 and via cutaneous production following skin exposure to UVR was compared.

2. Materials and methods

2.1. Mice and diet

All experiments were performed according to the ethical guidelines of the National Health and Medical Research Council of Australia with the approval from the Telethon Kids Institute Animal Ethics Committee (AEC #276). Mice were purchased from the Animal Resources Centre, Western Australia.

Female 6-week-old C57Bl/6 mice were fed semi-pure diets sufficient in vitamin D (SF05-34, Specialty Feeds, 2,280 IU/kg vitamin D_3, 1% calcium) or deficient in vitamin D (SF05-033, Specialty Feeds, 0 IU/kg vitamin D_3, 2% calcium).

Mice were housed under perspex-filtered fluorescent lighting, which emitted no detectable UVB radiation (290–320 nm) as measured using a UV radiometer (UVX Digital Radiometer, Ultraviolet Products Inc., Upland, CA, USA).

2.2. UV irradiation of mice

A bank of six 40 W lamps (Philips TL UV-B, Eindhoven, The Netherlands) emitting broadband UVR, 250–360 nm, with 65% of the output in the UVB range (280–315 nm), was used to irradiate mice and to deliver 1 kJ/m^2 of UVR onto clean-shaven 8 cm^2 dorsal skin. A new sheet of PVC plastic film (0.22 mm) was taped to the top of each perspex cage immediately before irradiation to screen wavelengths < 290 nm. Sunlamps were held 20 cm above the cages.

After 4 weeks on their respective diets, half of the mice from the vitamin D deficient group received 1 kJ/m^2 daily for four consecutive days. After this they were irradiated bi-weekly with 1 kJ/m^2 UVR for 4 weeks. Mice were sacrificed at Weeks 6, 7 and 9 (Fig. 1). Blood was drawn by cardiac puncture at the time of sacrifice. The experiment was repeated twice.

2.3. The pilot D-Health and PhoCIS trials

Serum samples were obtained from participants in two clinical trials, the pilot D-Health [15,16] and PhoCIS trials [17], to analyse $25(OH)D_3$ and C3-epi $25(OH)D_3$ metabolites. An amendment to the original D-Health ethics application was approved by the human research ethics committee at the QIMR Berghofer Medical Research Institute (trial 2010/0423). The PhoCIS trial (Phototherapy for Clinically Isolated Syndrome) was approved by the Bellberry Human Research Ethics Committee (2014-02-083) and endorsed by the Human Research Ethics Office of the University of Western Australia (RA/4/1/6796). Briefly, the D-Health trial was a population-based, randomized, placebo-controlled, double-blind chemoprevention trial of vitamin D_3 in older adults. In total, 644 individuals aged 60–84 in the eastern states of Australia were recruited and randomly assigned to monthly doses of placebo, 30,000 IU, or 60,000 IU vitamin D_3 for 12 months [18]. Blood samples were collected at baseline and within 2 weeks after the last dose of vitamin D. The initial analysis of $25(OH)D_3$ for the study was performed using the Diasorin Liaison platform immunoassay which does not distinguish the C3-epi $25(OH)D_3$ when reporting total 25(OH) D_3 levels. In this sub-study, serum samples were reanalyzed from 11 subjects who were treated with 60,000 IU Vitamin D_3 / month and had demonstrated at least a 30% rise in $25(OH)D_3$ according to the Diasorin assay, as these were most likely to demonstrate the differences in epimer metabolites.

The PhoCIS trial examined 20 people with clinically isolated syndrome, which refers to a first episode of neurologic symptoms lasting at least 24 h and caused by inflammation or demyelination in the central nervous system. This may be part of the multiple sclerosis disease course. Participants were randomized in a non-blinded 1:1 fashion to receive, or not receive, narrow band UVB phototherapy (24 sessions total, 3 exposures/week for 8 weeks) [19]. Blood samples were collected at months 1, 2, 3, 6 and 12. As published, three subjects in the phototherapy group and 5 subjects in the control group (no phototherapy) had a baseline $25(OH)D_3$ level < 80 nmol/L requiring supplementation [19]. If baseline $25(OH)D_3$ levels were < 80 nmol/L, then participants in both groups were supplemented with low dose oral vitamin D3 (800 IU/day) to achieve serum $25(OH)D_3$ levels > 80 nmol/L [17].

2.4. $25(OH)D_3$ and C3-epi $25(OH)D_3$ measurement

All blood samples from the murine and human studies were analyzed using liquid chromatography tandem mass spectroscopy at the University of Western Australia, Centre for Metabolomics. This assay has been certified by the Centre for Disease Control (NIH, USA) for both precision and accuracy of 25(OH)D3 measurement with a correlation (R^2) of 0.9979. For 3-epi-$25(OH)D_3$ measurement, the correlation was 0.9961 [20].

2.5. Real-time PCR for measurement of enzymes involved in vitamin D metabolism

Messenger RNA expression of key enzymes in the synthesis and metabolism of vitamin D were examined to determine if any differences with UV-irradiation compared to oral vitamin D supplementation exist. mRNA was extracted from snap-frozen kidney and liver tissue with cDNA synthesised and real-time assays performed as previously described [21]. Real-time PCR primers included *CYP27B1* cat # 301447280210/0&1, *CYP24A1* cat # 3014472802-20/0&1 (Sigma-Aldrich co., St. Louis, MO), *CYP2R1* cat # QT0005750 and *GC* cat # QT00267799 (Qiagen, Hilden, Germany), with elongation factor 1α (eEF1α) as the housekeeping gene for all samples (Sigma-Aldrich co., St. Louis, MO). Quantitect SYBRGreen was used for qPCR on the AB17900HT instrument. Fold-change in mRNA was determined by using the $2^{-\Delta\Delta Ct}$ method.

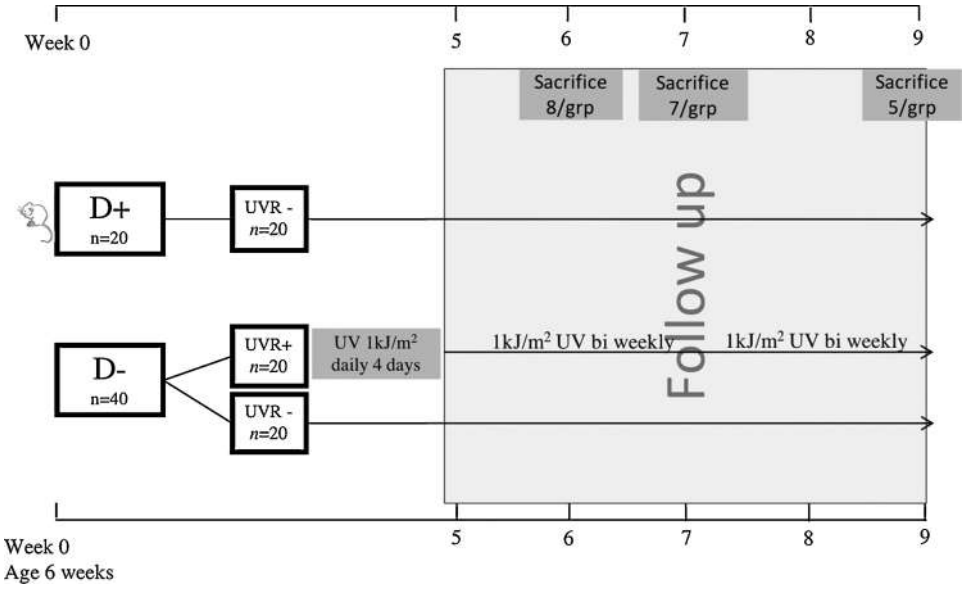

Fig. 1. The experimental approach. 6-week-old C57BL/6 female mice were fed diets with sufficient (D +) or no (D-) vitamin D_3 for 4 weeks. Half of the mice on the vitamin D deficient (D-) diet were UV-irradiated with 1 kJ/m^2 daily for 4 days, followed by biweekly UV exposures for the remainder of the study. Mice continued on their respective diets until 9 weeks, and were sacrificed at weeks 6, 7 and 9. The experiment was repeated twice. Total n = 20/group.

2.6. Statistical analysis

IBM® SPSS® Statistics Version 22 (IBM Corp. Armonk, NY) was used for statistical analysis of the animal model. Homogeneity of variances was tested with Levene's test. Means were compared using student's unpaired *t*-tests for murine data, and paired *t*-test analysis for paired human data. Pearson correlations were performed for relation between 25(OH)D_3 and its metabolites.

The change in absolute C3-epi 25(OH)D_3 and %C3-epi 25(OH)D_3 was calculated between baseline and subsequent visits (month-2, -3, and -12) for all participants in the Phototherapy group (PhoCIS trial) and between baseline and month-12 for participants in D-Health trial. The changes in both absolute and %C3-epi 25(OH)D_3 between baseline and subsequent visits were compared using generalized linear models, under the assumption that participants in the D-Health study reached their month-12 level of epimer and %C3-epi 25(OH)D_3 by month-2. Clinical data were analysed using SAS version 9.4. P-values < 0.05 were considered statistically significant.

3. Results

3.1. Murine serum 25(OH)D_3 levels

C57Bl/6 female mice were fed diets with sufficient (D +) or no (D-) vitamin D_3 for a total of 9 weeks. The mean serum 25(OH)D_3 concentration after 4 weeks on the vitamin D deficient (D-) diet was 6.4 ± 3.4 nmol/L. After daily UVR exposure at 1 kJ/m^2 for 4 days followed by biweekly treatment with 1 kJ/m^2, 25(OH)D_3 concentrations rose significantly to 33.1 ± 3.7 nmol/L (mean ± SD) at week 6 (Fig. 2A). The mean 25(OH)D_3 concentration was not significantly different to levels measured in the vitamin D-sufficient (D +) group, 33.4 ± 6.8 nmol/L (mean ± SD).

3.2. Epimer and percentage epimer levels in mice

The correlation between C3-epi 25(OH)D_3 and 25(OH)D_3 concentrations was 0.833 (p < 0.001) (Fig. 3). This indicates that a greater proportion of 25(OH)D_3 is measured in the epimerized form with increasing 25(OH)D_3 concentration.

Mice in the D-UVR + group had lower C3-epi 25(OH)D_3 and %C3-epi 25(OH)D_3 compared to D + UVR- at week 6 (p < 0.05 and 0.08, respectively) (Fig. 2A–C). These changes were also seen at weeks 7 and 9. These data suggest that either less C3-epi 25(OH)D_3 is produced, or it is more efficiently metabolized, when vitamin D_3 originates from cutaneous production compared to dietary sources.

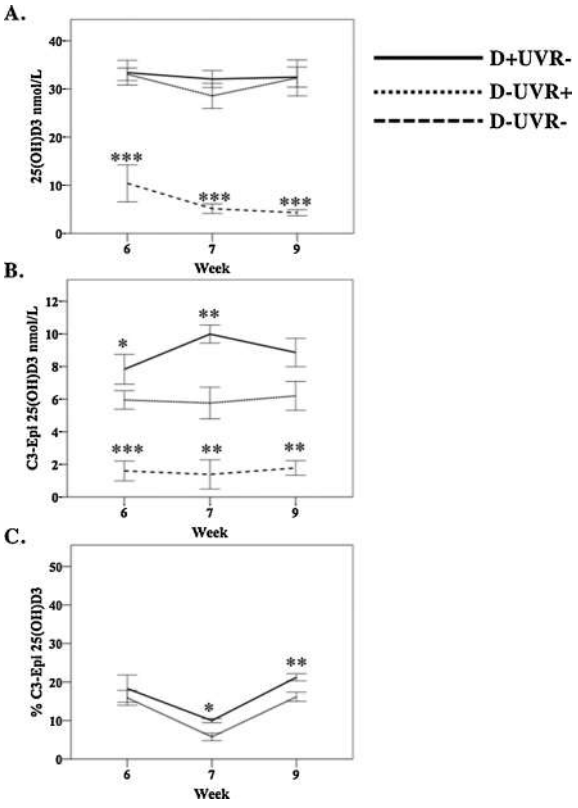

Fig. 2. Vitamin D metabolite levels. Vitamin D metabolites were analysed at weeks 6, 7 and 9. *A.* 25(OH)D_3 *B.* C3-Epi 25(OH)D_3 *C.* %C3-Epi 25(OH)D_3. For C, data for D-UVR- group were not plotted as the absolute values were close to limit of detection and thus percentages aberrantly high. Values are expressed as mean ± SEM, from at least two experiments, n = 5–8/group. Significance values are in reference to the D-UV + group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.3. mRNA expression of vitamin D enzymes

Given the lower absolute and percentage levels of C3-epi 25(OH)D_3

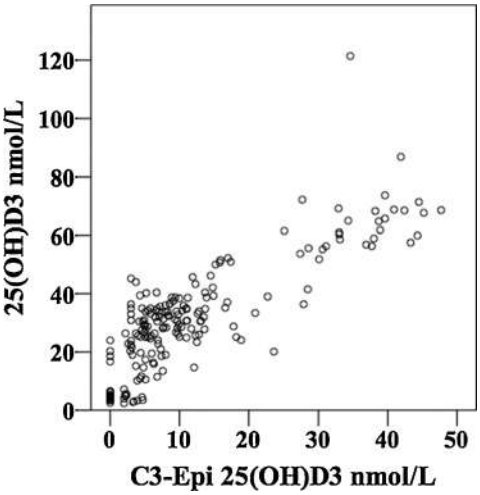

Fig. 3. Serum 25(OH)D_3 levels correlate with C3-Epi 25(OH)D_3 levels. Scatter plot of serum C3-Epi 25(OH)D_3 and 25(OH)D_3 from collected samples from all groups during the experiment period (n = 60).

among the D-UVR + group compared to D + UVR- group, differences in mRNA expression of key enzymes in the synthesis and metabolism of vitamin D were explored. Among UV-irradiated mice (D-UVR+), CYP2R1 mRNA levels were 0.5 ± 0.1 (mean ± SEM) fold that of vitamin D-supplemented, non-irradiated mice (D + UVR-), p = 0.003 (Fig. 4A). Vitamin D_3 and 25(OH)D_3 are predominantly bound to the vitamin D binding protein (VDBP) in the circulation. VDBP is encoded by the *GC* gene and synthesised in the liver. The liver *GC* mRNA expression among UV-irradiated mice (D-UVR+) was a 0.5 ± 0.1 (mean ± SEM) fold that of D + UVR- mice, p = 0.003 (Fig. 4B). The circulating concentration of VDBP, however, was not different between the two groups (Fig. 4C). Renal CYP27B1 was expressed similarly by mice in the D-UVR + and D + UVR- groups (Fig. 4D). 1,25$(OH)_2D_3$ and 25(OH)D_3, including their epimer forms, induce the expression of renal CYP24 A1 which is the main catabolic enzyme which completes a classical endocrine negative feedback loop. Renal CYP24 A1 was expressed 1.4 times more in the D-UVR + group compared to the D + UVR- group, p = 0.07 (Fig. 4E), but despite this similar 1,25$(OH)_2D_3$ levels were measured between the two groups (Fig. 4F). In summary, while mRNA expression of CYP2R1, *GC* protein and CYP24 A1 were altered with UVR exposure, an effect on VDBP and 1,25$(OH)_2D_3$ production was not observed.

3.4. Human data

After 12 months of supplementation with 60,000 IU of vitamin D_3/month, the 25(OH)D_3 concentration in the D-Health participants rose from a mean (± SD) of 68.9 ± 22.6 to 120.5 ± 16.1 nmol/L post supplementation, (p < 0.001); the change in mean 25(OH)D_3 concentration is shown in Fig. 5A. Similarly, there was a significant rise in the C3-epi 25(OH)D_3 (mean difference 8.5 units, 95% CI 4.3,12.8, p = 0.01) and %C3-epi 25(OH)D_3 (mean difference 3.9, 95% CI 1.6,6.1, p = 0.03) (Fig. 5B and C).

Among participants in the PhoCIS control group (no phototherapy), there was no significant change in the mean 25(OH)D_3 concentration between baseline and any time point to 12 months (Fig. 5A). As previously reported [19], after phototherapy the 25(OH)D_3 concentration was significantly higher by month 2 (p < 0.001) and month 3 (p < 0.01) compared to baseline, including after adjustment for season of blood draw (Fig. 5A).

In the phototherapy group, there was higher C3-epi 25(OH)D_3 (p < 0.001) and % C3-epi 25(OH)D_3 (p < 0.01) at month 2 compared to baseline (Fig. 5B & C). At 3 months, there was no longer any significant difference in absolute or %C3-epi 25(OH)D_3 levels compared to

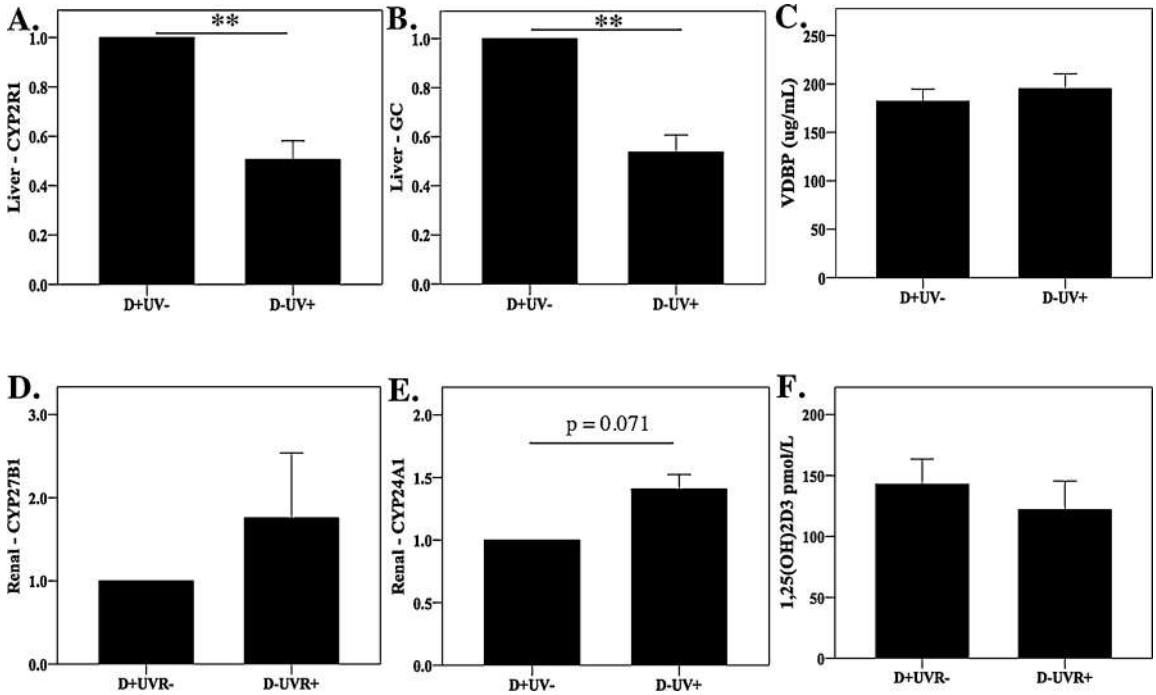

Fig. 4. Effect of UVR on mRNA expression of vitamin D metabolic enzymes, serum VDBP and 1,25$(OH)_2D_3$ concentrations at week 6. Female C57Bl/6 mice were sacrificed at week 6 after being established on vitamin D-sufficient (D+) or -deficient diets (D-). The D- mice were then either treated (UVR+) or not treated with UV irradiation (UVR-). Vitamin D deficient mice treated with UVR (D-UVR+), had similar 25(OH)D_3 levels to D + mice, and thus these two groups were compared to determine the effect of UVR on vitamin D metabolic pathway. Livers, kidneys and blood were harvested to determine *A.* Liver CYP2R1 mRNA, *B.* Liver *GC* protein mRNA, n = 3/group, *C.* Serum vitamin D binding protein (VDBP) concentration, n = 5–7/grp *D.* Renal CYP27B1 mRNA, *E.* Renal CYP24 A1 mRNA, n = 3/group and *F.* Serum 1,25$(OH)_2D_3$ concentration, n = 3–6/group. mRNA gene expression by qtPCR was calculated using the $2^{-\Delta\Delta CT}$ method with eEF1α as the housekeeping gene. The D + UVR- group is shown as the reference control. Values are expressed as mean fold-change ± SEM. **P < 0.01.

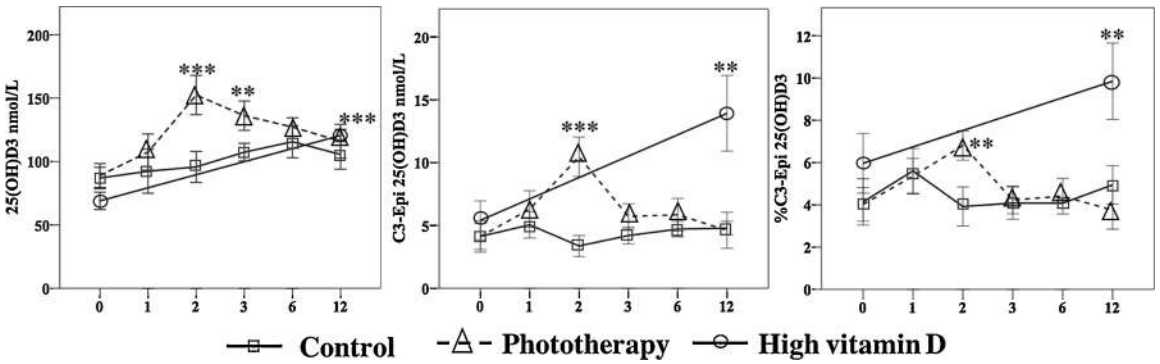

Fig. 5. Vitamin D and epimer levels in serum from participants in the D-Health and PhoCIS studies. Serum samples from the D-Health trial were collected at baseline and at 12 months after monthly dosing with 60,000 IU Vitamin D_3. In the PhoCIS trial subjects were treated, or not, with narrowband UVB phototherapy (3 sessions/week) for 8 weeks versus controls, and blood collected at baseline, 1,2,3,6 and 12 months. Vitamin D metabolites were analysed using the LC/MS/MS method to determine *A.* 25(OH)D_3, *B.* C3-Epi 25(OH)D_3 and *C.* %C3-Epi 25(OH)D_3. Data shown as mean ± SEM, n = 11in D-Health and 7–10/group in PhoCIS. Significance values represent mean difference relative to the baseline values for each individual group. *P < 0.05, **P < 0.01 ***P < 0.001.

baseline in the phototherapy group. No changes from baseline in absolute or % C3-epi 25(OH)D_3 were detected in the control group at any timepoint.

To compare the effect of phototherapy versus high dose oral supplementation, the data from the two trials were combined. Specifically, the effect of 12 months of Vitamin D_3 supplementation with 60,000 IU/month was compared to the 2 and 3-month data from the PhoCIS trial. These two timepoints in the PhoCIS trial were selected as representative of times close to maximal effect of phototherapy on vitamin D metabolism (see above).

Comparing the epimer levels from baseline to 12 months in participants from the two trials, both the absolute and %C3-epi 25(OH)D_3 concentrations were greater with high-dose oral supplementation compared to phototherapy, even after adjusting for the baseline concentration, and in the case of %C3-epi 25(OH)D_3 after adjusting for baseline 25(OH)D_3 and the change in 25(OH)D_3 concentration (Table 1). This is expected as the effect of phototherapy had diminished by 12 months. Comparing 12 months of oral supplementation to 2 months of phototherapy (i.e. at the time of maximal effect of both therapies), there was no difference in absolute C3-epi 25(OH)D_3 levels or %C3-epi 25(OH)D_3 between the two groups. At 3 months, when the acute effects of phototherapy would have diminished, the C3-epi 25(OH)D_3 levels and %C3-epi 25(OH)D_3 unadjusted, adjusted for baseline levels and adjusted for both baseline and change in 25(OH)D_3, were significantly higher with high dose oral vitamin D supplementation at 12 months compared to phototherapy (Table 1).

Table 1
Difference in mean change of Vitamin D epimer at different time points with Oral vitamin D supplementation versus Phototherapy.

Models	Baseline and month 2		Baseline and month 3		Baseline and month 12	
	Estimate (95%CI)	p-value	Estimate (95%CI)	p-value	Estimate (95%CI)	p-value
C3-epi 25(OH)D_3						
Unadjusted						
HighdoseD[a]	2.2 (−1.7 to 6.1)	0.27	6.9 (2.8–11.0	0.001	7.4 (3.3–11.5)	0.0005
Phototherapy	Reference		Reference		Reference	
Adjusted for baseline[b]						
HighdoseD	1.9 (−1.9 to 5.7)	0.33	7.0 (2.9–11.2)	0.0009	7.1 (2.9–11.3)	0.001
Phototherapy	Reference		Reference		Reference	
Adjusted for baseline[b] and change in vit D[d]						
HighdoseD	2.4 (−1.2 to 5.9)	0.19	6.3 (2.5–10.1)	0.001	5.0 (0.8–9.1)	0.08
Phototherapy	Reference		Reference		Reference	
%C3-epi 25(OH)D_3						
Unadjusted						
HighdoseD	1.1 (−1.5 to 3.7)	0.4	3.7 (0.8–6.6)	0.01	3.7 (0.6–6.8)	0.02
Phototherapy	Reference		Reference		Reference	
Adjusted for baseline[c]						
HighdoseD	1.6 (−1.0 to 4.1)	0.23	7.1 (2.8–11.3)	0.001	4.6 (1.5–7.6)	0.003
Phototherapy	Reference	0.23	Reference		Reference	
Adjusted for baseline[c] and change in vit D[d]						
HighdoseD	1.5 (−1.0 to 4.1)	0.24	4.2 (1.5–7.0)	0.003	4.4 (1.1–7.7)	0.009
Phototherapy	Reference		Reference		Reference	

[a] "HighdoseD" refers to the D-Health trial where participants are supplemented with Vitamin D_3 60,000 IU/month.
[b] Baseline C3-Epi 25(OH)D_3.
[c] Baseline %C3-Epi 25(OH)D_3.
[d] Change in vitamin D between the corresponding time points; values represents differences in mean changes (95%CI) between HighDoseD vs Phototherapy at corresponding time points.

4. Discussion

To date, it has been presumed that 25(OH)D_3 and 1,25$(OH)_2D_3$ acquired from diet or oral supplements are physiologically equivalent to that obtained by cutaneous production after sunlight exposure. This study has identified a difference in vitamin D epimer levels in mice depending on the source of vitamin D. Differences in epimer levels were not confirmed in humans, suggesting interspecies variation in vitamin D metabolism.

In the animal model, similar circulating 25(OH)D_3 and 1,25$(OH)_2D_3$ concentrations were measured after vitamin D_3 supplementation or exposure of shaved skin to "physiological" sub-erythemal UV radiation; however, higher absolute levels of the C3-epi 25(OH)D_3 and %C3-epi 25(OH)D_3 levels were observed with oral supplementation. This change persisted over time and is consistent with a study of hairless mice (Skh-1) fed vitamin D-containing diets or irradiated with UVB (2.24 kJ/m^2) three times weekly for 25 weeks [22]. The investigators of that study found less C3-epimer production in the UV-irradiated, compared to non-irradiated groups. In our study, the mechanism for reduced epimer levels with exposure to UV radiation is not clear, but a number of other changes were observed that may contribute to epimer production or breakdown. First, the expression of the liver 25-hydroxylase CYP2R1 was significantly less in the UV-irradiated compared to vitamin D-supplemented but not UV-irradiated mice. One hypothesis is that UVR-derived cholecalciferol (vitamin D_3) is hydroxylated with other, yet to be identified 25-hydroxylases to a greater degree than orally ingested vitamin D_3. The existence of unidentified hydroxylases has been

proposed by others, largely due to elimination of only 75% of the circulating 25(OH)D in CYP2R1 knockout mice [23,24]. One may then speculate that a change in hydroxylation reduces the susceptibility to epimerization. Secondly, the enzyme involved in epimerization is not known, and it is also not known whether it occurs before or after 25-hydroxylation or both. Epimerization occurs in different cell lines from bone, colon, kidney and liver tissue, though the amount of epimerization differs between different tissue types [7]. Thus, it is likely that epimerization also occurs in skin. It is possible that if pre-vitamin D_3 in the skin is epimerized, then this may be less susceptible to 25-hydroxylation, explaining reduced expression of CYP2R1 and production of C3-epi 25(OH)D_3. Finally, as in another study, a non-statistically significant increase in renal CYP24 A1 gene expression in UV-irradiated compared to vitamin D-supplemented but not UV-irradiated mice was observed [22]. As CYP24 A1 catabolises 25(OH)D_3, 1,25$(OH)_2D_3$ and its epimers, increased activity of this enzyme could explain the lower proportion of C3 epi-25(OH)D that was detected in our study, though the levels of 25(OH)D_3 and 1,25$(OH)_2D_3$ were similar and one would expect these also to fall with increased CYP24 A1 activity.

It must be acknowledged that our understanding of the vitamin D metabolic pathways are incomplete and continue to evolve. CYP11A1 can also hydroxylate vitamin D_3 and D_2, and the resultant products, 20$(OH)D_{3/2}$ and 20,22$(OH)_2D_{3/2}$ can be further hydroxylated by CYP27B1, CYP27A1 and CYP24 A1 [25,26]. CYP11A1 activity has been detected in different organs/ cell types including epidermal keratinocytes where it can produce 22(OH)D_3 and 20,22(OH)D_3 [27]. This represents an alternative pathway for vitamin D metabolism but the physiological importance of these products remains unknown. It is possible that if UVB exposure regulates the activity of CYP11A1, then there could be less substrate vitamin D_3 available for classical 25 hydroxylation in the skin or liver to form 25(OH)D_3, and the C3 epi-25(OH)D_3 measured by our assay. Further, the possibility cannot be excluded that alternative D3 hydroxy compounds have co-migrated with C3 epi-25(OH)D to interfere with the assay of C3 epi-25(OH)D. However, this is highly unlikely as the Centres for Disease Control and Prevention (CDC) certified assay used in this study has consistently demonstrated clean transition from 25(OH)D_3 to C3 epi-25(OH)D_3. Novel compounds would have to have the same retention time and fragmentation profile as C3 epi-(25)OHD_3 to co-migrate with it.

This study confirms in a prospective controlled trial setting that narrowband UVB phototherapy can induce vitamin D epimers. To our knowledge this is the first report of epimer production with phototherapy as an intervention. The results are consistent with previous reports including a post hoc analysis of vitamin D metabolites from a vitamin D_3 supplementation randomised-controlled trial [11]. The authors reported that during winter months, the 25(OH)D_3 and C3-epi 25(OH)D_3 levels fell among people randomised to the placebo group. This demonstrated that with reduced sunlight exposure, epimer levels will fall.

At the time of steady state with oral vitamin D supplementation (12 months) and peak effect of phototherapy (2 months), there was no significant difference in epimer concentration and %C3-epi 25(OH)D_3. These findings are in contrast to the differences seen in the murine studies. Further, the absolute and %C3-epi 25(OH)D_3 were significantly higher in the mouse, up to 20%, compared with 10% of total 25(OH)D_3 in the serum of human participants. The limited human studies available report that between 0 to 26% of 25(OH)D_3 can exist in the C3-epi form [13,14,28,29]. It is possible that in humans, epimerization was not sufficiently high to discriminate differences due to vitamin D supplementation or skin exposure to UVR. However, the current data suggest differences in vitamin D metabolism between rodents and humans, with higher circulating epimerized metabolite concentrations that are influenced in mice by the source of vitamin D. This is relevant as murine models have been used for the study of the effects of vitamin D and exposure to UVR in a number of non-skeletal disease states including inflammatory bowel disease and multiple sclerosis. While vitamin D therapy in a number of these studies have shown benefit, subsequent human interventional trials have failed to observe benefit [30,31]. There are fundamental differences between mice and humans that can affect vitamin D metabolism. These include a different skin structure, where human melanocytes are found at the basal layer of the epidermis while in the mouse melanocytes reside in the hair follicle and the dermis. Pelage density is much higher in rodents than humans, and the stratum corneum of rodents is generally more permeable and fragile [32–34]. Further, mice are nocturnal animals and rely predominantly on oral intake of vitamin D where as humans are diurnal animals that synthesize vitamin D in the skin on exposure to UVR.

Vitamin D epimer production in humans is relevant because of its biological activity and impact on the interpretation of total 25(OH)D_3 concentrations with current assays which often do not discriminate epimerized from non-epimerized 25(OH)D_3. Both C3-epi 25(OH)D_3 and C3-epi 1,25$(OH)_2D_3$ bind, albeit relatively weakly, to both VDBP and the vitamin D receptor. In two in vitro experiments, the epimers have shown both calcemic and non-calcemic effects [35]. In a large Norwegian study incorporating data from eight trials and over 2000 participants, the mean %C3-epi 25(OH)D_3 was 4.4% [36]. The investigators found a strong correlation between 25(OH)D_3 and C3-epi 25(OH)D_3. The %C3-epi 25(OH)D_3 increased with increasing serum 25(OH)D_3 but plateaued at ~7% when 25(OH)D_3 concentration reached ~120-140 nmol/L. Furthermore, the investigators reported no relationship between %C3-epi 25(OH)D_3 and serum VDBP level, VDBP phenotype or single nuclear polymorphisms related to serum 25(OH)D_3 levels. In the current study, while most people had %C3-epi 25(OH)D_3 levels of less than 10%, in four participants from the D-Health study, the epimer made up between 10–20% of total 25(OH)D_3. Thus, while at a population level, vitamin D epimers may not lead to significant misinterpretation, it appears there are people who are more predisposed to higher vitamin D epimer levels than others. It is in these people that interpreting current assays may be misleading. Additional work to understand the variability between people and production and/or breakdown of epimerized vitamin D metabolites is needed. Moreover, multiple lines of evidence have established the additional benefits of UVR that are independent of vitamin D. These include cutaneously induced cytokines, corticotropin-releasing hormone, urocortins, proopiomelanocortin-peptides, enkephalins, that when released into the circulation exert systemic effects, including activation of the central hypothalamic-pituitary-adrenal axis, opiodiogenic effects, and immunosuppression [34,37]. Thus, at a population level there is a strong argument to encourage “physiological” sun exposure rather than universal oral supplementation regardless of the effect on epimer production.

While this is the first study to examine the comparative effects of UV radiation in a prospective fashion in both humans and mice, several limitations warrant mentioning. First, the sample size of human subjects in the PhoCIS trial was relatively small, due to the stringent inclusion criteria requiring recruitment within the first 120 days of their demyelinating event. Despite the small sample size, a statistically significant increase in 25(OH)D_3 was detected with phototherapy. The second limitation was the analysis of data from two separate clinical studies, which enrolled different populations with different intervention duration (8 weeks in PhoCIS and 12 months in D-Health). In D-Health, an elderly general population was recruited, while PhoCIS enrolled younger participants with clinically isolated syndrome. In both populations, however, there was an expected increment in 25(OH)D_3 with the respective treatment, so one would expect changes in epimer levels to be detected. Finally, in the animal study, the involvement of only female mice limits the generalisability of the findings. Female C57Bl/6 mice were selected because in previous work from our group, exposure to UV radiation did not increase 25(OH)D_3 levels in male mice, likely due to reduced 7-dehydrocholesterol in the skin [38].

In conclusion, higher concentrations of vitamin D epimers were found in mice compared to humans, and in mice oral vitamin D supplementation compared to UV-irradiation was associated with a greater

proportion of epimers in serum, either due to increased epimer synthesis with oral vitamin D or greater breakdown with UV radiation. There is variability in the proportion of vitamin D epimer production in humans, so further work is needed to better understand the reasons for this variability and the role of vitamin D epimers in both health and disease.

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Chapter 7

Haifer C et al. **24,25- Dihydroxy vitamin D is lower with active Crohn's disease and spontaneously recovers with remission.** Unpublished. Abstract presented European Crohn's and Colitis Organisation Congress, Barcelona 2017.

24,25- dihydroxy vitamin D is lower with active Crohn's disease and spontaneously recovers with remission

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Abstract

Background

Vitamin D deficiency is associated with active Crohn's disease (CD). However, it remains unclear if lower 25-hydroxyvitamin D (25(OH)D) concentration is the cause, or consequence, of intestinal inflammation. Existing literature has focussed on the circulating metabolite 25(OH)D rather than the active $1,25(OH)_2D$ or its breakdown product, $24,25(OH)_2D$. We aimed to characterise vitamin D metabolism in a cohort of patients with active and inactive CD.

Methods

Fifty-four patients with CD and not on corticosteroids or vitamin D supplements, were enrolled in a 6-month prospective cohort study. Sera were collected on enrolment and at 6 months and tested for 25(OH)D, $1,25(OH)_2D$, $24,25(OH)_2D$ and vitamin D-binding protein. Validated questionnaires were used to quantify and control for vitamin D acquired from diet and sunlight exposure.

Results

At baseline, there were no differences in 25(OH)D or $1,25(OH)_2D$ levels between participants with active versus inactive disease. Levels of $24,25(OH)_2D$ were significantly lower (mean 3.9 vs 6.0 μmol/L p 0.007) and therefore the ratio of 25(OH)D:$24,25(OH)_2D$ was higher in patients with active disease (mean 17.3 vs 11.1 p 0.001). In those patients with active disease who achieved remission, there was a mean increase in 25(OH)D of 32.3 nmol/L and $24,25(OH)_2D$ of 2.1 μmol/L that was not seen in patients with persistently active, or inactive disease.

Conclusion

The levels of $24,25(OH)_2D$, but not 25(OH)D, fall in patients with active CD, and spontaneously rise with resolution of underlying inflammation. The utility of $24,25(OH)_2D$ as a biomarker of disease activity and vitamin D status in CD warrants further exploration.

Keywords: Vitamin D; Vitamin D binding protein; Inflammatory Bowel Disease; Crohn's Disease

INTRODUCTION

Active Crohn's disease (CD) and ulcerative colitis (UC) are associated with vitamin D deficiency in many[1-3] but not all studies [4, 5]. Vitamin D deficiency is defined by a 25(OH)D concentration in serum, or plasma, cut-off of <50 nmol/L [6, 7]. There are a number of factors that could account for the association between vitamin D deficiency and active inflammatory bowel disease (IBD) such as corticosteroid use, reduced sunlight exposure and reduced vitamin D oral intake and/or absorption [8].

Vitamin D is acquired from diet, or through dermal synthesis, predominantly in the form of vitamin D_3. Vitamin D_2 is produced by UV irradiation of ergosterols in plants but contributes modestly to the total circulating vitamin D, except in populations with food-supplementation with vitamin D_2. Vitamin D_2 and vitamin D_3 undergo comparable metabolism, so when vitamin D_3 metabolism is referred to, it also applies to vitamin D_2 metabolites. Vitamin D_3 undergoes 25-hydroxylation in the liver by the cytochrome p450 enzyme, CYP2R1, to produce 25(OH)D_3, which has little biological activity. The majority (85-90%) of 25(OH)D_3 circulates tightly bound to the vitamin D binding protein (VDBP) [9]. The remaining non-VDBP bound fraction (bioavailable 25(OH)D_3) is bound to albumin with less than 1% of 25(OH)D_3 unbound or free. It has been proposed that the free, or bioavailable, 25(OH)D_3 may be more physiologically relevant than total 25(OH)D_3, although this has not been formally evaluated [9, 10]. Circulating 25(OH)D_3 is further hydroxylated in the kidney and extra-renal tissues by the 1α-hydroxylase CYP27B1 to produce the metabolically active 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ exerts its actions principally through binding to a nuclear vitamin D receptor (VDR). Changing concentrations of 1,25$(OH)_2D_3$ and 25(OH)D_3 modulate the expression of the *CYP24A1* which is responsible for the production of 24,25$(OH)_2D_3$, 1α,25$(OH)_2D_3$-26,23S-lactone and calcitroic acid (1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin D_3). While 24-hydroxylase CYP24A1 is most abundant in the proximal and distal tubules of the kidney, it has also been detected in essentially all vitamin D target tissues including the colon.

The association between active IBD and vitamin D deficiency has led investigators to study vitamin D supplementation as a treatment for IBD in a series of small studies, which that have yielded mixed results [11-13]. In the largest study, a randomised double-blind placebo-controlled trial, 94 CD patients in remission were randomised to receive either 1200IU vitamin D_3, or placebo, once daily for 1 year. Treatment reduced the rate of clinical relapses from 29% to 13% (P=0.06) [11]. A further study in UC supplementing participants with 40,000 IU vitamin D_3 for 8 weeks found a reduction in faecal calprotectin and simple clinical colitis score, but not the partial Mayo score [12]. A pilot randomised open-label study treated 24 UC and 23 CD patients with 150,000 IU vitamin D_3 without improvement in disease activity scores [13].

We have recently shown in a mouse model, that circulating 25(OH)D_3 and the active 1,25$(OH)_2D_3$ acutely drop after inducing colitis with dextran sodium sulphate. This fall was associated with an increase in gene expression of the *CYP24A* [14]. Further, in a large Australian cohort of patients with CD in steroid-free remission, a low 25(OH)D level did not predict subsequent relapse which would otherwise be expected if vitamin D deficiency predisposed to intestinal inflammation [15]. Therefore, we hypothesise that the serum 25(OH)D falls in response to the inflammatory burden associated with IBD and that as the underlying disease improves, the 25(OH)D will subsequently recover.

The aim of this study was to determine if there is active catabolism of 25(OH)D or 1,25$(OH)_2$D in the setting of active CD.

MATERIAL AND METHODS

Patients and Design

Patients with CD were prospectively recruited from the IBD clinic at St. Vincent's Hospital, Sydney, Australia, between March and June 2017. All patients were diagnosed with CD according to standard clinical, endoscopic, and radiological criteria [16] and were phenotyped according to the "Montreal Classification". Patients were aged between 16 and 60 years and had either colonic (Montreal L2) or ileo-colonic (Montreal L3) disease. We included two groups of patients; those with moderate-severe disease activity and those in remission. Moderate-severe disease activity was defined by a CD activity index (CDAI) ≥ 220 in addition to an objective marker of active inflammation (C-reactive protein (CRP) ≥10 mg/L, faecal calprotectin >250μg/g or active ulceration seen at ileo-colonoscopy within 3 months). Inactive disease was defined by CDAI <150 and either CRP<10mg/L, faecal calprotectin <150μg/g; or no ulceration at ileo-colonoscopy within 3 months. Patients were excluded if they were on vitamin D supplements or corticosteroids within 4 weeks of recruitment, if they were pregnant, had short bowel syndrome, isolated small bowel CD or remission that was induced by colonic resection.

At enrolment, baseline data including demographics, disease and medication history were collected. Participants completed diet and sunlight questionnaires at baseline and at 6 months. Blood was collected, and serum stored from all participants at enrolment and after 6 months (Figure 1). Vitamin D metabolite testing was performed after the study period, effectively blinding treating physicians to the vitamin D results. The use of corticosteroids during the study period was left to the discretion of the treating physician and recorded in the study records. Participants were followed for a period of 6 months. If patients commenced on vitamin D supplementation during the study period, then they were not included in the follow up analysis. Disease relapse was defined as greater than 100-point rise in CDAI to at least 150 with associated objective markers of relapse (CRP ≥10 mg/L, faecal calprotectin >250μg/g or active ulceration seen at ileo-colonoscopy).

Peripheral blood was collected by venepuncture. Routine laboratory haematology and biochemistry tests were performed immediately, and a serum sample of 1-1.5ml was stored at -20^{0}C for later analysis of vitamin D metabolites.

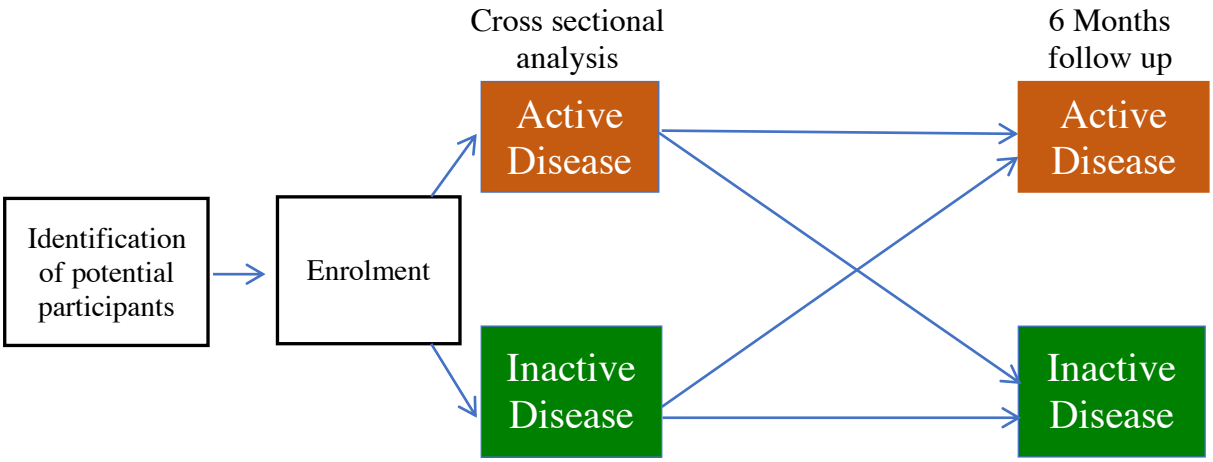

Figure 1. Study Design: Participants with active or inactive CD were enrolled. Active disease was defined by CDAI ≥ 220 in addition to an objective marker of active inflammation (C-reactive protein (CRP) ≥10 mg/L, faecal calprotectin >250µg/g or active ulceration seen at ileo-colonoscopy within 3 months). Inactive disease was defined by CDAI <150 and either CRP<10mg/L, faecal calprotectin <150µg/g or no ulceration at ileo-colonoscopy within 3 months. Cross sectional analysis of 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ and VDBP was performed at baseline. Participants were followed up for 6 months, with longitudinal analysis of vitamin D metabolites.

Biochemical measurements

Concentrations of serum 25(OH)D_3 were measured using liquid chromatography tandem mass spectroscopy (LC/MS/MS) at Metabolomics Australia, University of Western Australia, using two Agilent 1290 UPLC binary pumps coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). This assay is certified by the Centre for Disease Control (NIH, USA) for both precision and accuracy of 25(OH)D_3 measurement with a correlation of R^2 0.9979 [17]. 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ were assayed on the same LC/MS/MS, after sample derivatization with triazolinedione reagents [18]. VDBP was measured by immunonephelometry (Dade Behring, Liederbach, Germany) (inter-assay CV <6%), and albumin was measured by dye binding reaction to bromocresol green (inter-assay CV<5%). Free and bioavailable (free plus albumin-bound) concentrations of 25(OH)D were calculated using VDBP and albumin

concentrations, according to modified "Vermeulen" equations previously published and validated [19]. Albumin, C-reactive protein, white cell count and platelet count were analysed using routine laboratory techniques.

Questionnaires

Within 2 weeks of the baseline blood draw, participants completed a baseline questionnaire (See Figure 1, Appendix 1) including details of their demographics, ethnicity, smoking history, alcohol consumption, medication use, supplements, CD diagnosis and treatment. We used validated questions to measure sunlight exposure and dietary vitamin D intake [20]. Sunlight exposure assessment included questions related to skin colour and tanning characteristics, time spent outdoors between the hours of 10am and 3pm in the preceding month on weekdays and weekends, area of skin exposed and use of sunscreen. Vitamin D intake was measured using a food frequency questionnaire of foods with highest vitamin D content. At study exit, a follow up questionnaire (Appendix 1) was administered including details of symptoms, medications, smoking, sun exposure and dietary intake.

Data on ambient levels of ultraviolet (UV) radiation

Data on levels of ambient UV radiation were provided by the Australian Radiation Protection and Nuclear Safety Agency (ARPANSA) and measured as measured in Standard Erythemal Doses (available at www.arpansa.gov.au/our-services/monitoring/ultraviolet-radiation-monitoring/ultraviolet-radiation-dose)

Statistical Consideration and analysis

Using the results of the study by Harries et al [21] which showed lower levels of $25(OH)D_3$ in CD patients, the study was powered to detect an 8% difference in the $25(OH)D_3$ level between the two groups at baseline with α of 0.05 and β of 0.80. On this basis we determined a sample size of 27 per group was required. Longitudinal analysis of the 6-month data was exploratory and the study was not powered for this analysis.

Summary statistics include counts and percentages for categorical variables and mean (standard deviation (SD)) or median (interquartile range (IQR)) for continuous variables, depending on whether the distribution was normal or non-normal, respectively. Group comparison of continuous variables were assessed using Student's unpaired t-test for parametric data, or Mann-Whitney *U* and Kruskall-Wallis testing for nonparametric data. Categorical variables were compared using Pearson chi-square test or Fisher's exact test.

A score for vitamin D obtained from sunlight exposure was calculated using a modified method described by Cargill et al.[20] The reported time spent outdoors between 10am and 3pm during an average week was combined with a weighted UV multiplier based on the month the data was collected. Finally, a multiplier was applied based on the reported clothing coverage to estimate body surface area exposed. (See chapter 2.5.3 for details)

A score for vitamin D intake was generated from dietary sources using the dietary questionnaires combined with the reported vitamin D content of foods as described by the Nutritional Tables (NUTTAB) 2010 from Food Standards Australia New Zealand (available from http://www.foodstandards.gov.au).

The sunlight and dietary scores were then combined using the following process. Separate linear regression models assessing the change in measured 25(OH)D concentration in participants who remained with inactive disease throughout the study was computed using the sunlight or dietary scores as independent variables in their respective model. The calculated beta coefficients from each model (diet or sunlight) were then used as weights to calculate a combined score of external vitamin D determinants. This combined score was used as a controlling variable in the main analysis to ensure the difference in $25(OH)D_3$ and its metabolites were related to underlying disease activity rather than vitamin D intake or sunlight exposure.

A linear regression model was used in the cross-sectional analysis to test the association between vitamin D metabolites and disease activity; and in the longitudinal analysis to assess change in vitamin D metabolites in respect to changing disease activity. Outputs from the analysis included the marginal mean (95% confidence interval (CI)), marginal mean difference (95% CI) and the P-value.

All statistical analyses were completed on IBD® SPSS® Statistics version 25. A *P* value of <0.05 was considered statistically significant.

ETHICAL CONSIDERATIONS

The study protocol was approved by the St. Vincent's Hospital Human Research Ethics committee (LNR/17/SVH/26). Written informed consent was obtained from all participants.

RESULTS

Patient Characteristics

Fifty-nine consecutive patients with CD meeting the inclusion criteria were identified (Figure 2). Five patients were excluded either due to the inability to comply with required follow-up or current vitamin D supplementation that was not identified on screening. Fifty-four participants were included in the final analysis, 27 with active disease and 27 with inactive disease. Thirty-one patients (54%) were male and the mean age was 37 years (Table 1). All patients had colonic involvement, with 24 (42%) having isolated colonic disease (Montreal Classification L2) and the remaining 30 (58%) having ileo-colonic disease (Montreal Classification L3). Ten patients (17%) had a history of perianal CD though no patients had active perianal disease. As per the inclusion criteria, none of the patients were receiving vitamin D supplementation or corticosteroids for 4 weeks prior to enrolment.

Of the 27 patients with active disease, 24 (89%) were included based on a recent ileocolonoscopy showing active disease, with the remainder having an elevated faecal calprotectin.

There were no significant differences in age, disease phenotype, body mass index (BMI) or smoking status among those with inactive compared to active disease (Table 1). Patients with inactive disease were more likely to be on an immunomodulator ($p=0.029$) or biological therapy ($p=0.002$) and have higher mean serum albumin concentration ($p<0.001$).

Patients with active disease had higher dietary intake of vitamin D as well as sunlight exposure, collectively represented as external determinants of 25(OH)D levels (Table 1).

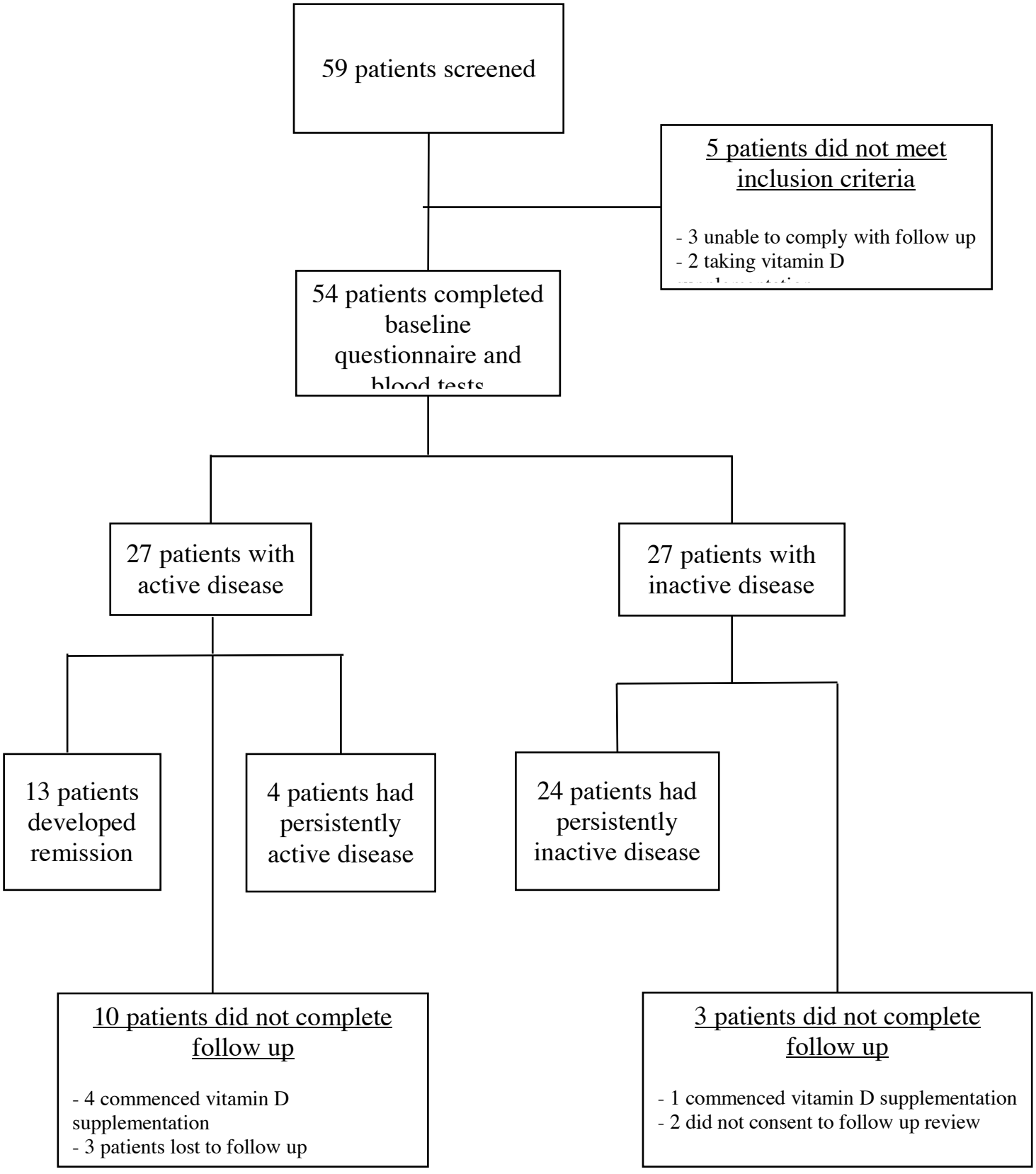

Figure 2. Patient enrolment and follow up

Table 1. Baseline Characteristics. Comparison of variables between participants with active disease and inactive disease.

	Active disease	Inactive disease	P value
Sex			
Male, n (%)	17 (63)	14 (52)	0.40
Age, mean (SD), years	39.3 (14.3)	36.4 (9.0)	0.39
Age at diagnosis, n (%)			0.14
A1	3 (11)	1 (4)	
A2	18 (67)	24 (89)	
A3	6 (22)	2 (7)	
Disease Location, n (%)			0.58
L2	11 (41)	13 (48)	
L3	16 (59)	14 (52)	
History of perianal disease, n	3 (11)	7 (26)	0.16
Smoking status, n (%)			0.89
Never	14 (52)	15 (56)	
Current	3 (11)	2 (7)	
Ex-Smoker	10 (37)	10 (37)	
Immunomodulator, n (%)	9 (33)	17 (63)	0.03
Biological Therapy, n (%)			<0.001
Tumour Necrosis Factor	7 (26)	22 (82)	
Vedolizumab	3 (11)	1 (4)	
Albumin, mean (SD), g/L	41.0 (4.6)	46.7 (3.1)	<0.001
C-Reactive Protein, median	5.3 (19.8)	0.7 (2.4)	<0.001
BMI, mean (SD), kg/m^2	24.7 (7.6)	24.0 (4.4)	0.68
Skin Tone, n (%)			0.07

Very Fair	4 (15)	5 (19)	
Fair	9 (33)	14 (52)	
Light Olive	13 (48)	6 (22)	
Brown	1 (4)	2 (7)	
External determinants of vitamin			
Diet	336 (201)	245 (125)	0.59
Sunlight	63 (78)	39 (52)	0.18
Combined	127 (48)	83 (48)	0.04

Levels of vitamin D metabolites - Baseline

Based on the concentration of $25(OH)D_3$, only 7 (13%) patients were in the vitamin D sufficient range ($25(OH)D_3$ ≥75nmol/L) as defined by the US Endocrine Society [6]. Twenty-nine (51%) had vitamin D insufficiency (50-74nmol/L) and 18 (32%) were in the deficient range (<50 nmol/L) (Table 2). There were no significant differences in $25(OH)D_3$ or $1,25(OH)_2D_3$ levels between those with active or inactive disease. However, participants with active disease had a significantly lower $24,25(OH)_2D_3$, and therefore had a higher 25(OH)D: $24,25(OH)_2D_3$ ratio (Table 2). In the active disease group, there was a trend to a higher VDBP and there was a lower calculated bioavailable $25(OH)D_3$ (Table 2).

Using linear regression modelling to control for external determinants of vitamin D, $24,25(OH)_2D_3$ and bioavailable $25(OH)D_3$ levels were significantly lower among those with active disease and the ratio of $25(OH)D_3$:$24,25(OH)_2D_3$ was significantly higher (Table 3). There were no significant differences in serum $25(OH)D_3$, $1,25(OH)_2D_3$, VDBP or free $25(OH)D_3$ levels between patients with active and inactive disease.

Table 2. Baseline vitamin D metabolite levels.

	Active disease n =27 (%)	**Inactive disease n=27 (%)**	**P value**
$25(OH)D_3$ (nmol/L)			0.51
0-49	11 (41)	7 (26)	
50-74	13 (49)	16 (59)	
75+	3 (11)	4 (15)	
$25(OH)D_3$, mean (SD),nmol/L	59.2 (26.3)	60.0 (22.0)	0.91
$24,25(OH)_2D_3$, mean (SD), □mol/L	3.9 (2.3)	6.0 (2.9)	0.007
Ratio of $25(OH)D_3$: $24,25(OH)_2D_3$ mean (SD)	17.3 (7.9)	11.1 (3.9)	0.001
$1,25(OH)_2D_3$, mean (SD), pmol/L	114.0 (56.0)	117.5 (39.6)	0.80
VDBP, mean (SD), □mol/L	5.6 (1.3)	5.0 (0.9)	0.07
Bioavailable $25(OH)D_3$ mean (SD), nmol/L	4.7 (2.5)	6.0 (1.9)	0.05
Free $25(OH)D_3$, mean (SD), pmol/L	14.3(5.8)	15.4 (4.8)	0.41

Table 3. Results from the multiple linear regression model testing baseline vitamin D metabolites across the active and remission groups, controlling for external sources of vitamin D.

Variable	**Active Disease Marginal Mean (95% CI)**	**Remission Marginal Mean (95% CI)**	**Mean difference (95% CI)**	**P value**
$25(OH)D_3$ (nmol/L)	57.9 (48.7 to 67.0)	61.3 (52.2 to 70.6)	-3.51 (-16.7 to 9.71)	0.60
$24,25(OH)_2D_3$ (□mol/L)	3.9 (2.9 to 4.9)	6.1 (5.1 to 7.1)	-2.3 (-3.7 to -0.8)	0.002
$25(OH)D_3$:$24,25(OH)_2D_3$	17.2 (14.9 to 19.6)	11.1 (8.7 to 13.5)	6.1 (2.7 to 9.6)	<0.001
$1,25(OH)_2D_3$ (pmol/L)	114.6 (93.7 to 135.5)	117.0 (98.8 to 135.2)	-2.4 (-30.7 to 25.9)	0.87
VDBP (□mol/L)	5.5 (5.1 to 5.9)	5.0 (4.6 to 5.5)	0.5 (-0.1 to 1.1)	0.13
Free $25(OH)D_3$ (pmol/L)	14.0 (12.0 to 16.1)	15.7 (13.7to 17.8)	1.7 (-4.6 to 1.2)	0.26
Bioavailable $25(OH)D_3$ (nmol/L)	4.7 (3.9 to 5.6)	6.1 (5.2 to 6.9)	-1.3 (-2.5 to -0.08)	0.04

Six Month follow up

Follow-up analysis, including repeated sunlight and dietary questionnaires and measurements of vitamin D metabolites, was completed for 41 patients. Of the 17 patients with active disease at enrolment that completed follow up, 13 (76%) developed remission during the follow-up period. Four patients had persistently active disease and no patients with inactive disease at the start of the study period developed a flare of disease (Figure 2).

In patients with active disease who developed clinical remission during the follow up period, there was a significant increase in the mean $25(OH)D_3$ from baseline to 6 months, after controlling for the change in external determinants of vitamin D. This was significantly more than the change in $25(OH)D_3$ levels in patients who had persistently active, or inactive, disease throughout the study period (Table 4). The change in $25(OH)D_3$ was accompanied by a significant increase in the mean $24,25(OH)_2D_3$ concentration, to a level that was similar to levels found in patients who remained in remission throughout the study (p=0.96) (data not shown).

Table 4. Results of a multiple Linear regression model of changes in vitamin D metabolites at 6 months follow up.

Variable	**Group**	**Mean difference between baseline and 6 months (95% CI)**	**Marginal Mean difference between groups of patients (95% CI)**	**P value**
$25(OH)D_3$ (nmol/L)[1]	Active to Remission	32.3 (16.6-47.90)	20.71 (1.4-40.0)	0.0
	Persistently active	6.9 (-20.7 – 34.6)	-4.6 (-34.7 to 25.4)	0.76
	Persistently inactive	11.6 (0.2 – 22.8)	Reference	Reference
	Active to Remission	2.1 (0.8 to 3.5)	2.3 (0.6 to 4.1)	0.008

$24,25(OH)_2D_3$ (μmol/L)[2]	Persistently active	1.0 (-1.4 to 3.3)	1.2 (-1.5 to 3.8)	0.38
	Persistently inactive	-0.2 (-1.2 to 0.8)	Reference	Reference
$25(OH)D_3$:$24,25(OH)_2D_3$[3]	Active to Remission	4.4 (0.2 to 8.6)	-0.5 (-5.9 to 4.9)	0.85
	Persistently active	-0.3 (-7.3 to 6.7)	-5.2 (-13.0 to 2.5)	0.18
	Persistently inactive	4.9 (1.9 to 7.9)	Reference	Reference
VDBP (μmol/L)[4]	Active to Remission	0.24 (-0.13 to 0.62)	0.24 (-0.23 to 0.71)	0.31
	Persistently active	-0.27 (-0.94 to 0.41)	-0.27 (-1.02 to 0.46)	0.46
	Persistently inactive	0.004 (-0.27 to 0.28)	Reference	Reference
Free $25(OH)D_3$ (pmol/L)[5]	Active to Remission	6.5 (2.8 to 10.19)	3.3 (-1.2 to 7.9)	0.15
	Persistently active	1.6 (-4.9 to 8.1)	-1.5 (-8.7 to 5.6)	0.67
	Remission	3.2 (0.5 to 5.8)	Reference	Reference
Bioavailable $25(OH)D_3$ (nmol/L)[6]	Active to Remission	1.7 (-0.1 to 3.4)	0.8 (-1.4 to 3.0)	0.46
	Persistently active	-0.1 (-3.2 to 3.0)	-0.9 (-4.4 to 2.5)	0.59
	Persistently inactive	0.8 (-4.4 to 2.1)	Reference	Reference

The change in vitamin D metabolite levels over the 6 months from baseline to follow up is shown for participants that remained in active disease, inactive disease, or were originally in active disease but achieved remission. Data were analysed using a multiple linear regression model with the following adjustments.
[1]Adjusted for baseline $25(OH)D_3$ and change in external determinants of vitamin D.
[2]Adjusted for baseline $24,25(OH)_2D_3$.
[3]Adjusted for baseline $25(OH)D_3$:$24,25(OH)_2D_3$.
[4]Adjusted for baseline VDBP.
[5]Adjusted for baseline free $25(OH)D_3$ and change in external determinants of vitamin D.
[6]Adjusted for baseline bioavailable $25(OH)D_3$ and change in external determinants of vitamin D

DISCUSSION

To date, there has been debate on whether vitamin D deficiency is causally linked to the development, activity and complications of IBD. Many of the association studies reporting this link do not adequately control for potential confounders such as corticosteroid use, vitamin D intake and sunlight exposure. This is the first study, to our knowledge, to prospectively examine the metabolism of vitamin D in a cohort of patients with CD not on corticosteroids and also carefully control for sunlight exposure and vitamin D intake to determine the independent effect of intestinal inflammation on circulating vitamin D metabolite levels.

Levels of $24,25(OH)_2D_3$ were significantly lower in participants with active disease. This was contrary to our original hypothesis which proposed that active inflammation would lead to catabolism of 25(OH)D resulting in increased $24,25(OH)_2D_3$ levels. One explanation could be that in the setting of active inflammation there is relative 25(OH)D deficiency which leads to reduced CYP24A1 activity to maintain levels of circulating $25(OH)D_3$, and the metabolically active $1,25(OH)_2D_3$, which were not different between the groups in our study. When disease activity improved, levels of $24,25(OH)_2D_3$ returned to levels that were similar to those seen in patients who remained with inactive disease throughout the study period. In one other study, $24,25(OH)_2D_3$ metabolites were measured using a radio-immunoassay in well-nourished and undernourished adult CD patients (n=40) compared to UC (n=20) and healthy controls (n=9) [3]. In that study, $25(OH)D_3$ but not $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$ levels were lower in those with active CD. The disparate results from that study compared to the current study could have been due to methodological differences. For example, in this previous study, there was a large variability in the $24,25(OH)_2D_3$ measurements likely indicating the limitations of the radioimmunoassay used. In addition, disease activity was defined using a simple clinical index without supporting inflammatory markers. Symptoms of CD correlate poorly with objective markers of inflammation and therefore in our study we included an objective marker of inflammation as part of the active disease criteria with 89% of participants having had a recent ileocolonoscopy showing active

disease and others had elevation of faecal calprotectin. These factors may explain the differing results found in our study.

In other chronic diseases such as chronic kidney disease, diabetes and genetically linked hypophosphataemia, aberrantly increased, rather than decreased, kidney CYP24A1 activity has been observed and proposed to be a major mechanism underlying accelerated degradation of $1,25(OH)_2D_3$ in these conditions. Whether appropriate *CYP24A1* expression in the human colon is maintained during colonic inflammation is not known. There is, however, overexpression of *CYP24A1* in colon cancer [22] and in murine DSS colitis with vitamin D sufficiency and deficiency; the functional significance of which is unclear [14]. Indeed, there may be differential tissue-dependent expression of key vitamin D enzymes such as *CYP24A1*, where expression is up-regulated in the colon in the setting of colonic inflammation.

Contrary to existing literature we did not find reduced $25(OH)D_3$ levels with active CD despite an appropriate sample size to detect this difference. There are several possible explanations for this. First, the location of the study, Sydney Australia (latitude 33^0 87' S), has a much higher UV Index compared to that of many North American and European cities from where the existing literature originates. For example the average summer UV Index in Sydney is 9-11, whereas in Denmark, where one of the earlier association studies was performed, the average summer UV Index is 5-6 [1, 23, 24]. Thus the Sydney population is likely to be more resistant to developing vitamin D deficiency, even in the face of active inflammation. Second, this study excluded patients on corticosteroids at baseline which is known to reduce $25(OH)D_3$ levels and has been a confounding factor in the published literature [8]. For example, in the previously mentioned cross-sectional Danish study, 25(OH)D levels were inversely correlated with disease activity as measured by CDAI and CRP. While the authors examined the effect of smoking and BMI as potential confounders, the use of corticosteroids was not discussed [1].

Participants who achieved clinical remission during the study period experienced a spontaneous and significant increase in $25(OH)D_3$ levels even after controlling for baseline levels and sunlight and

dietary intake. This is consistent with a study of 37 patients with CD, where an early increase in serum $25(OH)D_3$ was observed in those responding to tumour necrosis factor (TNF) inhibitors. This is an important observation as it supports the concept of $25(OH)D_3$ being a negative acute phase reactant rather than a driver of disease activity. Indeed, studies have suggested that vitamin D sufficiency improves the likelihood of achieving remission in CD with TNF inhibitors [25]. While this may be possible, it is also possible that those patients with higher $25(OH)D_3$ levels have less inflammatory burden and thus, are more likely to respond to biological therapy and by treating the underlying inflammation, $25(OH)D_3$ concentrations are able to spontaneously recover.

There was a trend for increased VDBP levels in the setting of active disease compared to inactive disease. This is consistent with our previous reports of elevated VDBP levels predicting subsequent disease relapse in patients with CD, as well as seen in murine models of colitis [14]. In contrast, VDBP levels fall in the setting of critical illness not specifically related to gastrointestinal disease [26]. One possible explanation for this difference in observations is that the rise in VDBP concentration in the setting of CD may be uniquely related a process that occurs in intestinal inflammation.

Several limitations warrant mention. First, the sample size was small, but we reached the pre-determined sample size for the baseline cross-sectional analysis. It is possible that with a larger cohort, differences in $25(OH)D_3$ levels may have been observed, however this is unlikely given the small, clinically insignificant difference seen between groups seen (0.08mmol/L) and p-value obtained (p 0.91). Further, the study was not powered to detect longitudinal changes in the vitamin D metabolites but, despite this, significant changes in several parameters were seen. Measuring external vitamin D sources using retrospective questionnaire data has limitations with UV-dosimeters remaining the gold-standard for measuring exposure to UV-B radiation. However, previous studies have shown that measurements from UV dosimeters explained only 8.3% of the variance in 25(OH)D levels [27]. The questionnaires used in this study have been validated against UV-dosimeter data and thus, are felt to be a reasonable estimate of sunlight exposure for the purpose of this study.

Conclusion

In the setting of active CD and no concurrent corticosteroids, $24,25(OH)_2D$ but not 25(OH)D levels were reduced. This may be related to an innate homeostatic mechanism to maintain circulating 25(OH)D and 1,25(OH)D levels in the setting of relative deficiency. Importantly, levels of 25(OH)D spontaneously rose with the treatment of underlying inflammation, suggesting that aggressive supplementation may not be necessary. The significance of $24,25(OH)_2D$ as a potential marker of vitamin D status and CD activity requires further exploration.

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Chapter 8

General discussion

GENERAL DISCUSSION

This thesis has examined the relationship between vitamin D and IBD. Specifically, the focus has been to determine if there is a causal association between vitamin D deficiency and IBD.

The table below summarises the main findings in relation to the original questions posed.

Table 8.1.1 Summary of main findings.

Does the serum 25(OH)D_3 level predict future disease relapse in patients with CD in remission?
Total, free and bioavailable 25(OH)D did ***not*** predict future disease relapse. Higher levels of the vitamin D binding protein (VDBP), lower serum albumin and medical (versus surgical) therapy to induce remission were associated with a shorter time to disease relapse.

What is the effect of vitamin D in protecting intestinal epithelium against DSS colitis and change in faecal microbiome in a mouse model?
Mice on the highest dose of vitamin D developed worst colitis as measured by endoscopy ($p<0.001$), histology ($p<0.05$) and weight loss ($p<0.001$) when compared to mice on vitamin D sufficient and deficient diets. Mice on the vitamin D sufficient diet experienced less weight loss compared to the other two groups. Importantly, serum 25(OH)D_3 fell by up to 63% among mice on high vitamin D containing diets after the development of colitis, and spontaneously increased as mice recovered from colitis. The microbial composition of faeces from mice on the highest vitamin D diet resembled that of mice after exposure to DSS.

Does ultraviolet radiation (UVR) protect against DSS colitis or alter faecal microbiome in a mouse model?
Treatment with daily UVR at 1 kJ/m^2 for 4 days followed by twice weekly treatment did not protect mice against DSS-induced colitis regardless of the type of vitamin D-containing diet administered. UV-irradiation of skin, however, did alter the faecal microbiome even prior to induction of the colitis, although the effect was most pronounced after DSS colitis had been induced.

Does the epimerisation of vitamin D metabolites differ with UVR exposure compared to oral vitamin D supplementation among mice compared to humans?
Serum vitamin D epimer concentrations were significantly higher among mice compared to humans. Oral supplementation was associated with higher epimer levels compared to UV-irradiation of skin in mice, but not humans.

What are the changes in vitamin D metabolites in a prospective cohort of CD patients with changing disease activity and no exposure to corticosteroids?

Serum 24,25$(OH)_2D_3$, but not 25$(OH)D_3$ or 1,25$(OH)_2D_3$ were lower among patients with active CD. 25$(OH)D_3$ and 24,25$(OH)_2D_3$ spontaneously increased with treatment of active CD by a mean of 32.3 nmol/L (95%CI 16.6-47.9, p=0.036) and 1.03nmol/L (95%CI 0.1-2.7, P=0.007) respectively, over a 6-month period after adjusting for baseline levels, sunlight exposure and vitamin D intake.

8.1 Findings in relation to other published studies

8.1.1 Circulating 25$(OH)D_3$ levels do not predict future disease relapse in CD

Cross-sectional and retrospective studies have found vitamin D deficiency is associated with active IBD [1-5]. Vitamin D may be low in active IBD disease due to a number of confounding factors such as reduced sunlight exposure, reduced oral intake, use of corticosteroid medication and reduced intestinal absorption of vitamin D. From a number of different lines of evidence this thesis proposes that intestinal inflammation itself causes a drop in circulating 25$(OH)D_3$.

If 25$(OH)D_3$ were able to modulate innate and adaptive gastrointestinal immune responses in a clinically significant way, a higher 25$(OH)D_3$ level would be expected to protect against future disease relapses. In the retrospective study, we did not find that 25$(OH)D_3$ levels predicted a subsequent disease relapse in CD patients in remission. A limiting factor of this study, however, was the small proportion of participants (12%) who were vitamin D deficient at baseline. This compares with a subgroup analysis of a North American cohort study of 449 IBD patients in remission at baseline where during a mean follow up of 3.4 years, 21.8% of patients had low 25$(OH)D_3$ levels and were more likely to require corticosteroids, undergo IBD-related surgery and had higher disease activity scores [6]. A methodological limitation of this study, however, was that low vitamin D was defined as the mean 25$(OH)D_3$ over the follow-up period rather than at baseline. As a result, patients may have been in remission at baseline and a subsequent relapse could have contributed to the emergence of vitamin D deficiency, and thus the mean 25$(OH)D_3$ level would be lower. This study, like many others, also could

not exclude the possibility that vitamin D deficiency was the result of a disease flare rather than the cause of a flare.

In the murine DSS-induced colitis model, we found that $25(OH)D_3$ levels dropped by up to 63% with a similar fall in $1,25(OH)_2D_3$ levels seen after the development of colitis, There was a subsequent slow recovery over the next 4 weeks of both levels. There was, however, no change in UVR exposure during this time, and the speed by which $25(OH)D_3$ fell suggests that this fall is not due to reduced oral intake or impaired intestinal absorption. A drop in $1,25(OH)_2D_3$, but not $25(OH)D_3$ has been observed by another group using the same colitis model [7], although, to our knowledge, this is the first study to use LC/MS/MS technology on mouse sera to analyse $25(OH)D_3$ levels and is also the first study to examine diets very high in vitamin D. These are possible reasons why the acute drop in $25(OH)D_3$ levels have not previously been reported in murine colitis models. The drop in $25(OH)D_3$ levels after inducing DSS colitis also supports the idea that $25(OH)D_3$ fell in response to inflammation and may not be initiating the inflammatory process. This is consistent with the results from our first clinical study which observed that low $25(OH)D_3$ levels did not predict a subsequent CD flare.

When we examined a prospective cohort of patients with CD in either clinical remission or with active disease, we did not find a difference in serum $25(OH)D_3$ or $1,25(OH)_2D_3$ levels at baseline. Importantly, participants were not on corticosteroid therapy, or vitamin D supplements, which have confounded results in prior publications and was a unique feature in our study's design. In the subgroup of patients with active disease at baseline where remission could be achieved, serum $25(OH)D_3$ levels spontaneously increased by a mean of 32.3 nmol/L even after correcting for sunlight exposure and dietary intake. This is consistent with a study of 37 CD patients, where an early increase in serum $25(OH)D_3$ was observed in those responding to TNF inhibitors, supporting the concept that treating underlying intestinal inflammation can lead to a spontaneous increase in $25(OH)D_3$ levels [8].

Taken together, these prospective and retrospective clinical studies, as well as mouse model experimentation, demonstrate that circulating $25(OH)D_3$ levels fall in the setting of acute intestinal inflammation and can thus be considered a "negative acute phase reactant".

8.1.2 What is the effect of vitamin D in protecting intestinal epithelium against chemical colitis and on faecal microbiome in a mouse model?

Despite $25(OH)D_3$ being a negative acute phase reactant, the question remained whether vitamin D supplementation could treat IBD and/or alter the faecal microbiome.

The murine studies identified that high dose vitamin D supplementation predisposed mice to worse DSS colitis and significantly altered the faecal microbiome even prior to DSS treatment. Mice on vitamin D sufficient diets lost less weight than vitamin D deficient mice, although the disease severity at colonoscopy was not different.

By contrast, the existing literature has described increasing severity of DSS-induced colitis with vitamin D deficiency when compared to vitamin D sufficiency. Also Cyp27B1 knockout mice, which cannot produce $1,25(OH)_2D_3$, have more bacteria from Bacteroidetes and Proteobacteria phyla and fewer bacteria from the Firmicutes and Deferribacteres phyla in their faeces when compared to wild-type mice [9, 10]. The measurement of colitis severity in these studies was by clinical measures alone rather than by endoscopy, and in the knockout models treated with DSS there was absolute vitamin D deficiency with numerous complicating skeletal abnormalities, so that these mice may not be a good representation of vitamin D deficiency in humans.

Among human studies, seven CD patients and ten healthy controls were supplemented with vitamin D_3 targeting serum $25(OH)D_3$ levels between 100 and 150nmol/L. The investigators found there was a significant, but temporary, change between week 0 and 1 in the faecal bacterial composition, which reversed in weeks 2, 3 and 4. Overall, there was a decrease in the number of bacterial taxa by week 4

(p=0.0001) [11]. An open-label pilot study supplemented sixteen healthy volunteers with high dose vitamin D_3 (four weeks of 980 IU/kg body weight per week, maximum 68,600 IU per week, followed by further four weeks of 490 IU/kg per week, maximum 34,300 IU per week in total) [12]. Gastroscopy and colonoscopy were performed before and after the intervention and mucosa sampled from the stomach, duodenum, colon, appendiceal orifice and terminal ileum. Faecal samples were collected before each procedure. Vitamin D_3 supplementation altered the microbiome in the upper GI tract with decreased Gammaproteobacteria including *Pseudomonas spp.* and *Escherichia/Shigella* spp. Interestingly, no significant taxonomic changes were seen in the terminal ileum, appendiceal orifice, colon or in the faecal samples. Thus, our finding of an altered faecal microbiome with oral vitamin D supplementation is supported by existing human and murine studies.

Unfortunately, there has been no adequately powered controlled trial of vitamin D supplementation in IBD. In a small open-label study, eight patients with active UC and vitamin D deficiency ($25(OH)D_3$<50nmol/L) were treated with 40,000 IU vitamin D_3 once weekly for eight weeks and no other change in IBD therapy [13]. The authors reported a fall in the faecal inflammatory marker calprotectin from median 275 to 111 μg/g (p=0.02) and an improvement in one of two examined clinical indices of disease activity. Vitamin D treatment was also associated with an increase in Enterobacteriaceae abundance. A pilot randomised double-blind placebo-controlled trial of high-dose vitamin D_3 at 10,000 IU daily versus 1,000 IU daily failed to show a difference in relapse rates in 34 patients with CD in remission [14]. A placebo-controlled trial of 1,200 IU vitamin D_3 in 94 patients with CD remission found a lower relapse rate among treated patients (13% vs 29%) but this did not reach statistical significance (p=0.06). The published human data to date have not shown any adverse effects of targeting higher levels of $25(OH)D_3$ on intestinal inflammation but also have not demonstrated clinical efficacy, although there are yet no large-scale studies.

The ideal study design to establish the efficacy of vitamin D as a therapy in IBD requires vitamin D supplementation to be the only intervention against a placebo control. This may only be ethically justifiable in a cohort of patients with IBD in remission and the time to relapse examined as attempted by the Danish study mentioned above [15]. Compared to the Danish study, it would be important to include only participants with vitamin D deficiency at baseline and supplementation to a higher target $25(OH)D_3$ level. The alternative, and perhaps more clinically relevant design, is to examine vitamin D supplementation in active disease as an adjunctive treatment with currently established therapies such as monoclonal antibodies against TNF. Given the known efficacy of these agents, the additional effect of vitamin D supplementation is likely to be small and thus mandate a large study population to adequately power the study. This is unlikely to ever be practically achieved.

8.1.3 Does exposure to ultraviolet radiation (UVR) protect against DSS colitis or alter faecal microbiome in a mouse model?

The increased incidence of CD and UC at increasing latitudes has been described in a number of epidemiological studies [16-18]. Higher residential sun exposure has been associated with a lower risk of CD in adults [19] and lower UV exposures were associated with increased severity of IBD [20]. One explanation has been the increased likelihood of vitamin D deficiency at greater latitudes due to reduced UVR exposure predisposing these populations to IBD. As already described, the effect of oral vitamin D supplementation in IBD has been mixed, but the vitamin D-independent effect of UVR on intestinal immunity has not been well studied. Further, after establishing that oral vitamin D alters the faecal microbiome, we sought to determine whether systemically acquired $25(OH)D_3$ through dermal synthesis had a similar effect, or if this was a direct effect of oral vitamin D.

UV-irradiation of skin in mice on high dose, sufficient dose or no supplemental vitamin D did not protect against DSS colitis. As the serum $25(OH)D_3$ level did not rise in the high and sufficient vitamin D dietary groups with UVR treatment, these were the best groups to identify a vitamin D independent effect of UVR. As discussed in chapter 5, these results are in contrast to a study of phototherapy where low light (1000 lux) but not high light (2500 lux) reduced severity of DSS-induced colitis, though the

wavelengths were not described [21]. In another study in C57Bl/6 mice, irradiation with broadband UVB (280-350nm) at a dose of 1.5 kJ/m^2 for four days prior to DSS treatment, reduced colitis severity as measured by weight loss, faecal haemoccult blood and histological scores [22]. There are methodological differences in the dose of UVR used and the measurement of colitis severity in these studies compared to those presented in this thesis which may account for the difference in results.

After controlling for the vitamin D groups, UV-irradiation of skin was associated with a change in overall beta-diversity (p=0.009) with enrichment of *Coprococcus* and *Mucispirillum* both of which may have anti-inflammatory properties. The effect of UVR on the faecal microbiome is intriguing, but not as pronounced as with dietary vitamin D supplementation, likely due to the lack of direct contact with the intestine. UV-irradiation of skin is known to induce systemic immune suppression, through multiple mediators released from dermal antigen presenting cells (APCs) and keratinocytes, as well as through the effects of UV irradiation on mast cells, lymphocytes (including regulatory T-cells and natural killer T cells) in the draining lymph nodes, and on bone marrow derived DCs [23]. After UV irradiation, bone marrow derived DCs from mice are poorly immunogenic and can suppress immunity [24, 25]. Thus, it is conceivable that through its effect on innate and adaptive immune responses, UVR could influence the composition of the intestinal microbiome. Overall the DSS model of colitis may not be the ideal experimental model to determine the benefits of exposure to UVR, as it is a better model for studying innate immune responses in IBD, whereas UVR has effects on both innate and adaptive immune responses.

While UV-irradiation of the skin is known to alter the microbiome of the skin [26], to our knowledge this is the first study to demonstrate changes in the faecal microbiome.

The impact of changes to specific taxa are difficult to interpret, but as a proof-of-concept the effect of UVR on faecal microbiome is important as it may have broader implications for the development of gastrointestinal immunity. The first 1000 days from conception, including in-utero changes in maternal microbiome, are believed to have a lasting impact on immune system development in offspring [27].

For example, gestation-only colonization with *E.coli* altered the number of early postnatal intestinal innate leukocytes in wild-type C57Bl/6 mice a change that persisted even after weaning [28]. Thus, by extrapolation, it is possible maternal and early life UVR exposure could alter intestinal microbial composition and have lasting effects on gastrointestinal mucosal immune responses. This may impact susceptibility to conditions such as IBD, coeliac disease and other food allergies.

The differences in faecal microbiome between mice acquiring vitamin D through UV-irradiation (D-UVR+) compared to diet (D+UVR-) were more striking after exposure to DSS with overall microbial composition (β-diversity) being statistically different between the two groups (p=0.021). This is because some bacteria are induced by the inflammatory process such as the mucin degraders *Akkermansia* and *Bacteroidales S-24-7,* both of which were enriched in the D+UVR- group, but not the D-UVR+ group. This demonstrates that to determine the effect of an intervention (i.e UVR in this case), it is not only the resting microbiome that is of relevance, but it is also the dynamic response to an external insult, such as DSS, as this reflects the stability or behaviour of the microbiome. While it is was not possible to determine whether UVR had a positive effect on the post-DSS treated faecal microbiome, there were distinct differences. Further study could examine this in other models of colitis and include metabolomic analysis of bacterial products such as short-chain fatty acids which are more reflective of the function of the microbiome.

8.1.4 Does the epimerisation of vitamin D metabolites differ with UVR exposure compared to oral vitamin D supplementation among mice and humans?

During the analysis of vitamin D metabolites from the animal studies, it became apparent that the serum vitamin D epimer levels were higher in mice than they had been in our initial retrospective human study. This is relevant as mice are often used in vitamin D experiments, but fundamental differences in vitamin D metabolism may limit their translatability to humans. Thus, we explored whether there was a true difference in vitamin D epimer levels between mice and humans, and moreover did exposure to UVR affect the proportion of vitamin D circulating in the epimerised form. A comparison of outcomes from two clinical trials, one with narrowband UVB irradiation and another with high dose oral vitamin D

supplementation, to our murine data, confirmed that mice have a greater proportion of C3-epi 25(OH)D_3, frequently measured between 10-20% whereas in humans, this was most frequently measured at <10%. Mice acquiring vitamin D exclusively through diet had proportionally greater C3-epi 25(OH)D_3 compared to mice acquiring vitamin D through UV-irradiation of their skin. This finding could not be confirmed in humans when comparing the effect of exposure to narrowband UVB radiation, 3 times weekly for 2 months, compared to a year of daily high dose vitamin D supplementation.

There are few animal studies where C3-epi 25(OH)D_3 has been measured. This is because until recently the assays to measure this required larger volumes of serum. A study of hairless mice (Skh-1) reported the %C3-epi 25(OH)D_3 in the order of 10-30% of total 25(OH)D_3 which increased with the dose of oral vitamin D as was found in our murine data, and UVR attenuated the amount of measured C3-epi 25(OH)D_3 [29]. There was a trend for male Skh-1 mice to have greater C3-epi 25(OH)D_3 levels ($p=0.06$). In a study of Sprague Dawley rats fed diets with 1, 2 or 4 IU of vitamin D_3, high proportions of C3-epi 25(OH)D_3 were again observed, and a sex dependent difference noted, but in the opposite direction with female rats exhibiting higher epimer levels [30]. Our study only included female mice, so it is possible that the epimer changes were a gender-dependent effect. Further studies should be carried out in male mice and other species of mice to confirm our findings.

The biological activity of C3-epimeric forms of vitamin D are lower than their native forms, yet they may contribute a significant proportion of circulating total 25(OH)D_3. A minority of participants in the clinical human study (D-Health) had a high %C3-epi 25(OH)D_3 (>10%), which may have been significant in interpreting the total 25(OH)D_3 level. Of relevance, a study of 1727 individuals who were genotyped, found that two SNPs in the 7-dehydrocholesterol reductase/nicotinamide-adenine dinucleotide synthetase 1 (*DHCR7/NADSYN1)* and Group specific component (*GC*) genes explained the variation in C3-epimer levels [31]. These studies suggest C3-epimers may only be detected as a significant proportion of 25(OH)D_3 in certain sub-populations with a genetic predisposition, and it is possible that any effect of UV-irradiation on epimer levels will only be seen in this subgroup.

8.1.5 Serum $24,25(OH)_2D_3$ and bioavailable $25(OH)D_3$, but not $25(OH)D_3$ or $1,25(OH)_2D_3$, are lower among patients with active CD. $25(OH)D_3$ and $24,25(OH)_2D_3$ spontaneously increase with treatment of active CD.

Few studies have examined vitamin D metabolites other than $25(OH)D_3$ during the course of IBD. We found that serum $24,25(OH)_2D_3$, but not $25(OH)D_3$ or $1,25(OH)_2D_3$, were lower among patients with active CD. This was contrary to our original hypothesis which proposed that active inflammation would lead to catabolism of $25(OH)D_3$ resulting in increased $24,25(OH)_2D_3$ levels. One explanation could be that in the setting of active inflammation, there is relative $25(OH)D_3$ deficiency, by another yet to be determined mechanism, leading to reduced CYP24A1 activity to maintain levels of circulating $25(OH)D_3$, and the metabolically active $1,25(OH)_2D_3$, which were not different between the groups. This is supported by the finding in our DSS model [32], where the group on high vitamin D diets showed the greatest drop in $25(OH)D_3$ and did not have an increase in kidney CYP24A1 activity, suggesting the fall in $25(OH)D_3$ in active intestinal inflammation must be through another mechanism. Approximately 20% of $25(OH)D_3$ is oxidised by the C23 oxidation pathway catalysed by CYP24A1 to produce $23,25(OH)_2D_3$ [33]. Thus, it may be possible that in the setting of inflammation there is preferential oxidation by this pathway leading to more $23,25(OH)_2D_3$ and less $24,25(OH)_2D_3$. Further, other enzymes, such as the mitochondrial CYP3A4, have also been shown to catabolise $25(OH)D_3$ and their role in the setting of inflammation has not been examined [34]. Another mechanism to explain the reduced $24,25(OH)_2D_3$ could be increased catabolism of this metabolite rather than reduced production, it is known that the side chains of $24,25(OH)_2D_3$ are further oxidised by CYP24A1 in the C24-oxidation pathway with the C-C bond between C23 and C24 being cleaved, ultimately producing C23 acid [35].

$25(OH)D_3$ and $24,25(OH)_2D_3$ in the clinical study spontaneously increased with treatment of active CD. One other study of adults published in 1985, examined the $24,25(OH)_2D_3$ metabolite using a radio-immunoassay in well-nourished and undernourished CD patients compared to patients with UC and healthy controls [1]. $25(OH)D_3$ but not $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$ levels were lower in those with active CD. There were methodological differences to explain the disparate results. First, there was a

large variability in the $24,25(OH)_2D_3$ measurements likely indicating the limitations of radioimmunoassay to measure this metabolite at the time. In addition, disease activity was defined using a simple clinical index without supporting inflammatory markers, and this may have limited the opportunity to detect differences between the two groups. Numerous studies have shown the association between low $25(OH)D_3$ levels and active IBD. The exclusion of participants on corticosteroids which is known to lower $25(OH)D_3$ levels, may have resulted in the disparate findings. Importantly however, $25(OH)D_3$ and $24,25(OH)_2D_3$ increased with treatment of underlying inflammation, again supporting the concept that both of these metabolites may be "negative acute phase reactants". It is also possible that $24,25(OH)_2D_3$ is a more sensitive marker of overall vitamin D status in IBD, or may reduce in concentration before $25(OH)D_3$. These possibilities warrant further evaluation in larger studies.

Bioavailable $25(OH)D_3$ is the proportion of $25(OH)D_3$ that is free or bound to albumin, but not bound to the vitamin D binding protein. This fraction may be more physiologically relevant than total $25(OH)D_3$ [36, 37]. Our study showed that bioavailable $25(OH)D_3$ was lower among participants with active CD. This is consistent with a study of 71 IBD patients which found an inverse correlation between bioavailable $25(OH)D_3$ and faecal calprotectin, but not systemic inflammatory markers [4]. Albumin was lower and the VDBP was elevated with active CD. These findings are consistent with the earlier work presented in this thesis, where lower albumin and higher VDBP levels were better predictors of disease flare in CD than $25(OH)D_3$. In addition, the murine studies found VDBP levels increased with the induction of DSS colitis. Thus, VDBP is likely to increase as an acute phase reactant in the setting of intestinal inflammation, while albumin is a negative acute phase reactant.

8.2 Significance of the study

- Vitamin D status is a reflection of current disease state. Serum $25(OH)D_3$ levels alone may not be the best reflection of vitamin D status, and other indices such as the ratio of $25(OH)D_3$ with $24,25(OH)D_3$ may be more meaningful, particularly in geographical areas with high UV index.

- Overzealous vitamin D replacement in the setting of active disease may not be necessary, as treatment of underlying inflammation is likely to lead to increasing 25(OH)D levels, and the safety of high dose supplementation cannot be confirmed.
- The consequences of oral supplementation with vitamin D are not equivalent to those acquired through UV-irradiation of skin – with different effects on the faecal microbiome and epimer levels in animal studies. While the impact of these changes on disease is not clear, existing data suggest there are likely to be additional benefits from healthy physiological UVR exposure compared to oral vitamin D supplementation.
- Whether vitamin D deficiency is a causally related to IBD and it complications, and whether vitamin D supplementation can modulate disease activity, are still not known and will require large-scale studies.

8.3 Strengths and Weaknesses of the work in this thesis

The strength of the studies reported in this thesis is the use of multiple research methods to explore vitamin D metabolism in the setting of IBD. This work started with a retrospective clinical study using stored sera and DNA samples, followed by a series of animal experiments and finally a small prospective study of IBD patients. Large-scale prospective clinical trials would be difficult to conduct, and small cross-sectional analyses provide association data but do not help elucidate causation. Thus, by utilising multiple methods this work has provided further insights into the relationship between vitamin D metabolism and intestinal inflammation.

The main limitation of the clinical studies was statistical power. In the retrospective study there was only a small vitamin D deficient group and this may have limited the ability to detect the impact of $25(OH)D_3$ levels on subsequent disease course. Similarly we did not identify smoking as a predictor of disease relapse which has been shown in other studies, again possibly reflecting reduced statistical power with only 88 current smokers in the cohort. This study was also limited by the retrospective design and reliance on chart review to designate disease remission and disease flare rather than using objective markers. This had the potential to under report the incidence of disease flare, however we believed that significant disease relapse would have always warranted a change in therapy. Finally the cohort studied here was almost exclusively Caucasian, and the results may not be generalisable to Asian populations who also may be at higher risk of vitamin D deficiency.

The prospective clinical study was limited by a relatively small sample size given the difficulty in recruiting patients with active disease and not on corticosteroids. Despite this, important findings were clearly demonstrated. The main limitation of the murine studies is that animal models do not reflect all aspects of human IBD, and so the findings are not necessarily directly translatable to humans.

8.4 Unanswered questions and future research

The studies reported in this thesis concentrated on determining the impact of intestinal inflammation on vitamin D metabolism. The murine studies also sought to determine the effect of vitamin D

supplementation and exposure of skin to UVR on the severity of intestinal inflammation, but this is an area requiring further clinical investigation. While some published data suggest vitamin D supplementation may reduce intestinal inflammatory markers in human UC [38], the clinical significance of this is not known.

1. Can oral vitamin D supplementation help induce remission in patients with CD or UC?
2. Is there a benefit in using oral vitamin D supplementation as an adjunctive treatment with other conventional treatments to either induce or maintain remission?
3. At a population level, can IBD be prevented by maintaining adequate vitamin D levels?

Large-scale clinical trials would be needed to answer these questions but are these unlikely to be feasible due to the magnitude of the study required.

The effect of exposure to UVR on intestinal inflammation and faecal microbiome in humans largely remains unexplored. Further experiments in animal models apart from DSS-induced colitis, are needed to determine if intestinal inflammation can be attenuated with this treatment strategy. The next step would be to examine the effect of UVR exposure in a clinical pilot study. A study examining the effect of narrowband UVB in IBD, which is known to be safe in patients with psoriasis and as was performed in the PhoCIS study of early multiple sclerosis [39], would be a feasible and important study. These studies should incorporate detailed analysis of intestinal microbiota, by examining microbial composition in faeces and mucosa, but also look at metabolomic factors to determine the effect of UV and vitamin D on the function of the intestinal microbiome. A limiting factor in human IBD studies is that CD and UC are a spectrum of diseases, as has been demonstrated in GWAS studies [40]. The different forms of CD behave and evolve differently over time, for example ileal CD, compared to colonic CD, is more likely to harbour NOD2 mutations and develop stricturing complications over time [41]. As such, it is important that future clinical studies target a particular type of IBD where the microbial changes are more likely to be consistent across the patient cohort and thus increase the chances of detecting differences after the intervention.

8.5 Final comments

This study has demonstrated that $25(OH)D_3$ falls in response to intestinal inflammation in mice and spontaneously rises with resolution of inflammation in both mice and humans with IBD. This information is useful for clinicians when interpreting $25(OH)D_3$ levels, as it may help guide the need for vitamin D supplementation and the likely doses that may be needed – i.e. higher if more severe inflammation, and in difficult to control and prolonged disease. We could not demonstrate a causal relationship between vitamin D deficiency and IBD activity. Caution should also be exercised with use of very high doses of oral vitamin D supplementation as this may have deleterious effects on intestinal inflammation. These data have also shown that exposure to UVR is not equivalent to oral vitamin D supplements in their effect on the faecal microbiome in animals and on vitamin D epimer levels in animals and humans, though the clinical implications of this are not yet clear.

Finally, while animal model experiments have been useful to further understand the relationship between vitamin D, intestinal inflammation and mucosal immune responses, the ultimate question of whether treatment with vitamin D supplements or UVR can ameliorate or prevent disease relapse in human IBD will require further carefully designed, large-scale clinical trials.

8.6 References

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Appendix

Appendix 1

Baseline and Follow up questionnaires for study published in chapter 7

Vitamin D in Crohn's Disease
Baseline Questionnaire

Patient ID - _____ Date - _____

Section 1 – Demographics and Employment

1. Gender? Male ☐ Female ☐

2. What is your date of birth? (dd/mm/yyyy) - _____

3. In what country were you born? _____
   - If you were NOT born in Australia – what year did you come to live in Australia? _____

4. In what state/territory do you live? _____
   - For how many years have you been living in this state or territory? _____

5. What are your parents ETHNIC origin (that is, the place were most of their ancestors came from) and the COUNTRY they were born in?

   MOTHER
   - Country of Birth? _____
   - Ethnicity? (Tick ONE that best describes)

□ Australian	□ English	□ Croatian
□ Aboriginal/Torres straight islander	□ Vietnamese	□ Greek
	□ Italian	□ Lebanese
□ Chinese	□ German	□ Polish
□ Scottish	□ Maltese	□ Other_____
□ Dutch	□ Indian	
□ Filipino	□ Irish	

   FATHER
   a. Country of Birth? _____
   b. Ethnicity? (Tick ONE that best describes)

□ Australian	□ Filipino	□ Indian
□ Aboriginal/Torres Straight Islander	□ English	□ Irish
	□ Vietnamese	□ Croatian
□ Chinese	□ Italian	□ Greek
□ Scottish	□ German	□ Lebanese
□ Dutch	□ Maltese	□ Polish

□ Other_____

6. What is the highest technical, professional or academic qualification that you have completed? (Tick ONE)

☐ Did not complete primary school
☐ Primary school
☐ High School - Year 11 or under
☐ Year 12 senior certificate
☐ Trade/Apprenticeship
☐ Certificate or diploma
☐ Bachelor degree
☐ Postgraduate degree

7. Which of the following best describes your occupation?
   - ☐ Manager or administrator
   - ☐ Professional (e.g. engineer, doctor, teacher, nurse, police etc.)
   - ☐ Tradesperson
   - ☐ Clerk
   - ☐ Salesperson or personal service worker (e.g. teller, insurance rep etc.)
   - ☐ Plant or machine operator or drive
   - ☐ Farmer
   - ☐ Laborer or related worker
   - ☐ Member of defense force
   - ☐ Other _____

8. What is your current employment status?
   - ☐ Unemployed
   - ☐ Home duties
   - ☐ Part time work
   - ☐ Full time work
   - ☐ Student
   - ☐ Disability pension
   - ☐ Sole parent pension
   - ☐ Retired
   - ☐ Other _____

9. Which of the following best describes your current occupation?
   - ☐ Mainly indoors
   - ☐ Half indoors/half outdoors
   - ☐ Mainly outdoors

Section 2 – Smoking and Alcohol

1. Were you ever a regular smoker? Y ☐ N ☐
   a) How old were you when you first became a regular smoker? ____
   b) What age did you stop? _____ I never stopped ☐

2. Are you currently a regular smoker? Y ☐ N ☐
   a) Over the past month, on average, how many do you smoke per week?

   □ Cigarettes - _____
   □ Cigars - _____
   □ Pipes - _____

3. How many standard drinks do you have each week? _____
   (1 std drink = a glass of wine, pot of beer or nip of spirits)

4. On how many days each week do you usually drink alcohol? _____

Section 3 - Medical History and Medications

1. Do you have any of the following medical conditions? (Tick all appropriate)
   □ High blood pressure
   □ High cholesterol
   □ Osteomalacia
   □ Osteopenia
   □ Rickets
   □ Osteoporosis
   □ Other_____

If you are a male, please go to Question 6

2. Are you currently taking Hormone Replacement Therapy (HRT)?

3. Are you currently taking any contraceptive medications? Y ☐ N ☐
   - If so, what is the name of the medication? _____

4. Are you currently pregnant? Y ☐ N ☐
5. Are you currently breast feeding? Y ☐ N ☐

6. Please list all medications you are currently taking.

Name of medication	Strength of medication on packet?	How many taken at each time?	How often do you take it?

7. Please list any supplements, vitamins over the counter medications you are currently taking. (these include, ostelin, vitamin D, calcium, fish oil)

Name of medication	Strength of medication on packet?	How many taken at each time?	How often do you take it?

SECTION 4 - Crohn's Disease History

1. At what age did you 1st have symptoms of Crohn's disease? ____yrs
2. At what age were you diagnosed with Crohn's Disease? ____yrs
3. Which parts of your bowel is affected? (Tick all appropriate)
   - □ Mouth
   - □ Stomach
   - □ Small bowel
   - □ Colon
   - □ Peri-anal

4. What medicines for your Crohn's disease have you been on in the past?

Name of medication	Strength	Frequency	Year started	Year ceased

5. Have you had any surgery for your Crohn's disease? Y ☐ N ☐
   a. If so how many? ____

6. Have you ever been hospitalized for your Crohn's disease? Y ☐ N ☐
   a. If so, how many times? _____
   b. When were you last admitted? _____

7. Have you had any setons inserted? Y ☐ N ☐

Section 5 - Current Crohn's Disease symptoms

Think about the last week….

1. How many liquid stools did you have on each of the 7 days?

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

2. Rate your daily pain on each of the 7 days
   0 – no pain, 1 – mild ache, 2 = moderate pain 3 = severe pain (doubled over in pain)

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

3. Rate your general well being
   0 – well, 1- slightly below par, 2-poor, 3- very poor, 4 - Terrible

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

4. Have you taken any anti-diarrhoeal agents (e.g. gastro-stop, codeine or loperamide) in the past week? Y ☐ N ☐

5. What is your current height? _____ cm

6. What is your current weight? _____ kg

SECTION 6 - Skin and the Sun

1. What best describes the colour of the skin on the inside of your upper arm, that is your skin colour without any tanning? (Tick one)
   - ☐ Very fair
   - ☐ Fair
   - ☐ Light olive
   - ☐ Dark olive
   - ☐ Brown
   - ☐ Black

2. What would happen if your skin was repeatedly exposed to bright sunlight during summer without any protection? (Tick one)
   - ☐ Very tanned
   - ☐ Moderately tanned
   - ☐ Mildly tanned
   - ☐ Occasionally tanned
   - ☐ Never tan
   - ☐ Only get freckled

3. In the last month, about how many hours per day would you usually spend outdoors between 10am and 3pm?
   a) During your usual work week? _____hrs

   b) During the weekend? _____hrs
   (or days off if you work weekends)

4. Over the previous month, there have been periods of a week or more when your sun exposure is very different from their 'usual' days as reported above?

   Y ☐ N ☐

   If Yes, Why?
   - ☐ Illness
   - ☐ Holiday
   - ☐ Other _____

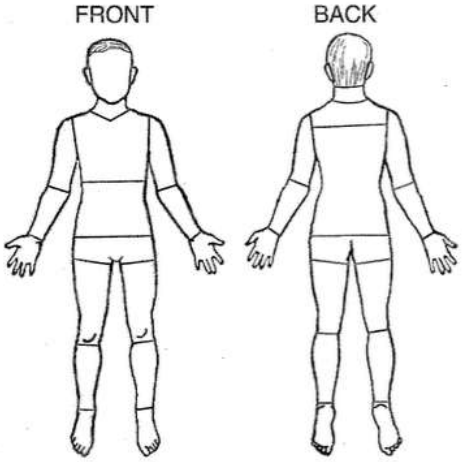
5. In the past month, when outside did you wear the following?
   (mark an "X" in the corresponding box in each row)

	Never/rarely	**Less than half the time**	**More than half the time**	**Almost always/always**
Long sleeve shirt				
Clothes that covers most of your legs				

6. Have you used sunscreen in the past month? Y ☐ N ☐
   a. What was the sun-protection factor (SPF) of the sunscreen? _____

7. On days that you have used sunscreen in the past month, how many times per day did you apply it? _____ x per day

8. Where do you apply it? (put an "X" on areas your applied)



*Patient ID*_____

SECTION 7 - Food and Dietary Supplements

1. In the LAST MONTH how often did you consume each of these foods/ drinks?
   (Put a cross in one box for each food. Each row **MUST** have a cross in it.)

	< 1 per month	1-3 per month	1-2 times per week	3-4 times per week	5-6 times per week	1 per day	2-3 per day	4 or more per day
Fresh salmon	☐	☐	☐	☐	☐	☐	☐	☐
Ocean trout	☐	☐	☐	☐	☐	☐	☐	☐
Barramundi	☐	☐	☐	☐	☐	☐	☐	☐
Canned red salmon	☐	☐	☐	☐	☐	☐	☐	☐
Canned pink salmon	☐	☐	☐	☐	☐	☐	☐	☐
Canned tuna	☐	☐	☐	☐	☐	☐	☐	☐
Canned sardines	☐	☐	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐	☐	☐
Beef	☐	☐	☐	☐	☐	☐	☐	☐
Pork	☐	☐	☐	☐	☐	☐	☐	☐
Lamb	☐	☐	☐	☐	☐	☐	☐	☐
Chicken	☐	☐	☐	☐	☐	☐	☐	☐
Margarine (exclude butter)	☐	☐	☐	☐	☐	☐	☐	☐
Butter	☐	☐	☐	☐	☐	☐	☐	☐
Dairy blend spread (exclude butter)	☐	☐	☐	☐	☐	☐	☐	☐
Cheese (eg. cheddar, cottage, brie)	☐	☐	☐	☐	☐	☐	☐	☐
Milk as a drink	☐	☐	☐	☐	☐	☐	☐	☐
Milk on cereal	☐	☐	☐	☐	☐	☐	☐	☐
Milk in coffee (latte, cappuccino, flat	☐	☐	☐	☐	☐	☐	☐	☐
Milk in other coffee and tea	☐	☐	☐	☐	☐	☐	☐	☐
Yoghurt	☐	☐	☐	☐	☐	☐	☐	☐

In the <u>last month</u>….

1. Each time you drank milk, how much did you drink?
   - □ did not drink milk as a beverage
   - □ About ½ cup
   - □ About 1 cup
   - □ About 1½ cups or more

2. Each time you put milk on cereal, how much did you use?
   - □ I did not use milk on cereal
   - □ About ½ cup
   - □ About 1 cup
   - □ About 1½ cups or more

3. In the last month did you usually use milk that has been fortified with added calcium and/or vitamin D?

Examples include: PhysiCAL , Anlene, Devondale Smart , Pura Boost, Soy Life, VitaSoy Calci Plus

□ Yes □ No (go to **Section 8)**

If YES,

a) What is the brand and type of <u>fortified milk</u> that you used? _____

b) How did you usually use <u>fortified milk</u>? (tick all that apply)

- □ As a beverage
- □ On cereal
- □ In coffee (latte, cappuccino, flat white,)
- □ In other tea / coffee

SECTION 8 – PHYSICAL ACTIVITY

1. In the past week, how many times did you do each of these activities?

a) Walking continuously for at least 10 minutes - _____times
(for recreation or exercise or to get to or from places)

b) Moderate physical activity - _____times
(like gentle swimming, social tennis, vigorous gardening or work around the house)

c) Vigorous physical activity - _____times
(that made you breathe harder or puff and pant, like jogging, cycling, aerobics, competitive tennis but not household chores or gardening)

2. If you add up all the time you spent doing each activity in the past week, how much time did you spend altogether doing each type of activity

a. Walking continuously for at least 10 minutes _____Hrs _____Mins

b. Moderate physical activity - _____Hrs _____Mins

c. Vigorous physical activity - _____Hrs _____Mins

*Patient ID*_____

Vitamin D in Crohn's Disease

Follow up Questionnaire

Patient ID -_____Date - _____

Section 1 – Smoking and Alcohol

1. Are you currently a regular smoker? Y ☐ N ☐
   a) Over the past month, on average, how many do you smoke per week?
      - ☐ Cigarettes - _____
      - ☐ Cigars - _____
      - ☐ Pipes - _____

2. How many standard drinks do you have each week? _____
   (1 std drink = a glass of wine, pot of beer or nip of spirits)

3. On how many days each week do you usually drink alcohol? _____

Section 2 - Medications

If you are a male, please go to Question 5

1. Are you currently taking Hormone Replacement Therapy (HRT)? Y ☐ N ☐

2. Are you currently taking any contraceptive medications? Y ☐ N ☐
   - If so, what is the name of the medication? _____

3. Are you currently pregnant? Y ☐ N ☐
4. Are you currently breast feeding? Y ☐ N ☐

5. Please list all medications you are currently taking.

Name of medication	Strength of medication on packet?	How many taken at each time?	How often do you take it?

6. Please list any supplements, vitamins over the counter medications you are currently taking. (these include, ostelin, vitamin D, calcium, fish oil)

Name of medication	Strength of medication on packet?	How many taken at each time?	How often do you take it?

*Patient ID*_____

Section 3 - Current Crohn's Disease symptoms

Think about the last week….

1. How many liquid stools did you have on each of the 7 days?

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

2. Rate your daily pain on each of the 7 days
   0 – no pain, 1 – mild ache, 2 = moderate pain 3 = severe pain (doubled over in pain)

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

3. Rate your general well being
   0 – well, 1- slightly below par, 2-poor, 3- very poor, 4 - Terrible

   Day 1 ___ Day 2 ___ Day 3 ___ Day 4 ___ Day 5 ___ Day 6 ___ Day 7___

4. Have you taken any anti-diarrhoeal agents (e.g. gastro-stop, codeine or loperamide) in the past week? Y D N D

5. What is your current height? _____cm

6. What is your current weight? _____kg

SECTION 4 - Skin and the Sun

1. In the last month, about how many hours per day would you usually spend outdoors between 10am and 3pm?
   a) During your usual work week? _____hrs

   b) During the weekend?
   _____hrs (or days off if you work weekends)

2. Over the previous month, there have been periods of a week or more when your sun exposure is very different from their 'usual' days as reported above?
   Y □ N □

   If Yes, Why?
   - ❑ Illness
   - ❑ Holiday
   - ❑ Other _____

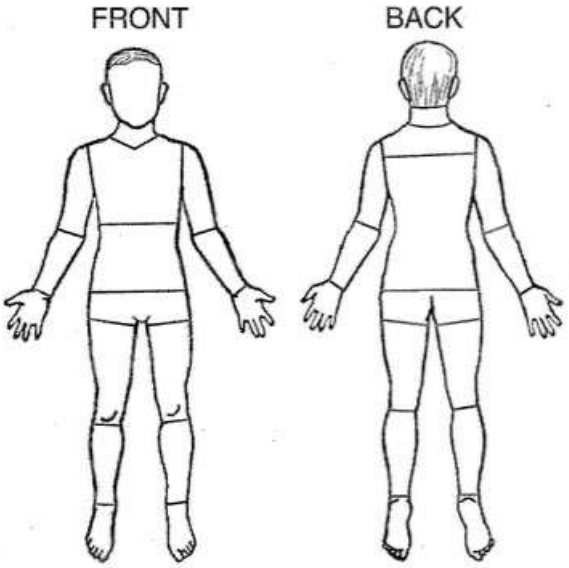
3. In the past month, when outside did you wear the following?
   (mark an "X" in the corresponding box in each row)

	Never/ rarely	**Less than half the time**	**More than half the time**	**Almost always/always**
Long sleeve shirt				
Clothes that covers most of your legs				

4. Have you used sunscreen in the past month? Y D N D
   a. What was the sun-protection factor (SPF) of the sunscreen? _____

5. On days that you have used sunscreen in the past month, how many times per day did you apply it? ____x per day

6. Where did you apply it? (put an "X" on areas your applied)



SECTION 5 – PHYSICAL ACTIVITY

1. In the past week, how many times did you do each of these activities?

   a) Walking continuously for at least 10 minutes - _____times
   (for recreation or exercise or to get to or from places)

   b) Moderate physical activity - _____times
   (like gentle swimming, social tennis, vigorous gardening or work around the house)

   c) Vigorous physical activity - _____times
   (that made you breathe harder or puff and pant, like jogging, cycling, aerobics, competitive tennis but not household chores or gardening)

2. If you add up all the time you spent doing each activity in the past week, how much time did you spend altogether doing each type of activity

   a. Walking continuously for at least 10 minutes ____Hrs_____Mins

   b. Moderate physical activity - ____Hrs_____Mins

   c. Vigorous physical activity - ____Hrs_____Mins

*Patient ID*_____

SECTION 6 - Food and Dietary Supplements

1. In the LAST MONTH how often did you consume each of these foods/ drinks?
(Put a cross in one box for each food. Each row **MUST** have a cross in it.)

	Less than 1 per month	1-3 times per month	1-2 times per week	3-4 times per week	5-6 times per week	1 per day	2-3 per day	4 or more per day
Fresh salmon	☐	☐	☐	☐	☐	☐	☐	☐
Ocean trout	☐	☐	☐	☐	☐	☐	☐	☐
Barramundi	☐	☐	☐	☐	☐	☐	☐	☐
Canned red salmon	☐	☐	☐	☐	☐	☐	☐	☐
Canned pink salmon	☐	☐	☐	☐	☐	☐	☐	☐
Canned tuna	☐	☐	☐	☐	☐	☐	☐	☐
Canned sardines	☐	☐	☐	☐	☐	☐	☐	☐
Eggs	☐	☐	☐	☐	☐	☐	☐	☐
Beef	☐	☐	☐	☐	☐	☐	☐	☐
Pork	☐	☐	☐	☐	☐	☐	☐	☐
Lamb	☐	☐	☐	☐	☐	☐	☐	☐
Chicken	☐	☐	☐	☐	☐	☐	☐	☐
Margarine (exclude butter)	☐	☐	☐	☐	☐	☐	☐	☐
Butter	☐	☐	☐	☐	☐	☐	☐	☐
Dairy blend spread (exclude butter)	☐	☐	☐	☐	☐	☐	☐	☐
Cheese (eg. cheddar, cottage, brie)	☐	☐	☐	☐	☐	☐	☐	☐
Milk as a drink	☐	☐	☐	☐	☐	☐	☐	☐
Milk on cereal	☐	☐	☐	☐	☐	☐	☐	☐
Milk in coffee (latte, cappuccino, flat	☐	☐	☐	☐	☐	☐	☐	☐
Milk in other coffee and tea	☐	☐	☐	☐	☐	☐	☐	☐
Yoghurt	☐	☐	☐	☐	☐	☐	☐	☐

In the last month….

1. Each time you drank milk, how much did you drink?
   - ❑ did not drink milk as a beverage
   - ❑ About ½ cup
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   - ❑ About 1½ cups or more

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   - ❑ I did not use milk on cereal
   - ❑ About ½ cup
   - ❑ About 1 cup
   - ❑ About 1½ cups or more

3. In the last month did you usually use milk that has been fortified with added calcium and/or vitamin D?

Examples include:

- PhysiCAL
- Anlene
- Devondale Smart
- Pura Boost
- Soy Life
- VitaSoy Calci Plus

❑ Yes □ No

If YES,

a) What is the brand and type of fortified milk that you used?

_____

b) How did you usually use fortified milk? (tick all that apply)
   - ❑ As a beverage
   - ❑ On cereal
   - ❑ In coffee (latte, cappuccino, flat white,)
   - ❑ In other tea / coffee

Thank you!